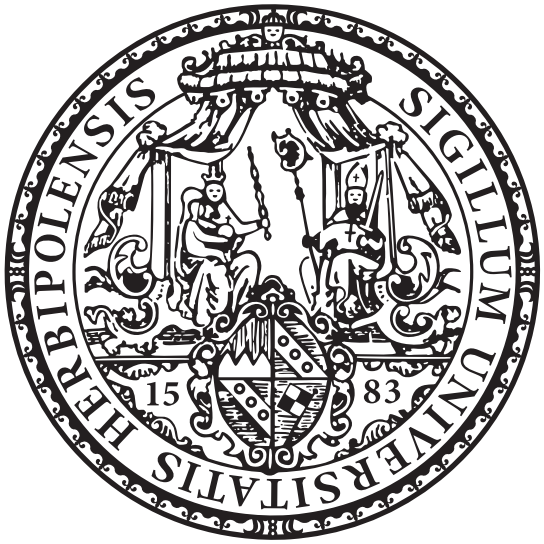
Evolution and diversity of cuticular hydrocarbon profiles of cuckoo wasps



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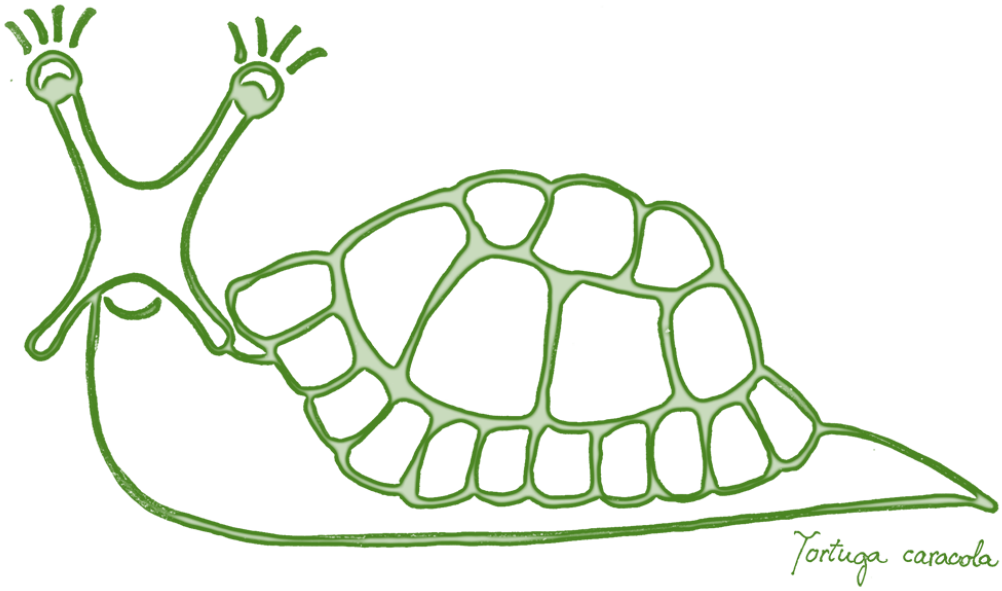
Believe you can and you are halfway there. (T. Roosevelt)



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Affidavit

I hereby declare that my thesis entitled: „Evolution and diversity of cuticular hydrocarbon profiles of cuckoo wasps" is the result of my own work.

I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation: „Evolution and diversity of cuticular hydrocarbon profiles of cuckoo wasps", eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Contents

Summary

Cuticular hydrocarbons (CHC) abound on the surface of arthropods. In spite of their simple structure (molecules of carbon and hydrogen atoms), they provide pivotal functions in insects: their hydrophobic properties confer the insects a means to regulate water balance and avoid desiccation, whereas their diversity has enhanced their use as signals and cues in a wide range of communication and recognition processes. Although the study of CHC in insects over the past two decades has provided great insight into the wide range of functions they play, there is still a gap in understanding how they diversify and evolve.

In this thesis, I have used members of the family Chrysididae to explore patterns of diversification of CHC. Most of the species of cuckoo wasps in this study are specialized parasitoids or kleptoparasites of mainly solitary hymenopteran hosts. Other hosts of the family include butterflies or stick insects. Cuckoo wasps are a particular interesting model to study the evolution of cuticular hydrocarbons because of their chemical adaptations that allow them to remain unrecognized by their hosts. Chemical insignificance (the reduction of the total amount of CHC on the cuticle) and chemical mimicry (the *de novo* production of CHC profiles resembling those of their female host) have been described in some representatives of the family and unpublished evidence suggests chemical deception is widespread in Chrysididae (Chapter 2). Nonetheless, to trace the evolution of any trait of interest, a reliable phylogenetic reconstruction of the family is required. Therefore, the first study of this thesis constitutes the largest and to-date most reliable phylogenetic reconstruction of the family Chrysididae, which includes representatives of 186 species of cuckoo wasps. While the results of this phylogenetic reconstruction are consistent with previous ideas on the relationships of subfamilies and tribes, it shows the existence of several non-monophyletic genera (Chapter 3).

CHC are involved in intraspecific recognition, often acting as contact sex pheromones. Nevertheless, it is not yet understood to what extent CHC profiles differ between the two sexes and whether some compound classes are more prevalent in one or the other sex. So far, no comparison of CHC profiles of males and females has been done for more than a dozen of related species. In Chapter 4, I describe and compare CHC profiles of females and males of 58 species of cuckoo wasps in order to evaluate whether and to what extent CHC profiles of these species differ between the sexes. I demonstrated that CHC profiles of cuckoo wasps are frequently (more than 90% of the species analyzed) and strongly dimorphic (both sexes of a given species tend to produce very different CHC compounds). Methyl-branched compounds tend to be more prevalent in males (especially dimethyl-branched compounds) and unsaturated compounds prevail in females. Moreover, a sex-specific pattern in the distribution of the double bond position of alkenes was evident: internal double bond positions (> 11) occur predominantly in males, whereas alkenes with the double bond at position 9 were more abundant and frequent in females (Chapter 4). In Chapter 5, I investigated how CHC profiles of cuckoo wasps differ across species. Are CHC profiles of cuckoo

wasps species-specific, enabling their use as cues for species recognition? How do CHC profiles resemble phylogenetic relatedness? In Chapter 5, I try to answer these questions by comparing CHC profiles of 59 species of cuckoo wasps. CHC profiles of cuckoo wasps are shown to be species (and sex-) specific. I show that CHC profiles are useful as a complementary tool to help delimiting taxonomically difficult sibling species. Moreover, the evaluation of CHC profiles of five commonly occurring species within a genus, showed little or no geographical variation. However, CHC profiles of closely related species may differ strongly among each other, not being useful to track the evolutionary history of species (Chapter 5). Sexual selection is generally credited for generating striking sexual dimorphism by causing changes in male traits. Most often, sexual selection has a stronger effect on males, who compete for access to and may be selected by females, thus male traits may rapidly evolve. Nevertheless, in cuckoo wasps, it appears that it is the female sex the one evolving faster changes, with females of very closely related species showing extremely divergent profiles. One plausible reason for this disparity is that natural selection acting on female's CHC profiles may be stronger than sexual selection on males (Chapter 6). Since females of cuckoo wasps are most probably engaged in an evolutionary arms race with their female hosts, CHC profiles of female cuckoo wasps are likely rapidly evolving, thus explaining part of the strong observed sexual dimorphism of CHC (Chapter 6). In fact, Chapter 7 shows evidence of a possible ongoing evolutionary arms race between five cuckoo wasps of the genus *Hedychrum* and their hosts. *Hedychrum* species parasitize either Coleoptera-hunting or Hymenoptera-hunting digger wasps. Since the coleopteran prey of the former digger wasps is naturally better protected against fungus infestation, these wasps do not embalm their prey with alkene-enriched secretions as do the Hymenoptera-hunting digger wasps. Thus, Coleoptera-hunting digger wasps can apparently diversify their profiles to escape chemical mimicry. Interestingly, only female cuckoo wasps of these hosts have started producing the same compound classes and even the same CHC compounds as those of their hosts. Male cuckoo wasps, however retain an alkene-enriched CHC profile that reflects the molecular phylogeny of the genus (Chapter 7). Whereas, a larger number of parasite-host comparisons may be needed to further conclude that an arms race between cuckoo wasps and their hosts is capable of generating sexual dimorphism of cuckoo wasps, this thesis constitutes the first effort towards this, providing a starting point for further studies.

Finally, I provide some methodological tools that may help in speeding up the sometimes cumbersome process of analyzing and identifying CHC profiles. One of the most time-demanding steps in the processing of CHC data is the alignment of CHC chromatograms. This process is often done manually, because alignment programs are mostly designed for metabolomics or are just recently being developed. I analyzed CHC profiles using a combined approach with two freely available programs. I used AMDIS (Automated Mass Spectral Deconvolution and Identification System, http://chemdata.nist.gov/mass-spc/amdis/) to deconvolute and automatically identify all CHC of interest present in a chromatogram. I then developed a series of R scripts to correct for potential, unavoidable errors while processing CHC chromatograms with AMDIS. Chapter 8 explains this procedure. In the next chapter, I developed a program that helps in the identification of one commonly occurring class of hydrocarbons. The limited number of linear alkanes (only one per carbon atom) and their characteristic diagnostic ion allows a rapid and unambigous identification of these substances. In opposition, unsaturated and methyl-branched com-

pounds are more difficult to identify, as a result of the much larger diversity of existing compounds. To identify unsaturated compounds a derivatization is necessary to determine the position of the double bond. Methyl-branched alkanes, however can be identified from the original chromatogram if their diagnostic ions are known. Nonetheless, polymethyl-branched alkanes (*e.g.*, compounds with two or more methyl groups along the chain) are often difficult to identify, because they may appear in mixes (*e.g.*, 3,7 diMeC27 and 3,9 diMeC27), and tables containing the diagnostic ions are not easily available. Therefore, I developed a program that creates a table with all possible methyl-branched compounds containing up to 4 methyl groups, and that provides their diagnostic ions and a calculated retention index. This may allow a much faster identification of the methyl-branched compound a researcher is dealing with, without having to lose time in the tedious calculations by hand. The program is able to correctly identify, or at least, greatly reduce the number of possible options for the identification of an unknown methyl-branched compound. Thus, using this tool, most methyl-branched compounds can be readily identified (Chapter 9). This thesis ends with a general discussion (Chapter 10). Overall, this work provides a comprehensive overview of the diversity of cuticular hydrocarbons of cuckoo wasps. The analyses presented here shed light on the emergence and evolution of interspecific diversity and intraspecific sexual dimorphism of CHC profiles. In addition, two technical methods have been developed that could greatly facilitate the CHC analysis of insects.

Zusammenfassung

Kutikulare Kohlenwasserstoffe (engl. „cuticular hydrocarbons“, CHC) sind Substanzen, die wir in größeren Mengen auf der Körperoberfläche von Arthropoden finden. Diese Moleküle aus Kohlenstoff- und Wasserstoffatomen haben trotz ihrer einfachen Struktur entscheidende Funktionen bei Insekten: Ihre wasserabweisende Eigenschaften geben den Insekten die Möglichkeit, den Wasserhaushalt zu regulieren und Austrocknung zu vermeiden. Darüber hinaus ermöglicht die Vielfältigkeit der CHC ihre Verwendung als Signale für eine breite Palette von Kommunikations- und Erkennungsprozessen. Obwohl die Erforschung von CHC in den letzten zwei Jahrzehnten einen großen Einblick in die Funktionen bei Insekten ermöglicht hat, gibt es immer noch Verständnislücken bezüglich der Evolution und Diversifizierung von CHC (Kapitel 1).

In der vorliegenden Dissertation habe ich anhand verschiedener Arten der Wespen-Familie Chrysididae die Diversifizierungsmuster von CHC erforscht. Die meisten der Goldwespenarten in dieser Studie sind spezialisierte Parasitoiden oder Kleptoparasiten von hauptsächlich solitären Hymenopteren. Wirte von anderen Goldwespen sind auch Phasmatodea und Lepidoptera. Goldwespen sind besonders interessante Modellorganismen, um die Evolution von CHC zu untersuchen. Denn sie haben auf ihrer Kutikula chemische Anpassungen an die chemischen Oberflächen ihrer Wirte entwickelt, um bei dem Wirt zu vermeiden, dass ihre eigenen chemischen Signale bei der Eiablage erkannt werden. Für einige Vertreter der Familie Chrysididae wurden chemische Unscheinbarkeit/Unsichtbarkeit („insignificance“) und chemische Mimikry beschrieben. Bei ersterem, handelt es sich um die Reduzierung der Gesamtmenge der CHC auf der Kutikula, bei letzterem um die Nachahmung des CHC Profils des Wirtes. Zudem, deuten unveröffentlichte Daten darauf hin, dass chemische Nachahmung unter den Chrysididae weit verbreitet ist (Kapitel 2). Eine zuverlässige phylogenetische Rekonstruktion der Chrysididae ist notwendig, um die Evolution eines Merkmales, wie z.B. die Ausbildung eines CHC-Profils, zu verfolgen. Daher stellt der erste Teil dieser Arbeit die größte und bis heute zuverlässigste phylogenetische Rekonstruktion der Familie Chrysididae dar, welche Vertreter von 186 Arten von Goldwespen umfasst. Die Ergebnisse dieser Phylogenie stehen in Übereinstimmung mit vorherigen Studien über die Beziehungen zwischen Subfamilien und Triben der Goldwespen. Die Phylogenie deutet jedoch auf die Existenz mehrerer nicht-monophyletischer Gattungen in Chrysididae hin (Kapitel 3).

CHC sind an der innerartlichen Erkennung beteiligt und fungieren manchmal als Kontakt-Sex-Pheromonen. Es ist jedoch noch nicht klar, inwieweit die CHC-Profile zwischen den beiden Geschlechtern differieren und ob einige Verbindungsklassen in dem einen Geschlecht häufiger als in dem anderen vorkommen. Bislang gibt es lediglich einen Vergleich von CHC-Profilen zwischen Männchen und Weibchen für weniger als ein Dutzend verwandter Arten. In Kapitel 4 werden die CHC-Profile von Weibchen und Männchen von 58 Goldwespenarten beschrieben und verglichen, um zu beurteilen, ob und in welchem Ausmaß, sich die CHC-Profile dieser Arten zwischen den Geschlechtern unterscheiden. Ich konnte zeigen, dass CHC-Profile von Goldwespen

stark sexuell dimorph sind (Männchen und Weibchen der gleichen Art neigen dazu, sehr unterschiedliche CHC-Verbindungen zu produzieren), und dass dieser Dimorphismus sehr häufig vorkommt (mehr als 90% der untersuchten Arten). Methylverzweigte Verbindungen (insbesondere dimethylverzweigte Verbindungen) waren tendenziel bei Männchen häufiger und bei Weibchen waren ungesättigte Verbindungen häufiger. Darüber hinaus war ein geschlechtsspezifisches Muster in der Verteilung der Doppelbindungsposition von Alkenen offensichtlich: interne Doppelbindungspositionen (>11) treten vorwiegend bei Männchen auf, während Alkene mit der Doppelbindung an Position 9 bei Weibchen häufiger vorkommen (Kapitel 4). Im darauf folgenden Kapitel meiner Arbeit, beschäftige ich mich mit der Frage wie unterschiedlich CHC-Profile von Goldwespen zwischen Arten sind. Sind CHC-Profile artspezifisch, wie es zu erwarten wäre, wenn sie zur Arterkennung dienen? Gibt es Ähnlichkeiten in Bezug auf die phylogenetische Verwandtschaft der Arten? In Kapitel 5, versuche ich diese Fragen zu beantworten, indem ich die CHC-Profile von 59 Goldwespenarten vergleiche. Ich zeige, dass CHC-Profile von Goldwespen art- (und geschlechts-) spezifisch sind, und dass CHC-Profile als ergänzendes Werkzeug zur Abgrenzung von taxonomisch schwierigen Geschwisterarten nützlich sind. Darüber hinaus zeigt die Beurteilung der CHC-Profile von fünf häufig vorkommende Arten innerhalb einer Gattung wenig oder keine geografische Variation, was bei der Abgrenzung der Arten hilft. Allerdings können CHC-Profile nah verwandter Arten sehr unterschiedlich sein. Somit sind sie kein geeignetes Merkmal um die Evolutionsgeschichte von Arten nachzuvollziehen (Kapitel 5).

Im sich daran anschließenden Kapitel, geht es darum, zu verstehen warum CHC-Profile der meisten Goldwespenarten so auffallend unterschiedliche CHC-Profile zwischen Geschlechtern aufweisen. Bei der sexuellen Selektion wird in der Regel erwartet, dass sie durch Veränderungen männlicher Merkmale zu einem auffälligen Sexualdimorphismus führt. Meistens wirkt die sexuelle Selektion stärker auf die Männchen aus als auf die Weibchen, weil sie um die Weibchen konkurrieren und von den Weibchen ausgewählt werden müssen. Daher wird erwartet, dass männliche Merkmale schneller evolvieren. Dennoch scheint das weibliche Geschlecht bei Goldwespen das Geschlecht zu sein, das schneller evolviert, was sich z. B. dadurch äußert, dass Weibchen sehr nah verwandter Arten extrem divergierende Profile zeigen (Kapitel 6). Ein plausibler Grund für diese Verschiedenheit zwischen den Weibchen nah verwandter Arten ist, dass die natürliche Selektion, die auf die CHC-Profile von Weibchen wirkt, stärker sein kann als die sexuelle Selektion bei den Männchen (Kapitel 6). Da die Weibchen der Goldwespen höchstwahrscheinlich in einem evolutionären Wettrüsten mit ihren weiblichen Wirten stehen, ist es möglich dass die CHC-Profile von Weibchen schnell evolvieren und somit den stark beobachteten sexuellen Dimorphismus von CHC in Goldwespen erklären (Kapitel 6). In Kapitel 7, werden Hinweise auf ein mögliches fortwährendes Wettrüsten zwischen fünf Goldwespenarten der Gattung *Hedychrum* und ihren Wirten aufgezeigt. Arten dieser Gattung parasitieren entweder Grabwespen die Coleoptera oder Hymenoptera als Nahrung für ihre Nachkommen jagen. Da die Coleoptera-Beute natürlicherweise besser gegen Pilzbefall geschützt ist, balsamieren diese Wespen ihre Beute nicht mit durch Alkene angereicherte Sekrete ein, im Gegensatz zu der anderen Gruppe der Grabwespen, die Hymenopteren als Futter verwerten. Daher diversifizieren Coleoptera-jagende Grabwespen offenbar ihre Profile stärker, um der chemischen Mimikry ihrer Parasitoiden zu entkommen. Interessanterweise haben nur weibliche Goldwespen dieser Coleoptera-jagende Wirte begonnen, die gleichen

Substanzklassen und sogar die gleichen CHC-Verbindungen wie die ihrer Wirte zu produzieren. Männliche Goldwespen behalten jedoch ein durch Alkene angereichertes CHC-Profil, das die molekulare Phylogenie der Gattung *Hedychrum* widerspiegelt. Um jedoch eindeutiger zu beweisen, dass ein Wettrüsten zwischen Goldwespen und ihren Wirten den Geschlechtsdimorphismus von Goldwespen hervorbringt, wäre eine größere Anzahl von Vergleichen zwischen Goldwespen und ihren Wirten nötig. Nichtsdestotrotz ist diese Arbeit ein erster Versuch, den Geschlechtsdimorphismus von CHC in Goldwespen zu erklären und ein Ausgangspunkt für weitere Studien.

Abschließend stelle ich einige methodische Werkzeuge vor, die helfen können, den bisher umständlichen Prozess der Analyse und Identifizierung von CHC-Profilen zu beschleunigen. Einer der zeitaufwendigsten Schritte bei der Verarbeitung von CHC-Daten ist die Alinierung von CHC-Chromatogrammen. Dieser Prozess wird oft manuell durchgeführt, da Alinierungsprogramme meist für die Metabolomik konzipiert sind oder gerade erst entwickelt werden. Meine CHC-Profile habe ich mit einem kombinierten Ansatz mit zwei frei verfügbaren Programmen analysiert. Ich benutzte AMDIS (Automated Mass Spectral Deconvolution and Identification System), um die CHC in einem Chromatogramm zu dekonvolutieren und automatisch zu identifizieren. Ich habe weiterhin eine Reihe von R-Skripten entwickelt, um mögliche unvermeidbare Fehler bei der Verarbeitung von CHC-Chromatogrammen mit AMDIS zu korrigieren. In Kapitel 8 wird dieses Verfahren erläutert. Im darauf folgenden Kapitel stelle ich ein Programm vor, das ich für eine erleichterte Identifizierung einer häufig vorkommenden Verbindungsklasse von CHC entwickelt habe. Die begrenzte Anzahl von linearen Alkanen (nur eines pro Kohlenstoffatom) und ihre charakteristischen diagnostischen Ionen erlauben die schnelle und eindeutige Identifizierung dieser Substanzen. Im Gegensatz dazu sind ungesättigte und methylverzweigte Verbindungen aufgrund der viel größeren Vielfalt möglicher Verbindungen deutlich schwieriger zu identifizieren. Für die Identifizierung ungesättigter Verbindungen ist eine Derivatisierung notwendig, um die Position der Doppelbindung zu bestimmen. Methylverzweigte Alkane können jedoch theoretisch vom ursprünglichen Chromatogramm unterschieden werden, sofern die diagnostischen Ionen bekannt sind. Trotz alledem sind polymethylverzweigte Alkane (z.B. Verbindungen mit zwei oder mehr Methylgruppen entlang der Kette) oft schwer zu identifizieren, da sie in Mischungen (z. B. 3,7 diMeC27 und 3,9 diMeC27) auftreten können. Ihre diagnostische Ionen müssen entweder berechnet werden oder in Tabellen, die nicht leicht verfügbar sind, gesucht werden. Ich entwickelte daher ein kleines Programm, das eine Tabelle erstellt mit allen möglichen methylverzweigten Verbindungen mit bis zu 4 Methylgruppen sowie deren diagnostischen Ionen und einem berechneten Retentionsindex. Dies erlaubt eine viel schnellere Identifizierung der richtigen methylverzweigten Verbindung, ohne dass ein Wissenschaflter Zeit für die mühsamen Berechnungen von Hand verlieren muss. Das Programm ist in der Lage, die Anzahl möglicher Optionen einer unbekannten methylverzweigten Verbindung korrekt zu nennen oder zumindest die Auswahl stark einzugrenzen und damit die Identifikation der Substanz stark zu erleichtern. Es ist daher zu erwarten, dass mit diesem Werkzeug die meisten methylverzweigten Verbindungen leicht identifiziert werden können (Kapitel 9). Ich schließe meiner Dissertation mit einer allgemeinen Diskussion (Kapitel 10). Die vorliegende Arbeit stellt einen umfangreichen Überblick der Diversität von kutikularen Kohlenwasserstoffen von Goldwespen dar. Dieser Einblick kann uns helfen, die Bedeutung von CHC-Profilen für Arthropoden im Allgemeinen besser zu verstehen. Konkret beleuchten die durchgeführten Analysen die Entstehung

und Evolution von interspezifischer Diverstität bzw. Ähnlichkeiten von CHC-Profilen und intraspezifischen sexuellen Dimorphismus von CHC-Profilen. Darüber hinaus wurden technische Methoden entwickelt, die zukünftige Arbeiten zu CHC Analysen von verschiedenen Insekten stark erleichtern könnten.

1. Introduction

1.1. Cuticular hydrocarbons

One common characteristic in all arthropods is the presence of an exoskeleton covering their body surface. This exoskeleton, also known as cuticle, is made up of two layers: an internal thick layer (procuticle) composed of proteins and chitin, and conferring strength and shape; and a thin outer layer (epicuticle) mainly composed of lipids, which provides the principal barrier to water loss from the animal body (Hadley, 1994). Although polar compounds (*e.g.*, wax esters, alcohols, fatty acids, glycerides, sterols, aldehydes and ketones) may occur in the epicuticle (Lockey, 1988; Buckner, 1993), this layer contains mostly nonpolar hydrocarbons. The synthesis of hydrocarbons takes place in the oenocytes (Lockey, 1988; Billeter *et al.*, 2009), from which an insect lipoprotein in the haemolymph transports them into the cuticle (Katase & Chino, 1982). There, hydrocarbons serve two important roles in an insect's life, the ancestral one is the avoidance of desiccation; the derived one, but not less important is their applicability as cues and signals in communication (Blomquist & Bagneres, 2010).

Cuticular hydrocarbons (CHC) can be simple aliphatic chains of carbon and hydrogen atoms (n-alkanes), or they may have a non-linear conformation due to the presence of one or more double bonds (*e.g.*, alkenes, alkadienes) and/or methyl groups (*e.g.*, methyl-branched compounds) attached to their chain. In addition, chain length varies. Hydrocarbons on the cuticle of insects start with 21 carbons since shorter chain compounds are too volatile. Molecules longer than 50 carbons are rarely found, possibly because of limitations in the detection thresholds of the analytical equipment used (Blomquist, 2010). Within the 30 carbons range, however, there is a countless number of possible hydrocarbon molecules, from which a subset varying between a few and sometimes more than 100 CHC may combine in the cuticle of one species (*e.g.*, Calderón-Fernández & Juárez, 2013). However, not only the number but also the relative abundance of each of these CHC molecules on the cuticle varies, enabling the possibility of having numerous and species-specific CHC profiles. In fact, CHC have been considered useful markers in chemotaxonomy (Kather & Martin, 2012) and have aided delimiting morphologically similar and cryptic species (*e.g.*, Collembola, Porco & Derharveng, 2009; orchid bees, Pokorny *et al.*, 2014).

The great diversity of CHC may have facilitated the appearance of their secondary role as signals in communication. CHC can carry a plethora of information about the sender (*e.g.*, sex, age, mating status, health condition, etc.) which can be intentionally transmitted to (signal) or inadvertently perceived by (cue) the receiver (Blomquist & Bagneres, 2010). In addition, if the sender belongs to a social species, CHC convey also information about caste and colony membership, kin relationship, fertility, etc., all of which can be important to facilitate division of labour in largely eusocial insect species (Leonhardt *et al.*, 2016).

The oldest, most important and widespread mode of communication of living organisms is chemical. Although visual or acoustic signaling may also be important for

specific cases of intraspecific communication in insects (*e.g.*, facial patterns in female *Polistes* social wasps indicating dominance status, Tibbets & Dale, 2004; or acoustic songs in grasshoppers, Simmons & Ritchie, 1996 and Römer, 2014), most of intraspecific communication, including mate recognition and choice process, is predominantly chemical in all arthropods (Greenfield, 2002). While a large number of volatile chemicals have been attributed a communicative role in arthropods (see for example Ando *et al.*, 2004; Millar, 2005; Keeling *et al.*, 2004; Francke & Dettner, 2005), CHC play an important role in short-range communication processes both at intra and interspecific level (Singer, 1998). Though largely unnoticed, they may constitute the most widely used language of the world (analogous to graphic ideograms of written languages), given that they are present in the vast majority of arthropods, whose species richness largely exceeds that of any other phylum (Zhang, 2013).

CHC composition is genetically determined (*e.g.*, Thomas & Simmons, 2008a). Although it has been shown to be stable across large geographical ranges (*e.g.*, Martin *et al.*, 2008b; Guillem *et al.*, 2016), it can nevertheless be plastic and subject to environmental influences such as climatic conditions (Wagner *et al.*, 2001; Rouault *et al.*, 2004), diet (Liang & Silverman, 2010; Fedina *et al.*, 2012), host species (Kühbandner *et al.*, 2012b). Moreover, changes in CHC composition do not only occur at early stages of life, but may also vary within a lifetime (*e.g.*, age, Kuo *et al.*, 2012; Vanickova *et al.*, 2012; mating status, Polerstock *et al.*, 2002; Everaerts *et al.*, 2010, breeding status, Steiger *et al.*, 2007, dominance status, Thomas & Simmons, 2011, see the recent review by Otte *et al.*, 2018). In all these cases, CHC composition varies mainly quantitatively (Menzel *et al.*, 2017a). Qualitative variation of CHC composition, may however occur within a species, when comparing both sexes (Menzel *et al.*, 2017a). Although a past review on the topic has shown that sexual dimorphism might be very common in insects (Thomas & Simmons, 2008b), the degree to which both sexes produce a qualitatively different CHC profile has not been consistently evaluated within a large group of species.

As shown above, despite being structurally simple, hydrocarbons constitute a relatively complex trait due to their multivariate nature: CHC profiles are complex mixtures of simple molecules, each of them possibly conveying different types of information. In addition, insects can perceive subtle differences in the quantitative composition of some compounds, so different messages can be encoded by the presence of, or variations in the abundance of one or many CHC compounds. On top of this, non-aliphatic CHC are associated with roles in recognition and communication, because their special features may facilitate perception and species-specific signaling (Dani *et al.*, 2001, Dani *et al.*, 2005) while n-alkanes are suggested to be involved in antidesiccation primarily (Gibbs, 1998, Dani *et al.*, 2001; Chung & Carroll, 2015). Because of their dual function, as signals in communication and as water-proofing entities, CHC are affected by natural and sexual selection (Chung & Carroll, 2015). For example, drier climatic conditions may select for CHC profiles with a preponderance of molecules of longer chain lengths and/or of linear alkanes, which are supposed to more effectively protect against desiccation in drier and hotter environments (Chung & Carroll, 2015, Menzel *et al.*, 2017a). Nevertheless, the degree to which these patterns may be observed, depends as well on the effects of selection acting on the communicative role. Understanding how the great diversity of CHC arises and how CHC profiles evolve remains still one of the exciting open questions to investigate.

1.1.1. Diversity and evolution of CHC

The importance of CHC in chemical communication has been recognized for more than 40 years, but it was in the last ten years that CHC have become more frequently studied traits and have helped us to understand different biological and evolutionary processes ranging from physiology to speciation. The growing ease of obtaining CHC data and the availability of powerful comparative analytical methods (see below) allow us to gain important insights about their evolution by studying many related species in a phylogenetic context. For example, the mode of evolution of several types of chemicals has been investigated several times. Two modes of evolution are possible. Signals may evolve gradually, via small changes, which may result in phylogenetic conservatism, by which closely related species share a similar composition (Symonds & Elgar, 2008). Or, they may evolve via saltational shifts, in which case, strong stabilizing selection acts against gradual changes in a signal (Symonds & Elgar, 2008). In theory, evolution via saltational shift is expected when signals need to be highly species specific (*e.g.*, those involved in species and mate recognition, Symonds & Elgar, 2008). Studies evaluating the evolutionary patterns of different chemicals have confirmed these theoretical expectations. For example, non-CHC pheromones of bark beetle species (Symonds & Elgar, 2004) and sex pheromones of flies (CHC included, Symonds *et al.*, 2009) evolve via saltational shifts. In contrast, the study by Symonds and Wertheim (1995) on aggregation pheromones of flies, discovered a gradual mode of evolution. Similarly, van Wilgenburg and colleagues (2011) found a gradual mode of evolution of CHC in ants and they explained this pattern as the result of CHC being used in caste and colony recognition. Nevertheless, the mode of evolution has not been yet tested considering differences between the sexes. In these cases, it would be interesting to explore whether the mode of evolution is the same for both sexes of the same species and if not, what selection pressures may be driving these differences.

Few studies have attempted to review patterns of CHC composition across many species. Three exceptions worth noting are those summarizing the diversity of CHC present in species of Hymenoptera (Martin & Drijfhout, 2009a; Kather & Martin, 2015, Menzel *et al.*, 2017a), which have provided interesting insights into the roles of CHC and what may be driving their evolution. In a comparison of published CHC profiles of 78 ant species, Martin and Drijfhout (2009a) found almost 1000 CHC compounds, the great majority of which were methyl-branched compounds (85%, most of them dimethyl-branched compounds). They found that ants possessed all main compound classes, even the so-far most complex methyl-branched alkenes (molecules combining both a double bond and a methyl group). Interestingly, they found that n-alkanes and monomethyl-branched compounds were relatively ubiquitous but dimethyl-branched compounds and unsaturated compounds were diverse and variable across species, possibly playing a role in species recognition processes. Moreover, they corroborated previous observations that compounds at odd-chain lengths were more frequent than those at even-chain lengths (Martin & Drijfhout, 2009a).

The study by Kather and Martin (2015) that compared CHC profiles of 241 species of eusocial and solitary Hymenoptera, went a bit further by trying to test if sociality could drive complexity of CHC. Complex CHC compounds are those showing a large number of disruptive features in the molecule (*e.g.*, trimethyl-branched are considered more complex than dimethyl-branched compounds, and the latter are, in turn, more complex than monomethyl-branched ones). It had been hypothesized that social in-

sects would require more complex CHC because individuals of a social species should be involved in a larger communicative complexity, by which they interact in many different contexts with different individuals (*e.g.*, a colony) in comparison to individuals of a solitary species (Freeberg *et al.*, 2012). However, the meta-analysis by Kather and Martin (2015) did not support this hypothesis. Surprisingly, CHC diversity in their analysis was instead larger in species of the Parasitica clade. Whereas the two previous studies used only presence/absence of CHC composition in their analyses, quantitative variation was included in a more recent study. Menzel and collaborators (2017a) compared a number of CHC profiles of 85 ants of two diverse ant genera from several biogeographic regions with the aim of understanding if there were phylogenetic constraints in the production of some classes of CHC and testing the influence of climatic factors and a parabiotic life style on CHC composition across species. Their results showed that chain length may indeed constrain CHC composition, that precipitation but not temperature shaped CHC composition and that there was a consistent difference in the CHC profile depending on the parabiotic lifestyle of the species used, emphasizing how different factors can affect the evolution of CHC profiles (Menzel *et al.*, 2017a).

1.2. Coevolution, cuckoo wasps and chemical mimicry

Arms races between natural enemies can result in the rapid evolution of extreme traits, high specialization and the origin of new species (Hanifin *et al.*, 2008). One of the first recognized and best studied examples includes that of cuckoos and its hosts (Davies, 2011). To exploit the parental care of their hosts, cuckoos have developed numerous adaptations, among which visual mimicry of their hosts' eggs, is well known (Brooke and Davies, 1988). In return, hosts' populations subject to high parasitism rates develop better discrimination abilities, which, in turn, results in better visual mimicry of the parasitic birds' eggs (Davies & Brooke, 1989a). A parallel similar model system in the chemical world is that offered by cuckoo wasps. These are kleptoparasitic and parasitoid solitary aculeate wasps (Hymenoptera: Chrysididae, see Chapter 2) that have been shown to employ some sort of chemical deception (*e.g.*, chemical mimicry, *de novo* synthesis of main CHC compounds of their hosts, Strohm *et al.*, 2008; chemical insignificance, a reduction in the absolute amount of CHC produced, Kroiss *et al.*, 2009a) in order to fool the recognition abilities of their hosts. As a result, chemical signatures of cuckoo waps may remain undetected when they oviposit in their host's nests, avoiding thus a potential removal of the foreign egg, or a complete abandonment of the nest, either of which may result in the death of the cuckoo wasp's developing larvae. On the contrary, if the cuckoo larvae remains undetected, it generally hatches before the host larvae, and either kills it immediately or after the host larvae has consumed the undigestable provisions, Ouayogode, 1979). It can then be expected that short-range recognition chemical signals (*e.g.*, CHC in this case), can rapidly evolve in such a system because of adaptations and counteradaptations. In fact, CHC profiles of female cuckoo wasps resemble those of their hosts in at least three species, and hosts seem to exert strong selection pressure on their parasites' CHC profiles (*e.g.*, Strohm *et al.*, 2008, Wurdack *et al.*, 2015). Whereas several examples of adaptations in CHC profiles driven by a coevolutionary arms race exist, most of them have been done using social parasites (*e.g.*, Brandt *et al.*, 2005, Lorenzi *et*

al., 2006, Kleeberg *et al.*, 2017). In these cases, social parasites usually agree with Emery's Rule (being phylogenetically closely related to their hosts, thus probably sharing most of the biosynthetic pathways to produce CHC), and/or they often employ chemical camouflage (the physical acquisition of their host's CHC profiles). Thus, these adaptations are probably easier to achieve since they generally involve primarily behavioural adaptations by which CHC may be acquired. The use of a system with *de novo* synthesis of CHC (such as that of cuckoo wasps), provides another interesting case to explore how CHC diversify and evolve. Specifically, since both sexes might be differentially affected by natural and sexual selection, the study of CHC profiles of cuckoo wasps allows exploration of how CHC are affected under different selection pressures.

1.3. Phylogenetic Comparative Methods (PCM)

The comparison of CHC profiles across several related species can provide insights for understanding the evolution of chemical signals when experimental manipulation of those species is impossible (*e.g.*, Menzel *et al.*, 2017a mentioned above).

The comparative approach is extremely powerful to explore the patterns of evolution and diversification of a group of (extant) species using two types of data: (1) a phylogenetic tree that shows how these species are related to each other and (2) the species contemporary trait values (Cornwell & Nakagawa, 2017). Since the first implementation of a PCM to correct for the statistical non-independence of the species data within a tree in 1985 (phylogenetic independent contrasts, Felsenstein, 1985b), the development of more sophisticated and diverse methods and the availability of robust phylogenetic trees have translated into an explosion of the applications and uses of PCM not only into the fields of evolutionary biology but also in anthropology, linguistics, and paleobiology (Pennel & Harmon, 2013; Cornwell & Nakagawa, 2017). Despite the many advantages of PCM, among them the possibility to explore evolutionary processes without the necessity to use experimental approaches, the employment of PCM requires that several assumptions are met to be able to interpret the results obtained by the method. PCM basically incorporate uncertainty via three sources: the methods employed to reconstruct the phylogenetic tree, the way the trait values are measured, and the model of evolution that is employed in the PCM (Cornwell & Nakagawa, 2017). Several reviews have summarized and emphasized how PCM can be rightly applied and how to overcome their limitations to make the best use of them (Martins & Hansen, 1996; Cunningham *et al.*, 1998; Losos, 1999; Freckleton, 2009; Boettiger *et al.*, 2012; Hansen, 2014; Cooper *et al.*, 2016b).

1.4. Aims of this thesis

The aim of this thesis was to study how patterns of CHC profiles across several related species diversify, to provide insights about how natural and sexual selection may affect the differential evolution of CHC profiles under a comparative analysis framework. Taking advantage of the biology of cuckoo wasps and the expected selection imposed by their hosts, I looked not only at species but also sex differences. Specifically, I attempted to:

a) shed light into the evolutionary history of the family Chrysididae by inferring a robust phylogeny with many representative species,

b) describe patterns of sexual dimorphism of CHC profiles in cuckoo wasps, and discover CHC compounds that could be sex-specific and potentially involved as putative contact sex pheromones,

c) provide insights into how sexual dimorphism of CHC profiles may have arisen,

d) evaluate the usefulness of CHC as complementary approach for CHC taxonomy, especially in cases of morphologically complex species and considering sexual dimorphism of CHC profiles,

e) explore CHC adaptations of hosts and parasites in an evolutionary arms race context.

1.5. Outline

Cuckoo wasps provide a good model to study the evolution of CHC profiles. Due to their parasitic behaviour, males' and females' CHC profiles are subject to differing strengths of natural and sexual selection, which has important implications on the mode of evolution of their CHC profiles. Chapter 2 starts with a brief introduction to the model. Cuckoo wasps are still relatively unknown. Therefore, a summary of the current knowledge with respect to their biology, ecology and classification of species is offered at the start of the thesis. This might enable a better understanding of the study subject used in all the remaining chapters. To conduct a comparison of CHC profiles in a large number of related species, a robust and reliable phylogenetic tree of these species is required. Chapter 3 refers to the most recent molecular phylogeny of cuckoo wasps, which has been done using more than 180 species, representative of the three most widespread subfamilies. This constitutes to date the most comprehensive and complete evolutionary reconstruction of the relationships of cuckoo wasps confirming and advancing previous findings (Niehuis & Wägele, 2004; Soon & Sarma, 2011) and rejecting hypothesis based on morphological characters especially on the species-rich tribe Chrysidini (*e.g.*, Kimsey & Bohart, 1991). CHC profiles of cuckoo wasps are sexually dimorphic. They do not only vary quantitatively in the proportion of shared compounds by both sexes, but in most of the species, the two sexes possess two very different CHC profiles, often with different compound classes prevailing in both sexes (*e.g.*, *Chrysis propinquata*, females possess mainly alkenes, males methyl-branched compounds). Chapter 4 describes and compares CHC profiles of males and females of 58 cuckoo wasp species and calculates indices of sexual dimorphism. This study constitutes the first comparison of sexual dimorphism in a large number of related species and reveals an interesting pattern of the prevalence of sex-specific alkenes with different double bond positions. This difference in alkene prevalence in males and females may provide hypotheses for the future testing of some of these substances as sex pheromones in these species. Chapter 5 compares CHC profiles among 59 species with the aim of illustrating the use of CHC as a tool in chemotaxonomy. CHC profiles of cuckoo wasps are not only sex-specific but also species-specific. Thus, CHC analysis can be complementary to molecular approaches to help differentiating closely related species, which are otherwise difficult to separate. Sexual dimorphism has been traditionally considered to originate as a result of sexual selection acting on males. In Chapter 6, I propose that in cuckoo wasps, natural selection acting on females has had

a preponderant role in causing sexual dimorphism. The mode of evolution of CHC profiles of cuckoo wasps strikingly differed between females and males, with females showing a faster pace of evolution and less phylogenetic signal, probably implying a much stronger selection on the CHC profiles. One plausible reason for this difference is that only female parasites may be under selection by a coevolutionary arms race with their female hosts, to achieve chemical deception. In fact, in Chapter 7, evidence for the evolution of adaptations and counteradaptations in CHC profiles that occur in a brood parasite-host system (*Hedychrum* cuckoo wasps and their Philanthinae digger wasp hosts) is presented. These adaptations are shown to occur in females, but not males of both hosts and cuckoo wasps, the sex that is directly involved in the chemical arms race. The last two chapters of the thesis are methodological. Chapter 8 is a brief description of the methodology used to analyze and align CHC chromatograms in this thesis, which slightly differs from commonly employed methods. In this thesis, I used the freely available software AMDIS to analyze CHC chromatograms. The result files were then curated in the widely used R programming language for posterior data exploration and statistical analysis. In chapter 9, I provide a small software tool, written in R, to help in the identification of methyl-branched compounds by providing the diagnostic ions and a calculated retention index. This simple tool, is expected to be especially useful in cases in which two or more methyl-groups are present. In the end, a discussion integrating all chapters is presented.

2. The family Chrysididae and their hosts: The study group

Cuckoo wasps is the general common English name for a group of parasitoid and kleptoparasitic wasps in the family Chrysididae. The scientific name of the family derives from the greek word "chrysos" (gold), from which the common name of jewel wasps or "Goldwespen" (used in languages such as German or Swedish) also originates (Paukunnen *et al.*, 2014). The golden adjective refers to the wasps' usual metallic reflective coloration in different tones (red, blue, green, copper, arranged in beautiful patterns, Figure 2.1), that shines like a drop of gold when the wasp is moving fast under bright sunlight (Kimsey & Bohart, 1991). Their beautiful appearance has rendered them one of the most beloved groups among collectors of wasps. Nevertheless, Chrysididae wasps still remain understudied with many taxonomic problems to solve and the biology of many species unknown (Paukunnen *et al.*, 2014; Paukunnen *et al.*, 2015).

2.1. Diversity and distribution

The family has a worldwide distribution and estimates of the number of described species in Chrysididae vary between 2500 (Aguiar *et al.*, 2013, who conducted a recent revision of the order Hymenoptera) and approximately 3000 species (Kimsey and Bohart, 1991, who did the last major thorough revision of the family). However, as with many other taxa, tropical regions are highly diverse and remain insufficiently studied (Kimsey & Bohart, 1991). In this sense, new species (and genera) are being described every year, especially from the southern Hemisphere and Central Asia (*e.g.*, Bohart, 1985a; 1985b; Kimsey, 1987; 1988; 1993; 1995; 1998; 2005; 2008; 2013; Rosa & Lotfalizadeth, 2013; Wei *et al.*, 2014; Rosa *et al.*, 2016a; 2016b).

The history of chrysidid research started with Linnaeus at the beginning of the 18th century who described three species (Linnaeus, 1758). A historical overview of chrysidid research in the world has been presented by Kimsey and Bohart (1991), and more recently for chrysidid research in northern Europe (Paukkunnen *et al.*, 2014) or when revising the large collections of famous former taxonomists (*e.g.*, Maximilian Spinola, Rosa & Xu, 2015; Walter Linsenmaier, Rosa *et al.*, 2015b; Anders Dahlbom, Rosa & Vardal, 2015), small historical accounts regarding the importance of their work have been provided.

2.2. Morphology

A detailed description of the morphological distinctive characteristics of Chrysididae is provided in the revision of the family by Kimsey and Bohart (Kimsey & Bohart, 1991). Here, the major morphological features that distinguish Chrysididae from

Figure 2.1.: Two examples of the beautiful coloration patterns of cuckoo wasps. a) Female of Chrysis longula; b) Male of Chrysis equestris. Photos by Oliver Niehuis.

other wasps are summarized. The family Chrysididae belongs to the Aculeata group within the Apocrita suborder, in which females possess a modified ovipositor that is also a sting. However, chrysidids have a highly reduced sting, which has no defense purposes, but that rather helps the female to guide her during the oviposition (Kimsey & Bohart, 1991). In replacement of the sting, two or more abdominal segments have been internalized and function as an ovipositor (Kimsey, 1992). Chrysidids tend to be small in size ranging between 1–12 mm (Bohart & Kimsey, 1980). Compared to other groups of wasps, chrysidids have reduced the number of visible abdominal segments to a maximum of five. The number of segments is also used to differentiate the different subfamilies and tribes of Chrysididae, and can also help to distinguish females from males in some of the tribes, because females tend to have one segment less than males. However, this is not the case in the most species-rich tribes of the family (Elampini and Chrysidini), in which females and males show the same number of segments. The antennae of all chrysidids have a pedicel, a scape and 11 flagellomeres. The wing venation is also extremely reduced with the fore wing possessing at most five closed cells and the hind wing lacking closed cells (Kimsey & Bohart, 1991). Chrysidids of one of the most species-rich groups (Chrysidinae) also possess sculptures and punctuations on the thorax that accentuate their brilliant metallic coloration. Although many species of the family have diverse metallic coloration, this is not a characteristic of the family, since several species (of at least two subfamilies) are completely black with no reflective coloration.

Their morphology presents strong correlations with the characteristics of the host that they parasitize. Species that parasitize relative harmless hosts show less sclerotized abdominal segments, whereas those parasitizing more dangerous hosts (subfamily Chrysidinae) have the ability to roll up into a ball, protecting their soft and vulnerable body appendages when they are threatened (Kimsey & Bohart, 1991, see figure 2.2). Additionally, the degree of modification and internalization of the abdominal segments into an ovipositor also correlates with the type of host they parasitize (Kimsey, 1992).

2.3. Classification and systematics

Based on analyses of a number of morphological characters, Kimsey and Bohart (1991) proposed a classification of the family that is used until now. The family is subdivided into four subfamilies: Cleptinae, Amiseginae, Loboscelidiinae, and Chrysidinae, the last of which is by far the most species-rich and further subdivided into five tribes (Allocoelini, Elampini, Kimseyini, Parnopini and Chrysidini) (Kimsey & Bohart, 1991; Antropov, 1995). Of all these, only species of the subfamilies Cleptinae and Chrysidinae (excluding Allocoelini and Kimseyini) occur in Europe accounting for about 500 species (Paukunnen *et al.*, 2014).

The evolutionary relationships among and within the different subfamilies and tribes, are still not well resolved, and the first phylogenetic tree based on molecular data of 33 species belonging to Cleptinae and Chrysidinae (Niehuis & Wägele, 2004) showed that the relationships for major lineages and especially for taxa of Cleptinae was supported, but found discrepancies with respect to the position of species of the *Euchroeus* group that apparently occupy a more basal position than previously suggested (Niehuis & Wägele, 2004). Soon and colleagues (Soon & Sarma, 2011; Soon

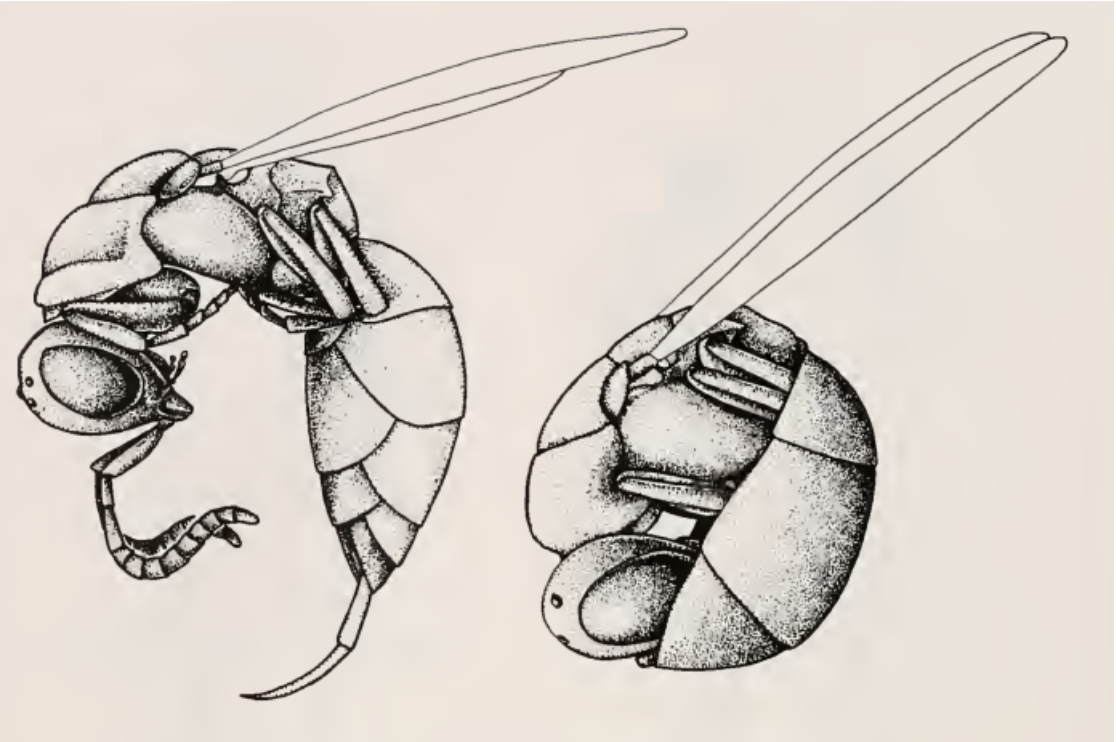

Figure 2.2.: Rolling up as protection from host attacks. Left in Cleptinae, right in Chrysidinae. From Kimsey, 1992 (Creative Commons License).

et al., 2014), have more recently inferred detailed molecular phylogenies for a large group of morphologically homogeneous species (*Chrysis ignita* species group), that are difficult to discriminate even for the experienced eye (*e.g.*, Kunz, 1994). Their results show that the group is monophyletic, that relationships proposed by the molecular data are only partly congruent with those based on morphology, and that many species may be cryptic (Soon & Sarma, 2011). Likewise, the use of molecular data may assist and complement morphological analysis in species delimitation (Soon *et al.*, 2014).

However, so far the largest and most reliable molecular phylogeny for the family is that inferred by Pauli and colleagues (Pauli *et al.*, accepted), which has been conducted on almost 190 species representing all but one of the main subfamilies (see Chapter 3). In this thesis, I have used this latest phylogeny and reduced it to contain the species used in the chemical analyses (~59 species).

2.4. Biology and behavior

Relatively little is known about the life history and the biology of chrysidids but most of this knowledge is related to their parasitic behavior and the interactions with their hosts. As mentioned above cuckoo wasps are all either parasitoids or kleptoparasites of other insects, and the host type is a defining characteristic of the subfamily. Amiseginae and Loboscelidiinae attack eggs of walking stick insects (Phasmatodea). Cleptinae have specialized on pupa of sawflies (Tenthrenidae, Diprionidae) while all tribes of Chrysidinae use the larvae of aculeate wasps and bees as hosts. However, one exception exists in Chrysidinae, with all species of one genus (*Praestochrysis*) hav-

ing specialized to parasitize prepupal caterpillars of moths of the family Limacodidae (Kimsey & Bohart, 1991).

The development of the cuckoo wasp larvae into an adult (see figure 2.3) almost always results in the death of the host larvae. Only occasionally, when enough provisions are laid by the host female, host and cuckoo wasp may emerge from a nest (*e.g.*, Martynova *et al.*, 2017). The distinction between parasitoids and kleptoparasites relies on the fact that the latter steal and consume the provisions for the host larvae as well, with both types generally directly or indirectly killing the host larvae. However, other small biological adaptations in relation to their specialized parasitic behavior may be observed. In the kleptoparasitic type, the cuckoo wasp egg is usually placed in close distance to the host larvae (or close to the provisions), the developing cuckoo wasp larva emerges first and proceeds immediately to eat the host larvae and their insect provision (Ouayogode, 1979, Krombein, 1967, Malyshev, 1968). In the parasitoid type, the egg of the cuckoo wasp is usually placed as far as possible from the host and the provisions (Ouayogode, 1979), and the developing cuckoo wasp larva emerges but remains inactive. In this way, the host is allowed to grow and consume most of the provisions (mainly pollen) which cannot be directly digested by the cuckoo larvae (Krombein, 1967). The parasitoid larva will just start consuming the host when this may have already developed its cocoon (this happens usually in species parasitizing bees, Kimsey and Bohart, 1991, Ouayogode, 1979). Adapting to the host or host types includes changes in morphology (*e.g.*, Tormos *et al.*, 2001), physiology, behaviour (*e.g.*, Rosenheim, 1987b), and probably also the production of chemical compounds (*e.g.*, Strohm *et al.*, 2008) that should be reflected in the evolutionary history of the family. For example, the most primitive groups of cuckoo wasps are parasitizing relatively "simple" and harmless hosts (sawflies, stick insects) whereas the different tribes of the most diverse Chrysidinae parasitize mainly wasps and bees and have evolved different adaptations to either deceive or protect themselves against possible attacks (Kimsey and Bohart, 1991).

The parasitic behavior of exploiting the parental care of their hosts is the origin of their main common name (cuckoo wasps), implying thus certain similarity with cuckoo birds. In fact, as cuckoo birds are able to visually mimic the eggs' color, size and even markings of their different hosts to avoid detection and rejection of their own eggs by their hosts (Davies and Brooke, 1989a, Davies and Brooke, 1989b), cuckoo wasps may employ some type of chemical deception to avoid detection of their eggs and their chemical cues inside the nests.

Few studies have been conducted on a handful of relative common species. Strohm and colleagues (Strohm *et al.*, 2008) demonstrated that females of the cuckoo wasp *Hedychrum rutilans* produce a similar CHC profile composition as that of their female hosts (European beewolf, *Philantus triangulum*). This chemical mimicry allows them to reduce recognition of their chemical cues when entering their host nests to oviposit (Strohm *et al.*, 2008). However, *H. rutilans* females not only produce a similar CHC composition but they also employ a strategy called "chemical insignificance", namely, they reduce the absolute amount of CHC on their cuticle. Kroiss and colleagues (Kroiss *et al.*, 2009a) have shown that *H. rutilans* produces comparatively only one fifth of the amount of CHC (corrected for size) that its beewolf host produces. Moreover, the chemical cues of *H. rutilans* may in this way be diluted within a nest that mostly smells to the odor of the beewolf (Kroiss *et al.*, 2008; 2009a). Although less frequently observed, *H. rutilans* may also avoid entering the host by

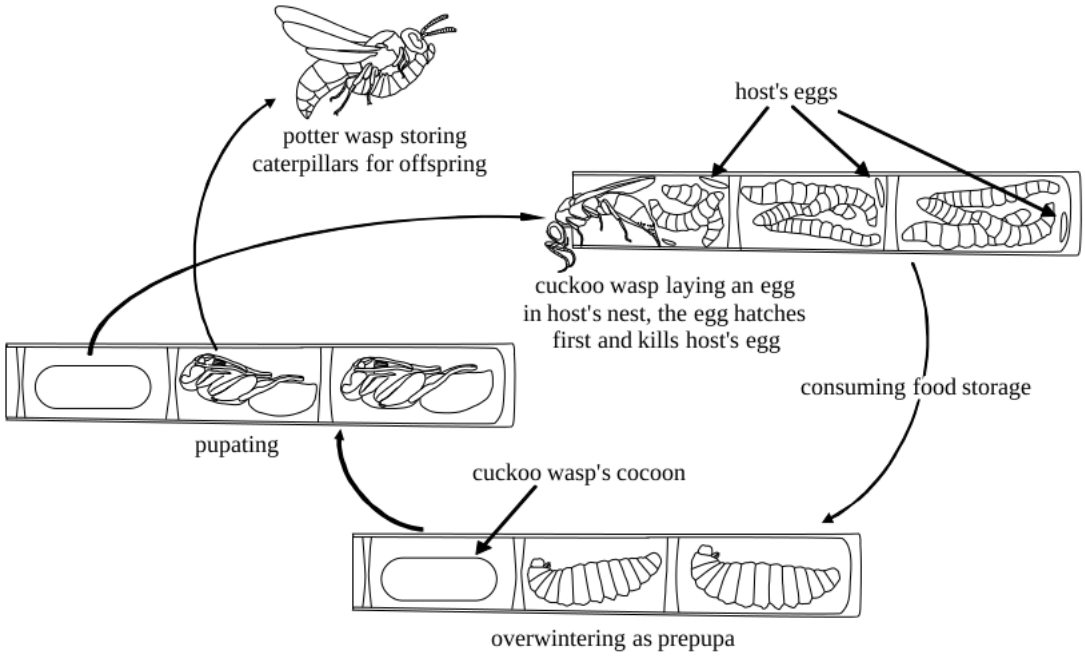

Figure 2.3.: Life cycle from a typical species of the Chrysis ignita group that often parasitize Eumeninae. From Soon, 2014 (with permission).

quickly ovipositing on the prey of the beewolf, while the last one is being brought to the nest (Simon-Thomas & Simon-Thomas, 1972, Veenendaal, 1987; Strohm *et al.*, 2008). Parasites that lay their eggs on the potential preys of their hosts are called 'Trojan horse' parasites (Strohm & Liebig, 2008), and this strategy may be common in cuckoo wasps of the Elampini tribe (*e.g.*, *Holopyga generosa* Veenendaal, 2012*, Pseudomalus auratus,* Paukkunen *et al.*, 2015*, Pseudolopyga carrilloi,* Carrillo & Caltagirone, 1970*, Omalus biaccinctus*, Winterhagen, 2015). By doing this, these cuckoo wasps avoid leaving their chemical traces in the nests and aggression from the hosts. As it has been shown, cuckoo wasps may employ more than one strategy to deceive their hosts, because the survival probability of their own offspring depends on their ability to remain undetected.

Hosts are detected visually and also chemically. Bioassays have shown that females of *H. rutilans* are able to discriminate between nest material of their host and of other non-host species, and that cuckoo wasps are attracted to the nest odour of their hosts (Kroiss *et al.*, 2008). Moreover, cuckoo wasps spend a lot of time in searching for adequate hosts and may explore a relative large area. Observations of released marked cuckoo wasps (*H. rutilans*) show that they are able to travel at least 100 m (Simon-Thomas & Simon-Thomas, 1972).

Working on another species of cuckoo wasp that is common in North America, Rosenheim (1987a) has shown that *Argochrysis armilla*, a kleptoparasite of *Ammophila dysmica* is able to distinguish and learn the spatial position of their host's nests during the excavation period, to oviposit some days later. Rosenheim (1987a) did not test for the use of chemical cues, but in his experiments the cuckoo wasps could not choose the right nest after certain landmarks had been misplaced, what led him to conclude that the use of visual cues and learning needed to play an important

role in the strategies used by this wasp. Unfortunately, no similar studies have been conducted on any other cuckoo wasp species.

As many other Hymenoptera, the activity period of a cuckoo wasp is restricted to the warm season. Cuckoo wasps emerge during spring/summer, reproduce, search for hosts, oviposit, and die. Their larvae molt and develop during winter and the cycle starts again. Few people have succeeded in rearing cuckoo wasps, but indirect evidence suggests that males emerge first (*e.g.*, Krombein, 1958) and live a much shorter life than females. For example, Kimsey and Bohart (1991) and Oliver Niehuis (ON, *pers. comm.*), suggested males may live for a couple of weeks, while females may be able to survive at least 3-4 months. Observations in the field have shown that males wait for females to emerge and copulate, whereas they are rarely spotted flying late in the season (ON, *pers. comm.*).

2.5. Ecology and their hosts

Cuckoo wasps are thermophilous, preferring warm and less humid habitats (Szczcepko *et al.*, 2013). Therefore, they are usually found in bright sunlight in open habitats, the surroundings of which may vary according to the host species, the cuckoo wasp is specialized in. For example, species parasitizing hosts nesting on the ground, are most commonly encountered in sandy open meadows, whereas species that parasitize hosts that nest in plant stems (above ground) are to be found close to woodlands and dry stems. They are mainly active under sunlight, and if not at nesting places looking for hosts, they may also be found around short vegetation looking for some nectar. Adult chrysidids may feed on some nectar (some species eventually on aphids honeydew), but their larvae are entirely carnivorous (O' Neill, 2001). Although the host-parasite relationships are not known for many of the species, recent studies derived from rearing cuckoo wasps and their hosts from trap-nests are showing that the host specialization of cuckoo wasps is generally high (Martynova *et al.*, 2015, Pärn *et al.*, 2015, Orlovskyté *et al.*, 2016). Most species have one, or maximum two hosts (usually of the same genus), although some others may have more than five species that they parasitize (*e.g.*, *Trichrysis cyanea*, Pärn *et al.*, 2015). As obligate parasites, the strict association and specialization of cuckoo wasps and their hosts is to be expected. Also, their presence, abundance and diversity indicates that their hosts are also abundant, and they may be used as indicators of biodiversity (González *et al.*, 2009; Szczcepko *et al.*, 2013). In general, the species diversity and abundance of chrysidids is higher in areas with high environmental and habitat heterogeneity (Szczcepkto *et al.*, 2013; Corcos *et al.*, 2017). This has important consequences for area and landscape conservation. A large number of cuckoo wasp species are under certain category of threat (Schmid-Egger, 2010) due mainly to habitat fragmentation and degradation. A recent study based on data from survey collections in Finland has shown that almost a fourth of the 48 species analyzed has declined between the two periods studied (a range span of 150 years, Paukkunen *et al.*, 2017). The decline of species that nest above ground has been stronger than those of more abundant cuckoo wasps that nest on open sand, probably because the latter were able to adapt to secondary habitats resulting from urban development more than species depending on woodlands (Paukkunnen *et al.*, 2017).

2.6. Cuckoo wasps as model organisms to study the evolution of CHC

Being specialized parasites of a number of solitary hosts, cuckoo wasps offer an interesting study system to explore how CHC profiles evolve and diversify. Both sexes inhabit similar habitats and hence abiotic factors such as temperature are not expected to affect CHC profiles differentially. However, both sexes are expected to utilize different CHC compounds with different communication purposes. CHC compounds of males may serve for species and sex recognition. CHC compounds of females however, may additionally be evolving under selection to deceive their hosts. The difference in the functions CHC play in each of the sexes, make the study and comparison of CHC profiles across the family Chrysididae very appealing.

In the present thesis, 59 species of cuckoo wasps and seven of their hosts have been used across all studies shown. The origin of the samples used, the respective host species, and few traits have been compiled for each of these cuckoo wasps and are presented in the Appendix.

3. Phylogeny and host associations of cuckoo wasps

3.1. Abstract

Cuckoo wasps (Hymenoptera: Chrysididae) are a species-rich family of obligate brood parasites (*i.e.*, parasitoids and kleptoparasites) whose hosts range from sawflies, wasps, and bees to walking sticks and moths. Their brood parasitic lifestyle has led to the evolution of fascinating adaptations, including chemical mimicry of host odours by some species. Long-term nomenclatural stability of the higher taxonomic units (*e.g.*, genera, tribes, and subfamilies) in this family and a thorough understanding of the family's evolutionary history critically depend on a robust phylogeny of cuckoo wasps. Here we present the results from phylogenetically analysing ten nuclear-encoded genes and one mitochondrial gene, all protein-coding, in a total of 186 different species of cuckoo wasps representing most major cuckoo wasp lineages. The compiled data matrix comprised 4,946 coding nucleotide sites and was phylogenetically analysed using classical maximum likelihood and Bayesian inference methods. The results of our phylogenetic analyses are mostly consistent with earlier ideas on the phylogenetic relationships of the cuckoo wasps' subfamilies and tribes but cast doubts on the hitherto hypothesized phylogenetic position of the subfamily Amiseginae. However, the molecular data are not fully conclusive in this respect due to low branch support values at deep nodes. In contrast, our phylogenetic estimates clearly indicate that the current systematics of cuckoo wasps at the genus level is artificial. Several of the currently recognized genera are para- or polyphyletic (*e.g.*, *Cephaloparnops, Chrysis, Chrysura, Euchroeus, Hedychridium, Praestochrysis, Pseudochrysis, Spinolia*). At the same time, our data support the validity of the genus *Colpopyga*, previously synonymized with *Hedychridium*. We discuss possible solutions for how to deal with the current shortcomings in the systematics of cuckoo wasp genera and decided to grant *Prospinolia* the status of a valid genus (*Prospinolia* **nov. stat.**) and transferring *Spinolia theresae* (du Buysson 1900) from *Spinolia* to *Prospinolia* (*Prospinolia theresae* **stat. restit.**). We discuss implications that the phylogenetic inferences have for understanding the evolution of host associations in this group. The results of our study not only shed new light on the evolutionary history of cuckoo wasps, but also set the basis for future phylogenomic investigations on this captivating group of wasps by guiding taxonomic sampling efforts and the design of probes for target DNA enrichment approaches.

3.2. Introduction

Chrysididae are a species-rich family of the superfamily Chrysidoidea. The approximately 3,000 species in the family are exclusively parasitoids or kleptoparasites,

namely of Hymenoptera (sawflies, wasps, and bees) and Phasmatodea (stick insects), but also, to a lesser extent, of Lepidoptera (moths) (Kimsey & Bohart, 1991). Due to their egg or brood parasitic lifestyle and bright metallic coloration, Chrysididae are commonly referred to as "cuckoo wasps" or "jewel wasps" (Paukkunen *et al.*, 2015). The family has a worldwide distribution (with the notable exception of Antarctica and New Zealand, where cuckoo wasps are not native; Tillyard, 1926; Kimsey & Bohart, 1991) and they are particularly species-rich in dry subtropical areas, such as the circum-Mediterranean region (*e.g.*, Linsenmaier, 1969).

Besides their attractive coloration, which has captivated entomophiles and entomologists alike, cuckoo wasps have also received scientific attention because of their morphological adaptations to the kleptoparasitic and parasitoid lifestyles (*e.g.*, the spikes at the distal end of the last gastral tergum, so-called anal teeth, which facilitate drilling an oviposition hole, Yamada, 1991; the wasp's ability to roll up their body into a ball to protect themselves from host attacks, Kimsey, 1992), their kleptoparasitic and parasitoid behaviour itself (Rosenheim, 1989Rosenheim (1989); Polidori *et al.*, 2010; Strohm *et al.*, 2001), and the intriguing chemical adaptations of some cuckoo wasp species (*e.g.*, chemical mimicry, Strohm *et al.*, 2008; Wurdack *et al.*, 2015). In fact, one of the most conspicuous morphological autapomorphies of cuckoo wasps, the internalization of terminal abdominal segments to form an extendible oviposition tube (Kimsey, 1992), is directly related to the kleptoparasitic and parasitoid lifestyles of the species in this family.

The family Chrysididae is currently classified into four subfamilies: Amiseginae, Chrysidinae, Cleptinae, and Loboscelidiinae. Of these, Chrysidinae is the most species-rich one and has been further subdivided into five tribes: Allocoeliini, Chrysidini, Elampini, Kimseyini, and Parnopini (Kimsey & Bohart, 1991; Antropov, 1995).

Most investigations that explored the phylogenetic relationships of the major lineages of Chrysididae cladistically analysed morphological characters of adults and larvae and/or host information (*e.g.*, Bohart & Kimsey, 1982; Kimsey & Bohart, 1991; Kimsey, 1992Kimsey (1992); Carpenter, 1999; Tormos *et al.*, 2001, 2009; Lucena & Melo, 2018). In the most frequently referred to of these studies, that by Kimsey & Bohart (1991), Cleptinae are considered to be the sister group of all remaining Chrysididae (*i.e.*, Amiseginae, Chrysidinae, and Loboscelidiinae), and Chrysidinae are hypothesized to be the sister group of Amiseginae plus Loboscelidiinae. Within the subfamily Chrysidinae, the tribe Elampini is currently considered to be the sister group of all remaining Chrysidinae, and Allocoeliini is thought to be the sister group of Chrysidini plus Parnopini. The phylogenetic position of the tribe Kimseyini (Antropov, 1995; Rosa *et al.*, 2015a) has so far remained unclear, and morphological similarity with Elampini was discussed (Carpenter, 1999).

The first phylogenetic investigation on cuckoo wasps based on the analysis of DNA sequence data (those of the mitochondrial genes LSU 16S rRNA and COI) was published by Niehuis & Wägele (2004) and considered representatives of 33 cuckoo wasp species, belonging to the subfamilies Cleptinae or Chrysidinae (representing the tribes Chrysidini, Elampini, and Parnopini). The results not only challenged previous ideas on the evolution of cuckoo wasps' anal teeth, but also questioned previously hypothesized phylogenetic relationships of cuckoo wasp genera in the tribe Chrysidini and the monophyly of the species-rich genus *Chrysis* Linnaeus 1761. However, the relatively low number of species included in this study and the small number of phylogenetically informative DNA sites did not permit solid conclusions about the phylogenetic rela-

tionships and the monophyly of major cuckoo wasp lineages (*i.e.*, subfamilies, tribes, and genera).

Recent molecular phylogenetic studies on cuckoo wasps have focused on the *Chrysis ignita* (Linnaeus 1758) species group within the tribe Chrysidini (Soon & Sarma, 2011; Soon *et al.*, 2014; Orlovskytė *et al.*, 2016). These studies helped to understand how species in this large species complex are related to each other and how they can be delimited and identified even in the absence of species-specific external morphological characters. However, a comprehensive molecular phylogenetic study on cuckoo wasps that includes a taxonomically wide array of species and considers a large number of preferentially nuclear encoded markers has been missing.

A thorough analysis of the phylogenetic relationships of the subfamilies, tribes, and genera of cuckoo wasps has become more and more important, as such information is essential for reaching long-term taxonomic stability and for tracing the evolution of traits of interest. For example, recent faunistic surveys and taxonomic studies on cuckoo wasps (*e.g.*, Rosa *et al.*, 2013; Rosa *et al.*, 2014; Paukkunen *et al.*, 2015) mostly relied on the genus-level systematics proposed by Kimsey & Bohart (1991), yet the study by Niehuis & Wägele (2004) indicated that the most species-rich genus, *Chrysis*, likely constitutes a polyphyletic assemblage. Furthermore, improved understanding of the phylogenetic relationships of cuckoo wasps could provide clues on which taxa may serve as hosts of species whose hosts have not been recorded yet. This is because closely related species are expected to attack closely related hosts (*e.g.*, species of the same family). Finally, a detailed assessment of the cuckoo wasps' phylogeny is paramount for any comparative analysis on cuckoo wasps (*e.g.*, for understanding the co-evolution between cuckoo wasps and their hosts).

We aim to shed new light on the phylogeny of cuckoo wasps. Our study covers most of the major cuckoo wasp lineages. However, our taxonomic sampling is focused on species occurring in the Western Palaearctic. The phylogenetic relationships between species of this region are of particular interest to us, since cuckoo wasps in this region are the subject of ongoing studies focusing on the co-evolution of cuticular hydrocarbons in cuckoo wasps and their hosts (Wurdack *et al.*, 2015). Nonetheless, many of the genera included in our study (*e.g.*, *Chrysis, Chrysidea* Bischoff 1913, *Elampus* Spinola 1806, *Hedychridium* Abeille de Perrin 1878, *Hedychrum* Latreille 1802, *Praestochrysis* Linsenmaier 1959, *Parnopes* Latreille 1796, *Pseudochrysis* Semenov 1891, *Trichrysis* Lichtenstein 1876) have a much wider geographical distribution, and our study also covers a considerable number of species from genera endemic to other biogeographical areas (*e.g.*, *Allocoelia* Mocsáry 1889, *Argochrysis* Kimsey & Bohart 1981, *Caenochrysis* Kimsey & Bohart 1981, *Ceratochrysis* Cooper 1952, *Chrysurissa* Bohart 1980, *Exallopyga* French 1985, *Exochrysis* Bohart 1966, Gaullea du Buysson 1910, *Ipsiura* Linsenmaier 1959). The present study is based on the targeted sequencing of ten nuclear-encoded genes and of one mitochondrial gene, all protein-coding, in 186 species of cuckoo wasps and two selected outgroup taxa. The results of our study challenge previous ideas on the phylogenetic relationships of genera, in particular within the tribes Chrysidini, Elampini, and Parnopini, with far-reaching consequences for the genus-level classification of cuckoo wasps and our understanding of the evolution of host group associations.

3.3. Material and Methods

3.3.1. Taxon sampling

We studied a total of 186 cuckoo wasp species (Hymenoptera: Chrysididae). Whenever applicable, we followed the systematic classification suggested by Kimsey & Bohart (1991) when referring to taxonomic units above the species level. We aimed at covering all major cuckoo wasp lineages. The sampled specimens represent the lineages Amiseginae (two species), Chrysidinae: Allocoeliini (two species), Chrysidinae: Chrysidini (127 species), Chrysidinae: Elampini (45 species), Chrysidinae: Parnopini (four species), and Cleptinae (six species). Our taxon sampling does not include members of the subfamily Loboscelidiinae and of the tribe Kimseyini (Antropov, 1995).

We added two outgroup species to our taxonomic sampling: *Cephalonomia tarsalis* (Ashmead 1893 (Bethylidae) and *Anteon* Jurine 1807 sp. (Dryinidae). Until recently, Bethylidae, Chrysididae, and Dryinidae plus some additional families had been united in the superfamily Chrysidoidea (Carpenter, 1999). However, a recent phylogenomic analysis revealed that Dryinidae (as well as Embolemidae and Sclerogibbidae) are possibly closely related to other families of Aculeata than to Bethylidae and Chrysididae (Branstetter *et al.*, 2017), and these families should consequently no longer be included in the superfamily Chrysidoidea. Two recent phylogenomic investigations indicated that contrary to what was previously thought (Carpenter, 1999), Bethylidae plus Plumariidae (and thus not Bethylidae alone) likely represent the sister group of Chrysididae (Branstetter *et al.*, 2017; Peters *et al.*, 2017).

Our complete taxonomic sampling, with specimen information, is listed in Table S1 (Appendix). Voucher specimens are deposited in the Biobank at the Zoological Research Museum Alexander Koenig, Bonn, Germany (voucher IDs are listed in Table S1, Appendix).

3.3.2. DNA extraction for sequencing

We extracted DNA from thorax and leg muscle tissue by using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Nucleotide sequence sections of ten nuclear target genes were PCR-amplified with oligonucleotide primers from the Hymenoptera primer tool box published by Hartig *et al.* (2012) and specified in Table S2 (Appendix). We additionally studied the mitochondrial gene Cytochrome c oxidase subunit I (COI), of which we PCR-amplified a major fragment by using the oligonucleotide primer pair LCO1490/Nancy (Folmer *et al.*, 1994; Simon *et al.*, 2006) or the oligonucleotide primer pair LCO1490-JJ/HCO2198-JJ (Astrin & Stüben, 2008) (Table S2, Appendix). All PCRs were performed in 20-µl reaction volumes and using a Multiplex PCR Kit (Qiagen, Hilden, Germany). We applied the touch-down PCR temperature profile given by Hartig *et al.* (2012) but used 25 instead of 20 cycles during the PCR amplification phase with a constant annealing temperature. We furthermore specified an annealing temperature of 50 °C instead of 65 °C during this phase.

We estimated the purity and size of all PCR products by size-separating the PCR products next to a molecular size marker (GeneRuler 100 bp Plus DNA Ladder; Fermentas, St. Leon-Rot, Germany) in 1.5% agarose gels. We used GelRed (Biotium, Cologne, Germany) in order to fluorescent-label the DNA and purified the PCR products with the Illustra ExoProStar Kit (GE Healthcare Life Sciences, Garching, Ger-

many). All cleaned PCR products were sent to Macrogen (Amsterdam, The Netherlands) for direct and bidirectional Sanger sequencing using the sequencing oligonucleotide primers HOG-Seq-A-F, HOG-Seq-A-R, HOG-Seq-B-F, and HOG-Seq-B-R (nuclear genes; Hartig *et al.*, 2012; Table S2, Appendix) or the PCR oligonucleotide primers LCO1490 and Nancy or LCO1490-JJ and HCO2198-JJ, (COI) to initiate the cycle sequencing reactions.

3.3.3. Assembly of DNA sequences

We visually inspected forward and reverse DNA strands and assembled them to contigs with the software Geneious (version 7.1.9; Kearse *et al.*, 2012). To facilitate the annotation of intronic and coding exonic nucleotide sequence sections (Fig. S1, Appendix), we added to our dataset transcripts of the ten analysed nuclear genes from an unpublished whole-body transcriptome of the cuckoo wasp *Chrysis terminata* Dahlbom 1854 (= *Chrysis ignita* form A *sensu* Linsenmaier, 1959; Soon et al., 2014) sequenced in the context of the 1KITE project (www.1kite.org). All orthologous nucleotide sequences were aligned with the L-INS-i algorithm of MAFFT (version 7.123; Katoh & Standley, 2013). We then annotated introns and coding exons by searching for canonical splice sites (*i.e.*, the dinucleotide sequence GpT-ApG) at the ends of the intronic nucleotide sequence sections. Since the intronic nucleotide sequences were difficult to align across all species, we removed them. Additionally, we trimmed the ends of the aligned coding nucleotide sequences, so that all nucleotide sequence alignments started with the first and ended with the third position of a codon. Finally, we concatenated all aligned coding exonic nucleotide sequence sections to a supermatrix (Files S1–S3, Appendix). All inferred nucleotide sequences have been deposited at Genbank (accession numbers KY430694–KY432298, see Table S3, Appendix).

3.3.4. Phylogenetic Analysis

We used five *ad hoc*-defined data partitions based on DNA-type and codon position (*i.e.*, first and second codon position of the mitochondrial gene COI; and first, second, third codon position of the ten nuclear genes and selected a nucleotide substitution model for each. We refrained from including the 3rd codon position of the mitochondrial gene COI in our analysis due to its high compositional heterogeneity across species (Niehuis & Wägele, 2004). We also desisted from analysing the supermatrix on the translational level due to the low level of amino acid sequence variation across species: the pairwise distance of amino acid sequences ranged from 0 to 10%, with a median of 1% across 1,724 amino acid positions. We used Modelfinder (Kalyaanamoorthy *et al.*, 2017), which is integrated in IQ-TREE (version 1.6.5; Nguyen *et al.*, 2015, Chernomor *et al.*, 2016), , to select the best partioning scheme and the best fitting nucleotide substitution model for each data partition (Sullivan & Joyce, 2005). We tested all nucleotide substitution models available in IQ-TREE and parameters for among-site rate variation (*i.e.*, E, G, I, R). We allowed Modelfinder to merge partitions (TESTMERGE) and used the corrected Akaike information criterion (AICc; Hurvich & Tsai, 1989) to choose between models.

We used IQ-TREE (version 1.6.5, Nguyen *et al.*, 2015, Chernomor *et al.*, 2016) to infer the phylogenetic relationships of cuckoo wasps under the maximum likelihood optimality criterion. We selected the best scoring tree (*i.e.*, the tree with the lowest

negative log-likelihood score) from a sample of 20 separate tree searches, each using a starting tree obtained by applying the maximum parsimony principle. Branch support was estimated with the bootstrap method (Efron, 1979; Felsenstein, 1985) from 1,000 non-parametric bootstrap replicates. We additionally assessed whether uncertainty in the phylogenetic placement of a given species lowered support of multiple nodes simultaneously. For this purpose, we conducted a rogue taxon analysis (Wilkinson, 1995; Sanderson & Shaffer, 2002) using RogueNaRok (version 1.0; Aberer *et al.*, 2013) on the best scoring phylogenetic tree.

To assess the impact of the specific tree inference method on the phylogenetic results, we also conducted a phylogenetic analysis in a Bayesian framework. For this purpose, we used the software MrBayes (version 3.2.6; Ronquist *et al.*, 2012). We again made use of Modelfinder, implemented in IQ-TREE (version 1.6.5), to select the best partitioning scheme **and** the best fitting nucleotide substitution model for each data partition (see above). However, we considered only those **substitution models** that are included in MrBayes. We started four parallel runs starting from a random starting tree with $2*10^7$ generations each. We sampled trees every 1,000 generations. We chose a suitable "burn-in" based on the parameter convergence metrics 'effective sample size' (ESS) and 'potential scale reduction factor' (PSRF). We verified that ESS was greater than 200 and that PSRF was approaching 1.000 ($\leq$1.001) for all parameters in all independent runs. Additionally, we used the software Tracer (version 1.6; Rambaut *et al.*, 2014) in order to assess parameter convergence visually. Based on this procedure, we discarded 30% of generations (i.e., $6 * 10^6$) of each run as "burn-in" and built a 50% majority rule consensus tree from the 56,000 sampled trees.

Even in large datasets, a high level of nucleotide heterogeneity can adversely weight the results of phylogenetic analyses and erroneously cluster groups with similar GC content (Bossert *et al.*, 2017). In order to assess a possible bias of phylogenetic results from heterogeneous nucleotide composition, we assessed the GC content of each concatenated nucleotide sequence using the seqinfo function of the EMBOSS software suite (version 6.6.0.0; Rice *et al.*, 2000).

To assess whether phylogenetic relationships proposed by preceding authors or suggested by genomic meta-characters are statistically significantly less well supported by our nucleotide sequence data than those phylogenetic relationships that we inferred, we conducted 'approximately unbiased' (AU) tests (Shimodaira, 2002). Specifically, we tested (1) the monophyly of Amiseginae, (2) a possible sister group relationship of monophyletic Amiseginae to Chrysidinae [Loboscelidiinae not included in the present study], and (3) a monophyly of those Chrysidini that lack a codon triplet at a specific site in the mitochondrial gene COI relative to all other Chrysidini). All tests were conducted using IQ-TREE (version 1.6.5; Nguyen *et al.*, 2015; Chernomor *et al.*, 2016), applying the same partition scheme as before (see above), specifying 10,000 bootstrap replicates, and de novo estimating substitution models and branch lengths.

3.3.5. Compilation of host usage information

To assess how often and where in the phylogeny of cuckoo wasps major host shifts occurred, we compiled a literature survey of host usage by those cuckoo wasp species (or by closely related cuckoo wasp species) included in our study. Unfortunately, the hosts of many cuckoo wasp species are still unknown. Furthermore, it often remains

unclear what evidence was used to conclude a possible host relationship and how such conclusions could have been compromised (*e.g.*, could the reported host species have superimposed the nest on another species that actually served as host?). We therefore consider that not all available host information is equally reliable. Particularly unfortunate is the fact that it is often unclear whether a cuckoo wasp acts as a kleptoparasite or as a parasitoid. A detailed listing of the reported hosts and of our assessment of the reliability of the host information is given in File S4 (Appendix) and is summarized in figures 3.1 and 3.2.

3.4. Results and Discussion

3.4.1. Dataset and tree inference statistics

Per species, we obtained the nucleotide sequences of between three and eleven (Table S3; median nine, lower quartile seven, upper quartile ten) of the eleven protein-coding genes, of which ten are encoded by the nuclear genome and one by the mitochondrial genome. The gene coverage across all species was 77%. Species with particularly low gene coverage are *Adelphe* sp., *Hedychridium femoratum* (Dahlbom 1854), and *Omalus* sp. (each represented by the nucleotide sequences of three genes only in our dataset) (Table S3). We used the obtained nucleotide sequence data, which were combined in a supermatrix of 4,946 nucleotide sites length, to inferred the phylogeny of the cuckoo wasps. We modelled the nucleotide substitution rates with independent substitution models for each partition as recommended by Modelfinder: 1st codon position of COI (TVM+R5), 2nd codon position of COI (GTR+R4), 1st codon position of the ten nuclear genes (GTR+R4), 2nd codon position of the ten nuclear genes (TVM+R2), and 3rd codon position of the ten nuclear genes (GTR+R6). Two taxa of the original sampling showed a rogue behaviour (*i.e.*, lowered bootstrap support values of multiple branches due to their highly unstable phylogenetic position) in the phylogenetic analysis: *Chrysis cavifacies* Linsenmaier 1999 and *Chrysura trimaculata* (Förster 1853). Excluding the nucleotide sequences of these species from the phylogenetic analyses did not change the result of what nucleotide substitution model to prefer for analysing the supermatrix. However, exclusion of the two rogue taxa altered the estimated rate parameters. We consequently used the rate parameters in the subsequent analyses that were estimated after exclusion of the two rogue taxa. The phylogenetic tree with the best log-likelihood score (–84474.070) inferred with IQ-TREE after excluding the nucleotide sequences of the above two rogue taxa from the supermatrix is shown in figures 3.1 and 3.2 (File S5, Appendix). The results from the Bayesian analysis are largely compatible with those from the inference with IQ-TREE (Figs. S2 and S3; File S6, Appendix).

The GC content of the nucleotide sequences that we phylogenetically analysed ranges from 42% to 54%. The median GC content is 46%, with a lower quartile of 45% and an upper quartile of 46%. The inferred phylogeny shows no conspicuous and unexpected clustering of species with low (*i.e.*, *Cephalonomia tarsalis*, *Holopyga generosa* (Förster 1853), *Holopyga austrialis* Linsenmaier 1959, *Holophris* Mocsáry 1890 sp. 2, *Hedychrum longicolle* Abeille de Perrin 1877) or high (*i.e.*, *Adelphe* Mocsary 1890 sp., Cleptinae, *Pseudochrysis neglecta* (Shuckard 1837), *Spinolia theresae* (du Buysson 1900)) GC content. The only possible exception is *Adelphe* sp., which

clusters with Cleptinae rather than with the second representative of the subfamily Amiseginae in our study, *Amisega* Cameron 1888 sp.) (Figs. 3.1 and 3.2; Table S4, Appendix). We therefore do not think that the observed GC content differences between the analysed coding sequences had a major impact on the phylogenetic estimates (except perphaps for *Adelphe* sp.).

3.4.2. Phylogenetic results and their implications for the current genus-level classification of cuckoo wasps subfamilies and tribes

Our inferred phylogeny of Chrysididae is mostly consistent with the current classification of cuckoo wasp subfamilies and tribes (*i.e.*, that by Kimsey & Bohart, 1991). Specifically, the topology is consistent with the assumption of monophyletic Cleptinae (node 1 [n. 1], 100% bootstrap support [100%]) and of monophyletic Chrysidinae (n. 2, 82%). However, we did not recover monophyletic Amiseginae: *Amisega* sp. is found as sister to all remaining Chrysididae (n. 3, 62%) and *Adelphe* sp. as sister lineage of the subfamily Cleptinae (n. 4, 100%), although support for a monophyly of those Chrysididae that *Amisega* sp. was inferred as sister to is low (n. 5, 79%). AU tests conducted to assess the monophyly of Amiseginae, with Amiseginae as sister lineage of (1) Cleptinae plus Chrysidinae [Loboscelidiinae not included in the present study], (2) Cleptinae, or (3) Chrysidinae, revealed only a statistically significant difference relative to the inferred topology (Figure 3.1) for a position of monophyletic Amiseginae as sister lineage of Chrysidinae [Loboscelidiinae not included in the present study] (AU test: (1) $p = 0.111$; (2) $p = 0.069$; (3) $p = 0.001$). While we cannot exclude the possibility that the comparatively high GC content of the analysed nucleotide sequences of *Adelphe* sp. (50%) and the representatives of the subfamily Cleptinae (52–54%) could have resulted in an erroneous placement of *Adelphe* sp. next to Cleptinae, the differences in the GC content to other species in our dataset are comparatively small (*e.g.*, *Amisega* sp.: 46%, Allocoeliini: 47%; Elampini: 43–49%; Parnopini: 45–46%). Within the subfamily Chrysidinae, the inferred topology provides support for the hypothesis of a monophyletic origin of each of the tribes Allocoeliini (n. 6, 100%), Elampini (n. 7, 100%), Parnopini (n. 8, 100%), and Chrysidini (n. 9, 86%). Our taxon sampling does not allow judging the monophyly of the subfamily Loboscelidiinae as it is not represented in our investigation. Our study furthermore does not include the monotypic Kimseyini (Antropov, 1995; Rosa *et al.*, 2015), whose phylogenetic position could render the tribe Elampini paraphyletic (Carpenter, 1999).

The inferred interrelationships of the major cuckoo wasp clades (*i.e.*, subfamilies and tribes) differ from those hypothesized by Kimsey & Bohart (1991). However, the branch support values for these specific relationships are low in our analysis. Based on morphological data, Kimsey & Bohart (1991) hypothesized the subfamily Cleptinae to be the sister group of all remaining Chrysididae, while in our study *Amisega* sp. was inferred as sister group of the remaining cuckoo wasps. The branch leading to all the remaining Chrysididae (n. 5) received only 79% bootstrap support, however. AU tests conducted to assess the monophyly of Amiseginae revealed that a monophyletic clade Amiseginae being sister of Chrysidinae [Loboscelidiinae not included in the present study] would be statistically significantly less well supported by our data than polyphyletic Amiseginae or monophyletic Amiseginae that are not

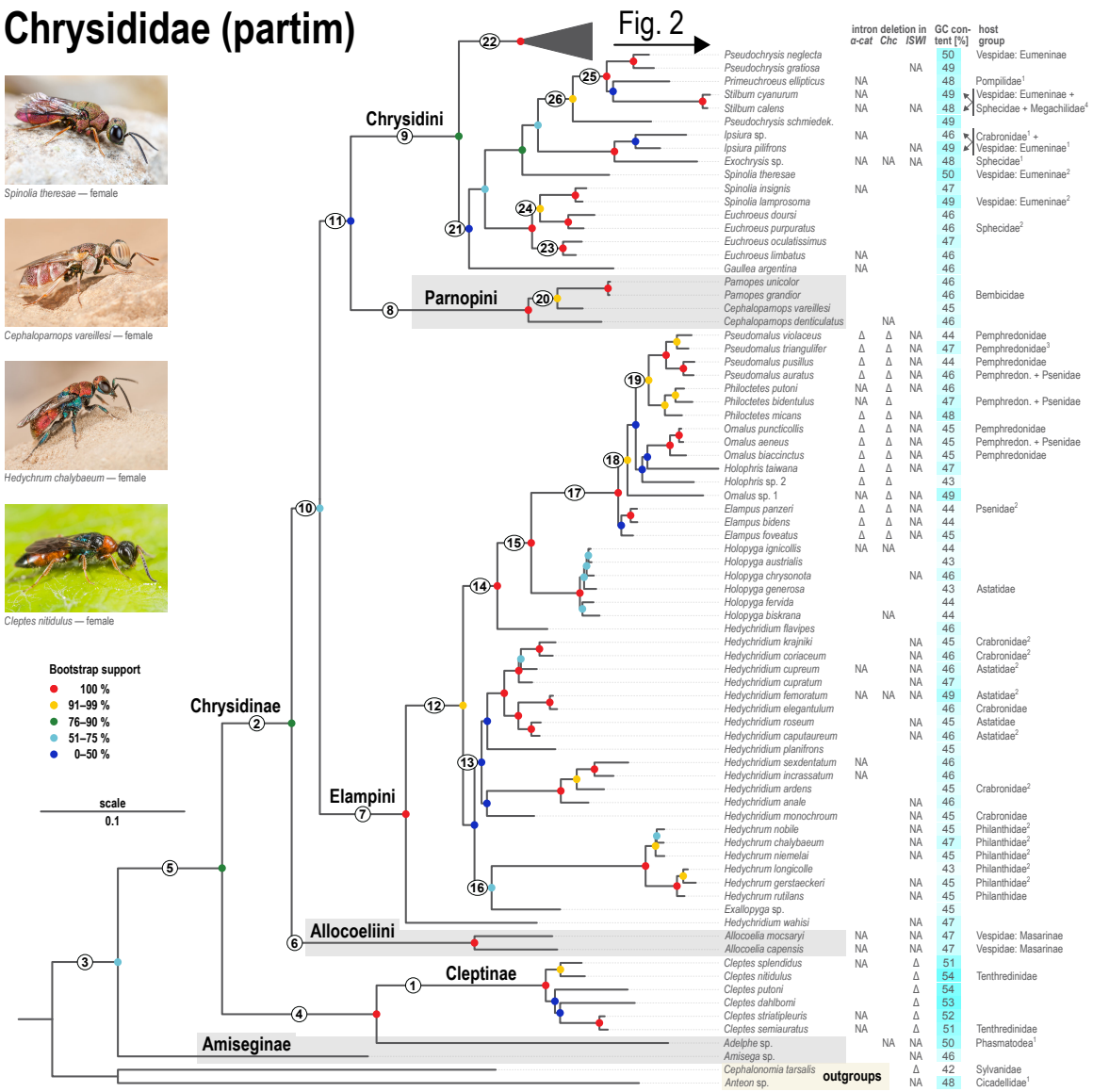

Figure 3.1.: Phylogenetic relationships between and within major cuckoo wasp lineages (continued in Fig. 3.2) and photographs of representative cuckoo wasp species. The tree was inferred with the software IQ-TREE by analysing a nucleotide supermatrix (4,946 sites) consisting of fragments of ten nuclear-encoded protein-coding genes and of a major fragment the mitochondrial protein-coding gene COI and by applying the maximum likelihood optimality criterion. The supermatrix was subdivided into five partitions (*i.e.*, the 1st and the 2nd codon position of COI; and the 1st, the 2nd and the 3rd codon position of the ten nuclear genes; 3rd codon position of ten nuclear genes). Nucleotide substitutions were modelled with the best-fitting model according to Modelfinder (Kalyaanamoorthy et al., 2017). Node support values were inferred from 1,000 non-parametric bootstrap replicates and are indicated in the tree by colour codes (percent values were rounded to the first digit before the decimal point). *Cephalonomia tarsalis* (Bethylidae) and *Anteon* sp. (Dryinidae) served as outgroups for rooting of the tree. The systematics of apoid wasps follows Sann *et al.* (2018), that of vespid wasps Bank *et al.* (2017). Foot notes: [1] host of congeneric species; [2] host information derived exclusively from co-occurrence of the potential host with the respective cuckoo wasp and the cuckoo wasp possibly having entered the potential host's nest; [3] possibly also Psenidae; [4] host information considered not reliable; [5] a representative of the *Chrysis decemdentata* Linsenmaier 1959 species group sensu Linsemaier (1959), merged by Kimsey & Bohart (1991) with the *Chrysis smaragdula* Fabricius 1775 species group. All photographs by O. Niehuis.

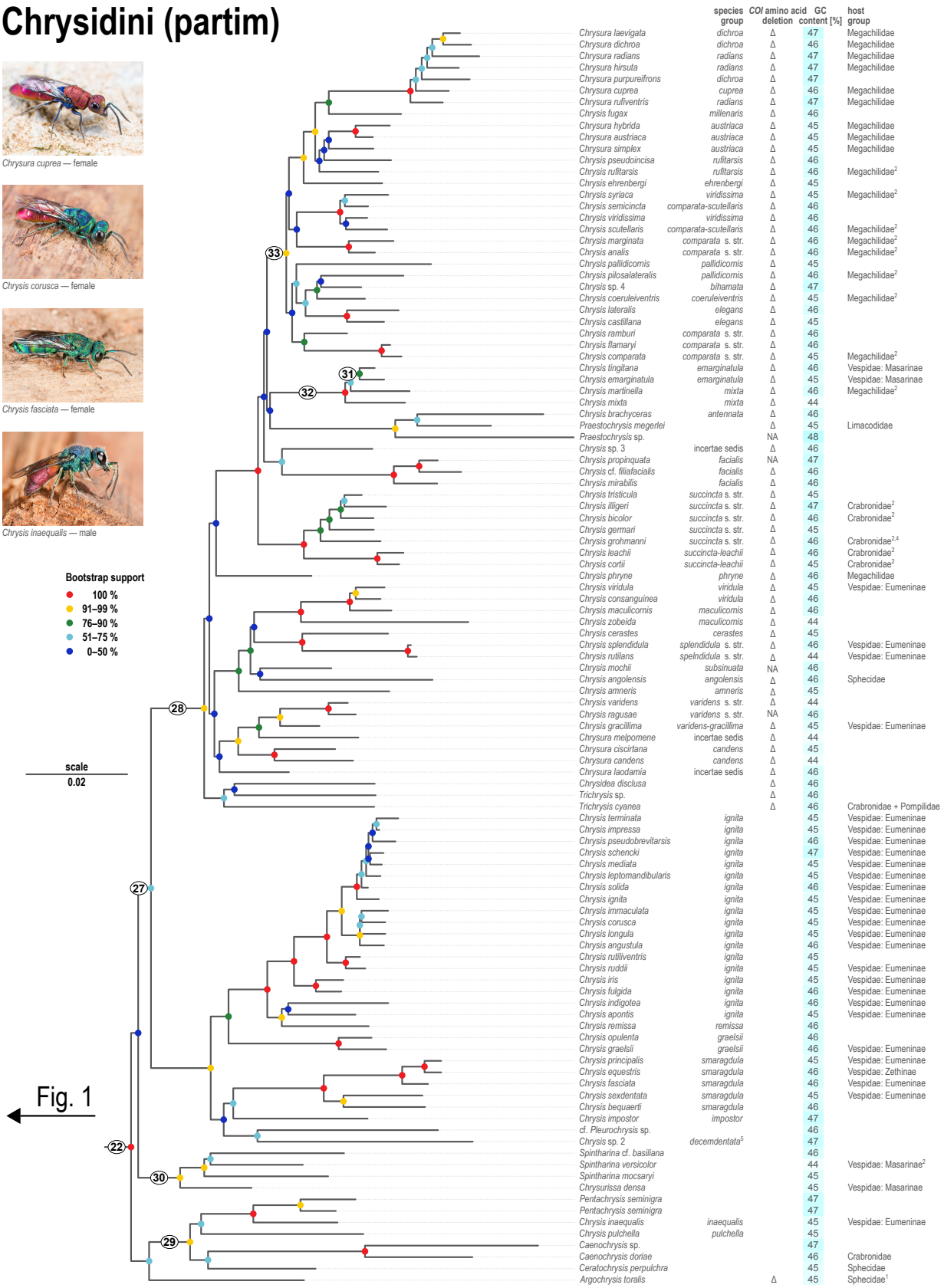

Figure 3.2.: Continuation of figure 3.1

sister to Chrysidinae (see above). A possibly in this respect informative genomic meta-character, the absence of an intron in the gene ISWI of *Cephalonomia tarsalis* (Bethylidae) and species of the genus *Cleptes* (Cleptinae), is unfortunately inconclusive, as we are lacking character state data from *Adelphe* sp. and *Amisega* sp. (both Amiseginae) and of Allocoeliini (Fig. 3.1). Note that the gene coverage by the two Amiseginae is also particularly low in our dataset (three and five gene only). Likewise, Kimsey & Bohart (1991) hypothesized the chrysidine tribe Elampini to be the sister group of all remaining Chrysidinae, while our analysis suggests Elampini as the sister taxon of Parnopini and Chrysidini. However, the bootstrap support for the latter clade (n. 10) is only 72%. Consistent with the inter-tribal relationships proposed by Kimsey & Bohart (1991) is the inferred sister group relationship of Chrysidini and Parnopini (n. 11, 50%) in our analysis. Note that an earlier molecular phylogenetic study on cuckoo wasps inferred Elampini instead of Parnopini as the sister group of Chrysidini, but with poor bootstrap support (< 60%; Niehuis & Wägele, 2004). The authors consequently did not consider this specific inference as conclusive. Given the substantial number of Sanger-sequenced data in the present investigation, a follow-up study should seek to resolve the phylogenetic relationships of cuckoo wasp subfamilies and tribes by applying a phylogenomic approach using transcriptome sequencing (*e.g.*, Peters *et al.*, 2017), or target DNA enrichment (*e.g.*, Mayer *et al.*, 2016; Branstetter *et al.*, 2017), or a combination of both (*e.g.*, Bank *et al.*, 2017; Sann *et al.*, 2018).

3.4.3. Elampini

The inferred phylogenetic relationships within the tribe Elampini strongly suggest that the species-rich genus *Hedychridium* is polyphyletic, comprising at least three distantly related lineages: (1) one of these lineages includes exclusively the species *Hedychridium wahisi* Niehuis 1998 and is inferred as the sister group of all remaining Elampini (n. 12, 99%). This result suggests that *Hedychridium wahisi* and very likely all remaining species of the *Hedychridium plagiatum* (Mocsáry 1883) species group (Niehuis, 1998) should be excluded from the genus *Hedychridium*, whose nominal species is *Hedychridium ardens* (Coquebert 1801) (in our analysis part of clade n. 13, 13%; Fig. 3.1), and to be united in a separate genus yet to be described. This lineage will be important for reassessing character transformations in the tribe Elampini. Its morphology and phylogenetic position suggest that the most recent common ancestor of the presently analysed representatives of the tribe Elampini likely matched most of the morphological features of the genus *Hedychridium (sensu* Kimsey & Bohart, 1991). (2) A second lineage of *Hedychridium* includes exclusively *Hedychridium flavipes* (Eversmann 1857). This species is robustly inferred as the sister group (n. 14, 100%) of a well-supported clade comprising the genera *Elampus, Holophris, Holopyga* Dahlbom 1845, *Omalus* Panzer 1801, *Philoctetes* Abeille de Perrin 1879, *Pseudomalus* Ashmead 1902 (n. 15, 100%). This result suggests excluding *Hedychridium flavipes* (as well as the remaining species of the *Hedychridium flavipes* species group) from the genus *Hedychridium*. The oldest available genus name is *Colpopyga* Semenov 1954 (Rosa, 2017), whose type species is *H. flavipes*. It should be mentioned that the genus *Colpopyga*, which was synonymized with *Hedychridium* by Kimsey & Bohart (1991), has been considered as a valid genus by several authors to this day (see Rosa, 2017). (3) All remaining analysed species of the genus *Hedychridium* (including the type species of the genus, *H. ardens*) are united in a third clade (n. 13). This clade

received only poor branch support (13%), however. While one of the species in this clade, *H. femoratum*, has a particular poor gene coverage in our dataset (three genes only), its inferred and well supported sister group relationship to *H. elegantulum* appears reasonable from a morphological point of view (Schmid-Egger, 1995). It remains to be tested whether the species of clade n.13 indeed represent a natural group that is possibly closely related to the genera *Exallopyga* and *Hedychrum* (n. 16, 66%). As before, the substantial number of Sanger-sequenced data in the present investigation suggests that for verification of some of the above weakly supported phylogenetic hypotheses, future studies should apply a phylogenomic approach.

We find an Elampini clade comprising the genera *Elampus, Holophris, Holopyga, Omalus, Philoctetes*, and *Pseudomalus* to be monophyletic with strong support (n. 15, 100%). This clade was inferred with the same topology also by Kimsey & Bohart (1991), who analysed morphological characters. We find the genus *Holopyga* to represent a natural group (100%) and to be the sister genus of the remaining five genera (n. 17, 100%). The close phylogenetic relationship of the genera *Elampus, Holophris, Omalus, Philoctetes*, and *Pseudomalus* is furthermore substantiated by two genomic meta-characters: the sequenced representatives of these genera are characterized by the lack of an intron in each of the genes clathrin heavy chain (chc) and α-catenin (α-cat) (Fig. 3.1). Within this group of five genera, we inferred *Elampus* as the sister group of a clade comprising *Holophris, Philoctetes, Pseudomalus*, and *Omalus* (n. 18, 97%). We furthermore find *Philoctetes* and *Pseudomalus* to constitute sister genera (n. 19, 91%). In contrast, we find species of the genus *Omalus* to be polyphyletic in respect of the genera *Holophris, Philoctetes* and *Pseudomalus*, and we find species of the genus *Holophris*, to be paraphyletic in respect of a clade comprising *Omalus aeneus* (Fabricius 1787), *Omalus biaccinctus* (du Buysson 1892), and *Omalus puncticollis* Mocsáry 1887. However, the branch support values for these relationships are low, partially possibly explained by the poor gene coverage of one of the species of clade n. 18 (i.e., *Omalus* sp., represented by only three genes in our dataset), and does not justify major conclusions.

3.4.4. Parnopini

Our phylogenetic analyses indicate that the genus *Cephaloparnops* Bischoff 1910 is paraphyletic with respect to *Parnopes* (n. 20, 98%). There are two possible ways how to resolve this taxonomical problem: synonymizing *Cephaloparnops* Bischoff 1910 (type species *Parnopes denticulatus* Bischoff 1910; sampled) with *Parnopes* Latreille 1796 (type species *Chrysis grandior* Pallas 1771; also sampled) or splitting *Cephaloparnops* into at least two genera, one of which (the one including *Cephaloparnops vareillesi* (du Buysson 1900)) would likely have to be described (unless it could be taxonomically included in the genus *Isadelphia* Semenov 1901, not included in our study). However, since our study does not comprise all species currently assigned to the genus *Cephaloparnops* and because our taxon sampling does not include species of the genus *Isadelphia* either, we refrain from explicitly applying taxonomic changes at this point.

3.4.5. Chrysidini

For describing the phylogenetic relationships within the tribe Chrysidini, we adapted the terminology applied by Niehuis & Wägele (2004). Specifically, we use the terms

"*Euchroeus* Latreille 1809 group" (n. 21, 14%) and "*Chrysis* group" (n. 22, 100%) to refer to the two major lineages into which the tribe Chrysidini splits at its base. The *Euchroeus* group (excluding *Gaullea*) has historically been given the rank of a tribe comprising the genera *Euchroeus*, *Spinolia* Dahlbom 1854, *Stilbichrysis* Bischoff 1910, *Stilbum* Spinola 1806, and *Neochrysis* Linsenmaier 1959 sensu lato (Kimsey, 1983). However, we refrain from re-establishing this tribal status, as we consider the monophyly of the *Euchroeus* group, even if *Gaullea* would not be included in it, as uncertain.

We find three of the genera in the *Euchroeus* group to be para- or polyphyletic. Specifically, we find a clade comprising *Euchroeus oculatissimus* du Buysson 1898 and *Euchroeus limbatus* Dahlbom 1854 (n. 23, 100%) to be the sister group of a clade comprising all other analysed species of genus *Euchroeus* (*i.e.*, *Euchroeus doursi* Gribodo 1875 and *Euchroeus purpuratus* (Fabricius 1787)) and two species of the genus Spinolia (*i.e.*, *Spinolia insignis* (Lucas 1849) and *Spinolia lamprosoma* (Förster 1853)) (n. 24, 95%). This renders the genus *Euchroeus* paraphyletic. The genus *Spinolia* itself is polyphyletic, as a third species (*Spinolia theresae*) is more closely related to other species of the Euchroeus group than to *S. insignis* and *S. lamprosoma*. Since Linsenmaier (1968) described *Prospinolia* as a subgenus of *Euchroeus*, with *Chrysis theresae* du Buysson 1900 as type species, we suggest granting *Prospinolia* the status of a valid genus (*Prospinolia* nov. stat.) and transferring theresae from *Spinolia* to *Prospinolia* (*Prospinolia theresae* stat. restit.; note that in the world catalogue of cuckoo wasps, Kimsey & Bohart, 1991, misspelled the specific epithet of *Prospinolia theresae*: *theresiae*). The current paraphyletic nature of the genus *Euchroeus* could be taxonomically resolved in two ways: (1) by synonymizing *Spinolia* (type species *Chrysis lamprosoma* Förster 1853; sampled) with *Euchroeus* (type species *Chrysis purpuratus* Fabricius 1787; sampled). Note that Linsenmaier (1959, 1968, 1969) already considered *Spinolia* as subgenus of *Euchroeus*. Alternatively (2), by transferring *E. oculatissimus* and *E. limbatus* and related species (*e.g.*, *Euchroeus singularis* (Spinola 1838), not sampled) into a new genus. While we have high confidence in the result that the genus *Euchroeus* is paraphyletic, we refrain from explicitly conducting additional taxonomic steps before we have a verification of the above results from phylogenomic investigations. A third polyphyletic genus within the *Euchroeus* group is *Pseudochrysis* (= *Pseudospinolia* Linsenmaier 1951, see Rosa *et al.*, 2017a). The genera *Primeuchroeus* (represented by *Primeuchroeus ellipticus* (Linsenmaier 1982)) and *Stilbum* (represented by *Stilbum calens* (Fabricius 1781) and *Stilbum cyanurum* (Forster 1771)) are more closely related to the species *Pseudochrysis gratiosa* (Mocsáry 1889) and *Pseudochrysis neglecta* (n. 25, 100%) than to *Pseudochrysis schmiedeknechti* Trautmann 1922, which is inferred as sister to all the above species of the genera *Pseudochrysis*, *Primeuchroeus*, and *Stilbum* (n. 26, 99%). While the inclusion of *Primeuchroeus* Linsenmaier 1968 (type species *Chrysis papuana* Mocsáry 1899; not sampled) into the genus *Pseudochrysis* (type species *Chrysura humboldti* Dahlbom 1845; see Rosa *et al.*, 2017a; not sampled) would be reasonable from a morphological point of view (species of the two genera are very similar), the morphologically highly derived nature of species in the genus *Stilbum* (type species *Chrysis calens* Fabricius 1781; sampled) would render merging species of *Stilbum*, *Primeuchroeus* and *Pseudochrysis* into a single genus with the name *Stilbum* difficult to justify. We therefore suggest excluding *Pseudochrysis schmiedeknechti* from the genus *Pseudochrysis* and placing it (and related species, *e.g.*, *Pseudochrysis marqueti* (du Buysson 1887, not sampled) into

a new genus. A similar step might be necessary with respect to *Pseudochrysis tertrini* (du Buysson 1898), whose current inclusion in the genus *Pseudochrysis* appears questionable from a biogeographical and morphological point of view (Linsenmaier, 1997). Note that Linsenmaier (1968) established the monotypic subgenus *Neospinolia*, whose type species is *Chrysis tertrini* du Buysson 1898, and subsequently raised it to genus level (Linsenmaier, 1997). Unfortunately, we were unable to sample DNA of this species.

We find that *Chrysis*, the most species-rich genus of cuckoo wasps comprising more than 1,000 described species (Kimsey & Bohart, 1991), is polyphyletic in relation to the genera *Argochrysis, Caenochrysis, Ceratochrysis, Chrysidea, Chrysura* Dahlbom 1845, *Chrysurissa, Pentachrysis* Lichtenstein 1876, cf. *Pleurochrysis* Bohart 1966, *Praestochrysis, Spintharina* Semenov 1892, and *Trichrysis*. Some of these genera are likely para- or polyphyletic themselves (*i.e.*, *Chrysura, Praestochrysis, Trichrysis*). This result is not too surprising given that most of the above-mentioned species-poor genera are characterized by at least one likely derived character, while the genus *Chrysis* lacks derived characters and received all remaining species (see Kimsey & Bohart, 1991). There are three reasonable solutions to deal with this result taxonomically: (1) splitting the genus *Chrysis* (and likewise *Chrysura*) into multiple monophyletic genera. By doing so, the taxonomic status of the species-poorer genera would (with few exceptions) remain unchanged. However, the identification of apomorphic characters to justify each of the numerous new genera would be a daunting task. We therefore think that this is not a practical solution in the short run. (2) Lumping all of the genera of the *Chrysis* lineage (n. 22) into a single genus *Chrysis* (which is the oldest available name; type species *Chrysis ignita*; sampled). While the taxonomic status of the species-poorer genera would change, those that delineate monophyletic units could still continue to be used as subgenera. (3) A compromise could be a combination of lumping and splitting. For example, one could unite all species of clade n. 27 (54%) in the genus *Chrysis* (by synonymizing *Chrysidea, Chrysura*, cf. *Pleurochrysis, Praestochrysis*, and *Trichrysis* with *Chrysis*). The remaining clades contain besides species of the genera *Argochrysis, Caenochrysis, Ceratochrysis*, and *Pentachrysis* only the species of two species groups currently included in the genus *Chrysis*: *Chrysis inaequalis* Dahlbom 1845 (*Chrysis inaequalis* group) and *Chrysis pulchella* Spinola 1808 (*Chrysis pulchella* group). Inclusion of the *Chrysis inaequalis* group into the genus *Pentachrysis* can easily be justified, since species of *Pentachrysis* and of the *Chrysis inaequalis* group all share a strongly bidentate mesopleuron (likely a synapomorphy) (Linsenmaier, 1959). The *Chrysis pulchella* group could be united in the genus *Gonodontochrysis* Semenov 1954 (type species *Chrysis flamma* Semenov 1954 [syn. *C. turceyana* Linsenmaier 1959], which belongs to the *Chrysis pulchella* group [Rosa *et al.*, 2017b]; not sampled), currently treated as a synonym of the genus *Chrysis*. There are three reasons why this last solution is not practical, however. First, we are not aware of any morphological synapomorphy that characterizes species of n. 27. Second, our taxonomic sampling does not include all species groups that are currently considered in the genus *Chrysis*. And third, the monophyly of clade n. 27 is poorly supported (54%). Therefore, it would remain unclear to which genus these species groups actually belong. We therefore suggest to synonymize *Argochrysis, Caenochrysis, Ceratochrysis, Chrysidea, Chrysura, Chrysurissa, Pentachrysis, Pleurochrysis, Praestochrysis, Spintharina,* and *Trichrysis* (and in the future possibly other genera not sampled by us) with *Chrysis*.

Within the *Chrysis* lineage, we identified a well-supported subordinated clade (n. 28, 99%) whose species are characterized by the deletion of a codon triplet in the mitochondrial gene COI. This lineage includes all representatives of the polyphyletic genus *Chrysura*, the possibly paraphyletic genus *Trichrysis*, the genus *Chrysidea*, the paraphyletic genus *Praestochrysis*, and various species groups of the polyphyletic genus *Chrysis*. The only other species in our dataset exhibiting a deletion of a codon triplet in the mitochondrial gene COI at (likely) exactly the same site is *Argochrysis toralis* Kimsey 1982, which we inferred as a sister lineage to clade n. 29 (95%, this clade includes species of the genera *Caenochrysis, Ceratochrysis, Pentachrysis* and species of the *inaequalis* and *pulchella* species groups of the polyphyletic genus Chrysis). However, bootstrap support for this sister group relationship is comparatively low (74%), rendering it possible (although not very likely, AU test: $p < 0.030$) for *Argochrysis* to be part of the above clade. Future phylogenomic studies using additional nucleotide sequence data will hopefully place *Argochrysis toralis* more confidently in the phylogenetic tree. Since COI is used as a barcoding gene and is sequenced in cuckoo wasps around the globe in barcoding initiatives, the presence or absence of this amino acid codon deletion allows a quick assessment of whether a species in the vast and polyphyletic genus *Chrysis* is part of the above clade n. 28.

3.4.6. Host associations

We used the inferred cuckoo wasp phylogeny to look for conspicuous patterns in host associations not previously discussed (*e.g.*, by Kimsey & Bohart, 1991). One particularly interesting phylogenetic result we obtained in respect of host associations is the inferred sister group relationship of the Old World genus *Spintharina* and the New World genus *Chrysurissa* (n. 30, 99%). Kimsey & Bohart (1991) hypothesized *Chrysurissa* to be the sister group of *Chrysura*, which our study proves to be polyphyletic. The close phylogenetic affinity of *Chrysurissa* and *Spintharina* inferred in the present investigation is thus unexpected. Intriguingly, however, species of *Chrysurissa* and *Spintharina* are well known for exploiting exclusively (as far as we know) pollen wasps of the vespid subfamily Masarinae as hosts (Hicks, 1929; Hungerford, 1937; Berland & Bernard, 1938; Blüthgen, 1961; Heinrich, 1964; Parker & Bohart, 1966). Only two other cuckoo wasp groups are known to use Masarinae as hosts: species of the genus *Allocoelia* (n. 6, Allocoeliini) (Gess & Gess, 2014) and species of the *Chrysis emarginatula* Spinola 1808 species group (n. 31, 86%) (Linsenmaier, 1968; Mauss, 1996). We therefore do not think that the exploitation of pollen wasps by the sister genera *Chrysurissa* and *Spintharina* is the result of convergent host exploitation. We rather assume that *Chrysurissa* and *Spintharina* are descendants of a common ancestor at lineage n. 30 that already exploited pollen wasps as hosts and that the exploitation of pollen wasps by cuckoo wasps consequently evolved only three times and not four times, as earlier phylogenetic considerations implied (Kimsey & Bohart, 1991).

One reason for pollen wasps being used by few cuckoo wasp species as host could be the fact that the pollen wasps represent a relatively species-poor group (Gess, 1996). However, an additional reason could be that a switch to Masarinae as hosts may have been difficult to achieve because cuckoo wasps are seemingly unable to digest pollen (Krombein, 1967; Ouayogode, 1979). Cuckoo wasps exploiting pollen-collecting species as hosts need to be parasitoids (*i.e.*, species that develop from the host itself

rather than from the host's provision for its offspring) rather than kleptoparasites (*i.e.*, species that primarily develop from the host provision for its offspring). Parasitoids should benefit from delaying their development relative to that of their hosts, while kleptoparasites should benefit from developing faster than their host. Specifically, the kleptoparasite has to kill the host larva prior to consuming its provision (Bordage, 1913), unless the host's offspring is killed by the ovipositing female. The latter behavior has, to the best of our knowledge, not been reported to occur in cuckoo wasps. Since the trajectories for the developmental speed of kleptoparasites and parasitoids are thus converse, an evolutionary switch from kleptoparasitism to parasitoidism and vice versa should consequently be uncommon. It is worth mentioning in this context that one of the three cuckoo wasp lineages that switched to use pollen collecting wasps as hosts (*i.e.*, the *Chrysis emarginatula* species group, n. 31) evolved from within a clade of species (n. 32, 100%) in which at least one species is a known parasitoid of pollen collecting bees (*i.e.*, the *Chrysis emarginatula* species group and *Chrysis martinella* du Buysson 1900).

While there is too little information about whether specific cuckoo wasp species act as kleptoparasite or parasitoid, there is circumstantial evidence in support of the idea that switches between kleptoparasitic and parasitoid lifestyles could indeed be rare. While pollen collecting bees of the family Megachilidae represent a major host group for cuckoo wasps, our phylogenetic inferences suggest that the exploitation of bees as hosts evolved less frequently than previous phylogenetic considerations implied (Kimsey & Bohart, 1991). This is because we find most of the bee parasitizing genera and species groups (*i.e.*, *Chrysura austriaca* (Fabricius 1804) group, *Chrysura cuprea* (Rossi 1790) group, *Chrysura dichroa* (Dahlbom 1854) group, *Chrysura radians* (Harris 1776) group, *Chrysis coeruleiventris* Abeille de Perrin 1878 group, *Chrysis comparata* Lepeletier 1806 group, *Chrysis pallidicornis* Spinola 1838 group, *Chrysis rufitarsis* Brullé 1833 group, *Chrysis viridissima* Klug 1845 group) to be united in a well-supported clade (n. 33, 97%). While this clade includes species of additional species groups (*i.e.*, *Chrysis bihamata* Spinola 1838 group, *Chrysis ehrenbergi* (Dahlbom 1845) group, *Chrysis elegans* Lepeletier 1806 group, *Chrysis millenaris* Mocsáry 1897 group), the hosts of the species in these species groups are still unknown and could also be bees. Furthermore, we consider it possible that the paraphyletic *Chrysis mixta* lineage (n. 32, 100%), which includes at least one species that also use bees as hosts and from within which the *Chrysis emarginatula* group (n. 31, 86%), which use pollen wasps as hosts, evolved, could be part of the above major clade of bee exploiting cuckoo wasps, as the bootstrap support for the branch separating the two lineages in the phylogenetic tree (Fig. 3.2) is poor (38%). There are only two additional lineages in our phylogenetic tree that have been reported to be possibly parasitoids of bees: *Chrysis phryne* Abeille de Perrin 1878 (Berland & Bernard, 1938) and species of the genus *Stilbum* (Mocsáry, 1889).

Another host group of cuckoo wasps worth discussing are spider wasps (Pompilidae). The use of a spider wasp (*Deuteragenia bifasciata* (Geoffroy, 1785), formerly *Dipogon hircanus* (Fabricius 1798)) as host of a cuckoo wasp (*Trichrysis cyanea* (Linnaeus 1758), formerly *Chrysis cyanea* (Linnaeus 1758)) was first suggested by Wolf (1971). Yet, the idea of spider wasps serving as hosts of cuckoo wasps was subsequently dismissed, as the possibility of a spider wasp having used the same nest space as species that are known to serve as a host (apoid wasps of the genus *Trypoxylon* Latreille 1796) could not be ruled out (Kunz, 1994). However, the extensive use of

trap nests during the last three decades has led to the accumulation of overwhelming evidence for *Trichrysis cyanea* using both apoid wasps of the genus *Trypoxylon* (Kunz, 1994) and spider wasps of the genera *Auplopus* Spinola 1841 (Theunert, 1997) and *Deuteragenia* Šustera 1912 as hosts (*e.g.*, Pärn *et al.*, 2015). We here report a second instance in which a cuckoo wasp uses a spider wasp as host: we reared *Primeuchroeus kansitakuanus* (Tsuneki 1970) multiple times from nests of spider wasps belonging to the genera *Auplopus* and *Deuteragenia* (File S4). All rearings were from trap nests originating in China. What specific factors could have facilitated a switch to use spider wasps as hosts remains unclear in the latter case. However, the use of apoid wasps of the genus *Trypoxylon* and spider wasps of the genus *Deuteragenia* as hosts by *T. cyanea* might provide a hint. Both, the apoid wasp and the spider wasp prey on spiders (Blösch, 2000; Wiśniowski, 2009). *Trichrysis cyanea* acts as kleptoparasite and thus its larvae primarily nourish on the hosts' provisions. It is therefore imaginable that the cuckoo wasps use chemical cues (*e.g.*, cuticular hydrocarbons) of the spiders hunted by the host for its offspring to identify appropriate and provisioned host nests. A switch from apoid wasps (which we here tentatively hypothesize to represent the ancestral condition) to spider wasps as hosts of *T. cyanea* would consequently have been a small evolutionary step that likely was not associated with any major temporal fitness reduction.

3.4.7. Conclusion

The present study helped to strengthen our confidence in the monophyly of the major cuckoo wasp lineages (with the notable exception of Amiseginae) and at the same time revealed that the current genus-level systematics of cuckoo wasps is highly artificial. We discussed options how to taxonomically resolve the current shortcomings in the classification of cuckoo wasp at the genus level. The lack of resolution especially at deeper nodes in the phylogenetic tree has prevented us from answering how exactly the subfamilies and tribes in the family Chrysididae are related to each other. Given the substantial number of Sanger-sequenced molecular markers in the present investigation, future studies should seek to address persisting uncertainties in the phylogeny with a phylogenomic approach. The present study lays the foundation for such approaches by guiding future taxonomic sampling (*e.g.*, the DNA sequences of what major lineages to include when designing DNA enrichment probes and the DNA of what species to include to infer a robust phylogenetic backbone tree; see Mayer *et al.*, 2016 and Bank *et al.*, 2017).

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4. Sexual dimorphism of cuticular hydrocarbons in Chrysididae

4.1. Abstract

The primary functions of cuticular hydrocarbons (CHC) are to protect insects from desiccation and to serve as barriers against fungi and bacteria. However, the role of CHC as signals in intraspecific and interspecific communication is as important as their protective function. In solitary insects, CHC are mainly involved in species and mate recognition whereas in social insects, they additionally mediate short-range recognition cues for colony and caste identification. However, whereas CHC are often used as mate recognition cues and sexual dimorphism in CHC may often occur, it is not well known how differences in CHC profiles between sexes are: whether differences in the relative amount of the same CHC compounds prevail or whether both sexes produce very different CHC compounds. In fact, there are still few cases in which a large group of species have been compared to evaluate the degree of sexual dimorphism. Here, I present the largest comparison of sexual dimorphism in CHC profiles of a group of closely related species of insects. Using CHC of both sexes of 58 species of cuckoo wasps (Chrysididae), a diverse group of solitary parasitoids and kleptoparasites, I show that in these wasps, CHC are highly dimorphic. Moreover, with only three exceptions, all studied species are qualitatively dimorphic, that is, major constituents of the profile of both sexes can be different, even belonging to different compound classes. Unsaturated compounds are more frequent in females while methyl-branched compounds occur more frequently in males, however, unsaturated compounds are more abundant than methyl-branched compounds in general. A sex-specific pattern in the position of the double bond of alkenes was found. In females, alkenes with double bonds at positions 9 and 7 abound while in males, double bonds are often located at more internal positions. Interestingly, these substances account for major differences between sexes and their possible significance as putative sex-pheromones in Chrysididae is discussed.

4.2. Introduction

The external layer of the cuticle of all insects is formed mostly of a mixture of long-chain non-polar hydrocarbons (CHC). Their hydrophobic characteristics confer the cuticle its primary anti-desiccation and protective functions (Blomquist & Bagnères, 2010b). In addition, CHC are also used in chemical communication (Blomquist & Bagnères, 2010b). Produced in the oenocytes and transferred to the cuticle, CHC occur in three main substance classes. CHC can form simple straight chains (linear alkanes), possess one or more double bonds (*e.g.*, alkenes, alkadienes, alkatrienes, etc., known altogether as olefins or unsaturated compounds), or present one or more

methyl groups (methyl-branched alkanes) at different positions along the carbon chain (Blomquist & Bagnères, 2010b). It is generally accepted that these latter two classes of CHC are preferred in a communication context because their spatial configuration can significantly increase the diversity and specificity of the signaling molecules that could be perceived and distinguished by insects (Dani *et al.*, 2001; Dani *et al.*, 2005 and Chaline *et al.*, 2005).

CHC profiles typically consist of a combination of 20–50 different CHC (although in some cases profiles with over 100 CHC have been described, Blomquist & Bagnères, 2010b; Calderón-Fernández & Juárez, 2013) of chain lengths usually between C21 and C35 of variable quantitative composition. The diversity of qualitative and quantitative CHC differences allows the (theoretical) existence of nearly infinite combinations of CHC compounds which can account for species-specificity. Therefore, CHC have been considered useful markers in chemotaxonomy (Kather & Martin, 2012) and are typically used as an additional tool to help distinguishing morphological similar (Collembola, Porco & Derharveng, 2009) and cryptic species (*e.g.*, *Laupala* crickets, Mullen *et al.*, 2007, orchid bees, Pokorny *et al.*, 2014). Although CHC have been found to be species-specific and to remain relatively stable across geographic regions (Guillem *et al.*, 2016), there are still a number of factors that can alter the expression of CHC profiles and introduce variation of CHC within species. For instance, CHC profiles may change with temperature (Gibbs *et al.*, 1997, Wagner *et al.*, 2001; Rouault *et al.*, 2004), age (Kuo *et al.*, 2012, Vanickova *et al.*, 2012), mating status (Polerstock *et al.*, 2002; Everaerts *et al.*, 2010), breeding status (Steiger *et al.*, 2007) and diet (Liang & Silverman, 2010; Fedina *et al.*, 2012), among others. Moreover, in solitary species CHC are involved in species and gender recognition, whereas in social species, they are additionally involved in caste and nestmate recognition (Wagner *et al.*, 2000; van Zweden & d'Etorre, 2010). For this reason, it has been suggested recently that sociality results in more complex CHC profiles (Freeberg *et al.*, 2012; Ord & Garcia-Porta, 2012), though there is no empirical support for this hypothesis so far (Kather & Martin, 2015).

CHC are often used as mate recognition cues (Singer, 1998; Barbour *et al.*, 2007; Ingleby, 2015) and are therefore expected to be different between the sexes. Nevertheless, still little is known about how often and in which cases CHC are sexually dimorphic and whether there is only quantitative (the same compounds are present in both sexes but vary in relative amounts) or additionally qualitative (sexes differ in the presence of specific compounds) dimorphism. A review on the topic revealed that sexual dimorphism was present in more than 70% of about 100 species analyzed representing the insect orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Heteroptera and Orthoptera (Thomas & Simmons, 2008b). However, the proportion of species displaying sexual dimorphism varied depending on the insect orders. Among the three orders that had been more intensively studied (Coleoptera, Diptera and Hymenoptera, 80 species) the percentage of species displaying sexual dimorphism varied between 55% in Coleoptera to 100% in Hymenoptera (Thomas & Simmons, 2008b). Nevertheless, although sexual dimorphism may be more common than expected, no study has compared sexual dimorphism in CHC profiles within a large group of closely related species yet. Moreover, most studies of CHC profiles between sexes reported dimorphism as an additional finding to other aspects evaluated in their studies (*e.g.*, Page *et al.*, 1997; Mullen *et al.*, 2007; Alves *et al.*, 2010, Jennings *et al.*, 2014, Pokorny *et al.*, 2015) and/or have involved comparisons of relatively few related species (< 12,

Diptera: Bartlet *et al.*, 1986, Cobb & Jallon, 1990, Coleoptera: Page *et al.*, 1997; Pattanayak *et al.*, 2014).

In Diptera, one of the best studied insect orders, both qualitative and quantitative sexual dimorphism in CHC are common, but in some species, females and males have undistinguishable CHC profiles (Bartlet *et al.*, 1986, Cobb & Jallon, 1990, Howard, 1993 and references therein, Alves *et al.*, 2010). In Hymenoptera, sexual dimorphism has rarely been compared for several related species, but varying degrees of dimorphism exist as well. For example, no sexual dimorphism of CHC has been found in a social wasp (*Ropalidia marginata*), and it has been suggested that these wasps may use other signal modalities for intraspecific communication (Mitra *et al.*, 2015). On the other hand, quantitative and qualitative differences in CHC composition have been found between females and males of orchid bees (Pokorny *et al.*, 2015). Unfortunately, sexual dimorphism of CHC profiles of orchid bees could only be studied in few species because females are more difficult to both sample in the field and identify (Pokorny *et al.*, 2015). Thus, CHC dimorphism has not been yet compared across many species in an hymenopteran family.

Here, I investigate patterns of sexual dimorphism and chemical diversity of hydrocarbons by describing and comparing CHC profiles in males and females of 58 species of parasitoid and kleptoparasite cuckoo wasps. The family Chrysididae is a widely distributed group of typically vividly coloured solitary wasps that exploit a wide range of hosts ranging from sawflies, apoid wasps and bees to slug moths and walking stick insects (Kimsey & Bohart, 1991). The phylogeny of the group with sequence data for more than 180 species representing three of the four subfamilies has been recently inferred (Pauli *et al.*, accepted, Chapter 3). Cuckoo wasps conform an interesting group to unravel patterns about the evolution of sexual dimorphism. Being solitary, their CHC profiles are likely involved in species and mate recognition but do not serve in other functions related to social lifestyles, such as caste or colony recognition. Due to their parasitic lifestyle, females are most probably being selected to evolve some sort of chemical deception. Thus, I hypothesize that both sexes are exposed differently and/or with varying strength to sexual and natural selection, and I expect major differences in CHC profiles between the sexes in this family of cuckoo wasps.

In particular, I ask 1) if CHC are sexually dimorphic in Chrysididae and 2) whether this dimorphism is quantitative or also qualitative. Furthermore, I ask 3) whether the differences between sexes vary among clades (*e.g.*, is sexual dimorphism stronger in certain clades?, are some clades more dimorphic than others?), 4) how this dimorphism relates to the composition and diversity of the CHC profiles, and 5) whether some CHC compounds are characteristic of one sex, so that some of these CHC compounds may also be sex-specific and might be candidates as sex pheromone (*e.g.*, are there sex-specific CHC that could eventually be hypothesized in playing a role as mate recognition signals?)

4.3. Material and Methods

4.3.1. Collection of samples

Insects were collected by netting between June 2005 and October 2014 in different locations in Europe and Northern Africa. A total of 1769 individuals belonging to

females and males of 58 species were collected. The origin, number and sex of the samples analyzed in this study are summarized in the Appendix. After collection, individuals were placed in a glass vial, transported to the lab, killed by freezing and stored at -20°C until the CHC extraction was conducted. After CHC extraction, all specimens were identified to species level by Oliver Niehuis.

4.3.2. GC/MS analysis

After thawing the frozen insects, CHC were extracted by adding n-hexane to each individual glass vial allowing for enough solvent to cover the wasp completely for 10 minutes. After CHC extraction, the wasps were stored in 100% ethanol. CHC extracts were placed in an insert, concentrated to a volume of ~ 80 mL using a gentle stream of CO_2 and subsequently analyzed with a gas chromatograph (GC) coupled to a mass selective detector (MS).

Analyses were conducted on either a HP 6890 GC coupled with a HP 5973 MS (Hewlett Packard, Waldbronn, Germany) or on an Agilent 7890/5975 GCMS System. The GC (split/splitless injector in splitless mode for 1 min, injected volume: 1 µL at 300°C injector temperature) was equipped with a DB-5 Fused Silica capillary column (30 m x 0.25 mm ID, df = 0.25 µm, J&W Scientic, Folsom, USA). Helium was used as carrier gas with a constant flow of 1 mL/min. Both GC/MS were run with the same temperature program: start temperature at 60°C, with an increase of 5°C/min until 300°C were reached, then and isotherm at 300°C for 10 min. An ionization voltage of 70 eV (source temperature: 230°C) was set for the acquisition of the mass spectra by electron ionization (EI-MS).

One to five extracts of each sex and species were pooled depending on the total amount of CHC and used to prepare dimethyl disulfide (DMDS) derivatives following the protocol of Carlson and colleagues (Carlson *et al.*, 1989). DMDS derivatives allow to determine the double bond position of alkenes. The double bond positions of alkadienes remained undetermined, and they were grouped according to their retention indices.

4.3.3. Characterization of cuticular hydrocarbons

AMDIS (Automated Mass spectral Deconvolution and Identification System) was used to extract CHC information from the chromatograms. AMDIS requires a mass spectral library to select target peaks. A mass spectral library with more than 900 identified mass spectra of common hydrocarbons and their retention indices was created previous to the analysis. Retention indices served to correctly identify methyl-branched alkanes in the library (Carlson *et al.*, 1998b). The parameters used in AMDIS were as follows: component width = 22, adjacent peak subtraction = 2, resolution = medium, sensitivity = low, shape requirements = medium). Refer to Chapter 8 for further explanations of the procedure applied in AMDIS to identify and quantify target peaks suitable for CHC analyses. CHC compounds that were infrequent (present in less than 50% of the individuals) or whose relative abundance was neglectable (the mean relative abundance fell below 0.1% of the total ion count) in any group (each sex and each species considered separately) were removed. Afterwards, I calculated the relative percentage of each peak relative to the total ion count between the selected peaks (in the range of C21–C35). Note that sometimes, more than one CHC com-

pound coelute at similar retention time (*e.g.*, 11-13-15 monomethyl-branched compounds), and are then difficult to separate. In these cases, these CHC compounds were considered a mix. Moreover, all analyses were conducted separately on females and males because the goal was to study sexual dimorphism of CHC profiles, having two datasets per species (one for each sex). In the text, tables and figures, I use a shorthand nomenclature to designate hydrocarbons, in which the total number of carbons in the chain is denoted by Cxx, and the location of methyl group or the bond position of alkenes precedes it (xMe for monomethyl, x,y diMe for dimethyl and x,y,z triMe for trimethyl-branched compounds or (Z)-x for alkenes; alkenes are assumed to be of (Z) configuration). For example 3-methyl-pentacosane becomes 3MeC25 and 9-heptacosene becomes (Z)-9-C27:1.

4.3.4. Selection of samples and CHC for the analyses

In this study, CHC extracts of 1769 individuals belonging to 58 species were analyzed. In common and abundant species (*e.g.*, *Hedychrum rutilans*, *H. gerstaeckeri*, *Pseudospinolia neglecta*, etc.), the sample size exceeded 40 individuals per sex. To simplify analyses, a maximum of 15 individuals per sex and species were randomly selected and used to calculate a mean CHC profile per species and sex. In a few instances, there were extracts of one (*e.g.*, *Chrysis laevigata, Hedychridium caputaureum*), two *(e.g.*, *Hedychrum longicolle, Hedychridium caputaureum, Omalus aeneus, Prospinolia theresae*), or three individuals (*e.g.*, *Chrysis propinquata*) for one of the sexes. Altogether however, the mean number of individuals used in the analyses was 10.2 individuals in the male sex and 10.7 individuals in the females sex.

4.3.5. General comparisons and patterns

The dominance of each hydrocarbon compound across species per sex was summarized using two simple metrics: prevalence (how often a cuticular compound occurs across species) and its mean relative abundance (calculated as the mean relative abundance of a CHC compound across all species), which gives an indication of how abundant each CHC compound is. Only a CHC compound that is overall very abundant will get a high value.

Additionally, to have an indication of whether the CHC profile is dominated by CHC compounds of longer or shorter chain, a mean chain length was estimated. This metric is calculated as the total sum of the weighted product of the retention index and the relative contribution of each peak in the chromatogram (one CHC or a mixture of coeluting CHC) and is expressed in retention index values. CHC compounds were grouped according to their homologous series and I compared the number of homolog groups between sexes. CHC belong to the same homologous series when they share a feature (double bond or methyl group inserted at the same position, but differing in carbon length) and, most probably, the same biosynthetic pathway (Martin and Drijfhout, 2009c, *e.g.*, 3MeC23, 3MeC25, 3MeC27 are members of the homolog series 3Me). The Shannon diversity index was calculated using all CHC compounds per sex and species to have an indication of chemical diversity. Shannon diversity increases with more compounds but also depends on the relative amount of those compounds. Extremely dominant compounds decrease the index. Pearson correlations were done between this diversity index and compound classes.

A phylogenetic tree inferred by Pauli and colleagues (Chapter 3) pruned to the species in this analysis was used to plot all graphs according to their phylogenetic relationships and for comparisons that take into account phylogenetic relatedness (see below). The R packages ape (Paradis *et al.*, 2004) and phytools (Revell, 2012) were used.

4.3.6. Calculation of sexual dimorphism

Three different methods were used to compare levels of sexual dimorphism. I first used the sexual dimorphism index employed by Alves and collaborators (Alves *et al.*, 2010). This sexual dimorphism index (SDI, in %) is estimated as the sum of the absolute values of percentage differences of each CHC compound between female and male in each species. Since differences may arise due to differences between shared (in both sexes) or unique compounds (in one of the sexes), the contribution of shared CHC compounds (quantitative differences) and the contribution of compounds exclusively present in one of the sexes (qualitative differences) to this index was also calculated. A downside of this index, however, is that SDI values may increase when the number of compounds in the CHC profile increases, thus, the index has no upper limit. Therefore, I additionally measured sexual dimorphism using an index established by Okamoto and colleagues (Okamoto *et al.*, 2013). This index (D) can be more easily compared among different species, because it does not depend on the number of CHC compounds. It is obtained by dividing the average of Bray-Curtis dissimilarity indices among all intrasex pairwise comparisons by the average of Bray–Curtis dissimilarity indices among all intersex pairwise comparisons (Okamoto *et al.*, 2013). It ranges from 0 (both sexes do not share any CHC compound and are dimorphic) to 1 (both sexes have identical CHC profiles and are monomorphic). I arbitrarily defined four regions with this index (0–0.25, dimorphic, 0.25–0.5, relative dimorphic, 0.5–0.75, relative monomorphic, 0.75–1, monomorphic). Using these different indices, I tested for differences in the degree of sexual dimorphism between the clades Elampini and Chrysidini, the two most species-rich clades in this study using a phylogenetic ANOVA based on the suggestion of Garland *et al.* (1993) using the function phylANOVA in the R package phytools (Revell, 2012). In addition to these two indices, I conducted a one-way ANOSIM (Analysis of Similarity, Clarke, 1993) in each species evaluating the differences between sexes (sexual dimorphism, 9999 permutations). In this case, a Bonferroni correction was done. The test statistic R ($1 >= R > = -1$) is a measure of the difference in the rank similarities between and within groups (Clarke, 1993) and indicates how separated the groups are. An R value of 1 indicates complete separation of groups: all individuals belonging to a group are more similar to each other than to any other individuals of any other group whereas $R = 0$ reflects the null hypothesis that there are no differences between groups. R values below 0 and close to -1 are rare because they may indicate that the dissimilarity between groups is smaller than that within groups (Clarke, 1993).

4.3.7. Identification of sex-specific differences

I did an analysis of percentage of similarity (SIMPER, Clarke, 1993) to calculate the individual contribution of each CHC to the main differences between sexes in each species with the purpose of discovering if some CHC compounds, homolog series or

compound classes contribute more to the differences in males or in females. The SIMPER analysis requires a minimum sample size of two in each tested group (both sexes). Therefore, this analysis could not be applied in *Chrysura laevigata, Elampus foveatus*, and *Hedychridium caputaureum*, but in the remaining 55 species. To identify the CHC compounds that contribute most to the differences between the sexes, I selected those CHC that contributed to a minimum of 75% of the differences between females and males in each species. I then assembled all CHC selected in each species into a single spreadsheet, containing 413 (repeated) CHC. Since the most abundant CHC can also be prevalent across many species, the same compound may be selected in different species, so that these 413 CHC represent 67 unique CHC (out of the 180 in the dataset). Across species, the minimum number of CHC accounting for 75% of differences between sexes was two (*Cleptes semiauratus* and *Philoctetes putoni*) and the maximum 16 (*Chrysura radians*). I assigned an order to each CHC per species, depending on its total contribution to the differences: the first compound that contributed the most to the differences in each species was assigned the order 1, and the last one 16 (occurring only in *C. radians*). The vegan package (Oksanen *et al.*, 2013) in R version 3.02 was used for the ANOSIM and SIMPER analyses.

4.4. Results

4.4.1. Cuticular hydrocarbon profiles of female and male cuckoo wasps

CHC profile information of 1214 individuals (592 males and 622 females) were used in the final dataset. A total of 180 cuticular hydrocarbons (or mixes of coeluting CHC) were identified, of which 149 were found in females and 140 in males. These CHC represented 15 alkanes, 72 alkenes, 26 alkadienes, 41 monomethyl-branched alkanes, 25 dimethyl-branched alkanes and one trimethyl-branched alkane (Appendix). Linear alkanes (C21–C29) were the most prevalent compounds in both sexes. Alkanes with an odd number of carbon atoms were abundant across all species, while alkanes with an even number of carbon atoms were present in most species, but were far less abundant, which represents a typical pattern of alkanes on insect cuticles (Martin & Drijfhout, 2009a; Kather & Martin, 2015, figures 4.1 and 4.2). Although no particular compound class was representative of any taxonomic group, there were differences in the frequency of occurrence of certain compound classes between sexes: olefins, especially alkadienes occurred more often in females (in 30 species vs. only 12 species in males), whereas methyl-branched alkanes were more prevalent in males (*e.g.*, dimethyl-branched alkanes occurred in males of 32 species, but were only present in females of 21 species).

After linear alkanes, alkenes with double bonds at position 9 and 7 represented the second most frequent and abundant CHC compounds in females, while monomethyl-branched compounds were the second most frequent substance class in males. Nevertheless, alkenes with double bond positions at 7, 11 or 14 were on average more abundant than monomethyl-branched alkanes in males (Figure 4.2). The most prevalent monomethyl-branched alkanes in both sexes were 11MeC23, a mix of 11 and 13MeC25, a mix of 11 and 13MeC27, and 3MeC27. Except for linear alkanes, which were either relatively abundant (odd-numbered n-alkanes) or relatively scarce (even-numbered

n-alkanes) across species, all other compounds varied strongly in their relative abundance among species. The most abundant compound in females (excluding n-alkanes) was (Z)-9-C25:1, which was present in 48 species and made up to half of the total abundance of the CHC profile in four species, whereas in males the most abundant compound was (Z)-14-C29:1 which was present in 17 species but contributed to at least 40% of the total CHC profile abundance in 7 of these species (Figures 4.1 and 4.2).

4.4.2. Patterns of CHC profile variation among species

4.4.2.1. Homolog series of alkenes and monomethyl-branched alkanes

CHC homolog series were evaluated in the two most common compound classes (alkenes and monomethyl-branched alkanes). There was a trend in males to be dominated by alkenes with double bond positions at 11, or more internal double bond positions (12-15), and the distribution of the double bond position shows phylogenetic signal, with the latter double bond positions dominating in species belonging to the *Chrysis ignita* group and the former in species of Elampini. On the other hand, females are dominated by alkenes with the double bonds at positions 9 and 7 (Figure 4.3). Interestingly, monomethyl-branched alkanes replaced the dominance of alkenes in certain species and some show phylogenetic signal in males as well (*e.g.*, 9Me in species of the *Chrysis comparata* group, or large proportions of internally branched methyl-alkanes in species of *Hedychridium roseum* group, figure 4.3).

4.4.2.2. Total number of CHC compounds, homolog series and chemical diversity

The average total number of cuticular compounds varied per species and sex and ranged from 13 cuticular compounds in females of *Chrysis propinquata* to 66 cuticular compounds in males of *Chrysura radians*, with an average mean number of 34 CHC per species and sex (both sexes have a similar average number of compounds; females: 34.6 ± 11.13 CHC, males: 34.1 ± 10.34 CHC, figure 4.4). The total number of CHC compounds per species (including both sexes together) ranged between 25 (in *Chrysis graelsii* and *Omalus aeneus*) and 84 (*Chrysura radians*). On average, the number of CHC compounds was larger in Chrysidini than in Elampini (mean number of CHC in Elampini: 42, in Chrysidini: 53), but this was not significant when correcting for phylogenetic relatedness (phylogenetic ANOVA $F=12.12$, $p = 0.655$). The number of different homolog series of CHC compounds (*e.g.*, 3Me, 5Me, (Z)-9 alkenes, etc.), was larger in Chrysidini than in Elampini (mean number of homolog series in Elampini: 10.6, in Chrysidini, 15). However, this was also not significant when phylogenetic relationships were considered (phylogenetic ANOVA $F=19.8$, $p = 0.559$). Compounds shared by both sexes represented as little as 13% (*Chrysis propinquata*) to 76% (*Hedychridium roseum*) of the total number of compounds per species (both sexes together).

The Shannon diversity index varied between 0.79 (a female of *Chrysis illigeri*) and 3.36 (a male of *Chrysura radians*). It was positively correlated with the number of methyl-branched compounds (Pearson's correlation: 0.70) but not with the number of unsaturated compounds in both sexes (Pearson's correlation: 0.21). Similarly, it was weakly but positively correlated with the relative proportion of methyl-branched

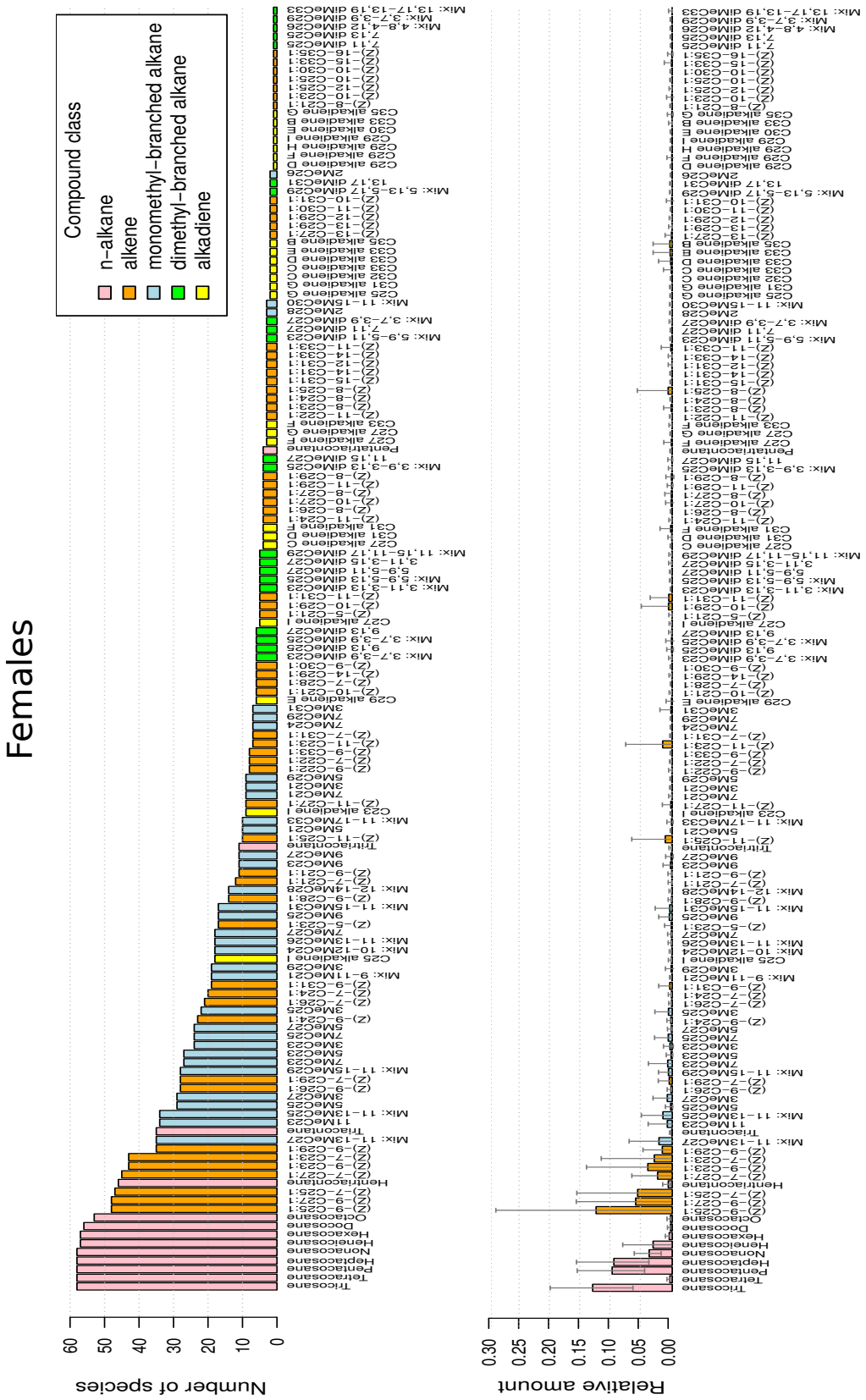

Figure 4.1.: Prevalence and average relative amount of cuticular hydrocarbons across all species in females. Prevalence refers to the total number of species that produce a given compound. Relative proportion in the CHC profile across species was calculated as the mean across all species. Compounds are arranged in descending prevalence. Hence, the order and the identity of CHC compounds differ in graphs referring to the two sexes. Colours indicate the different main classes of compounds.

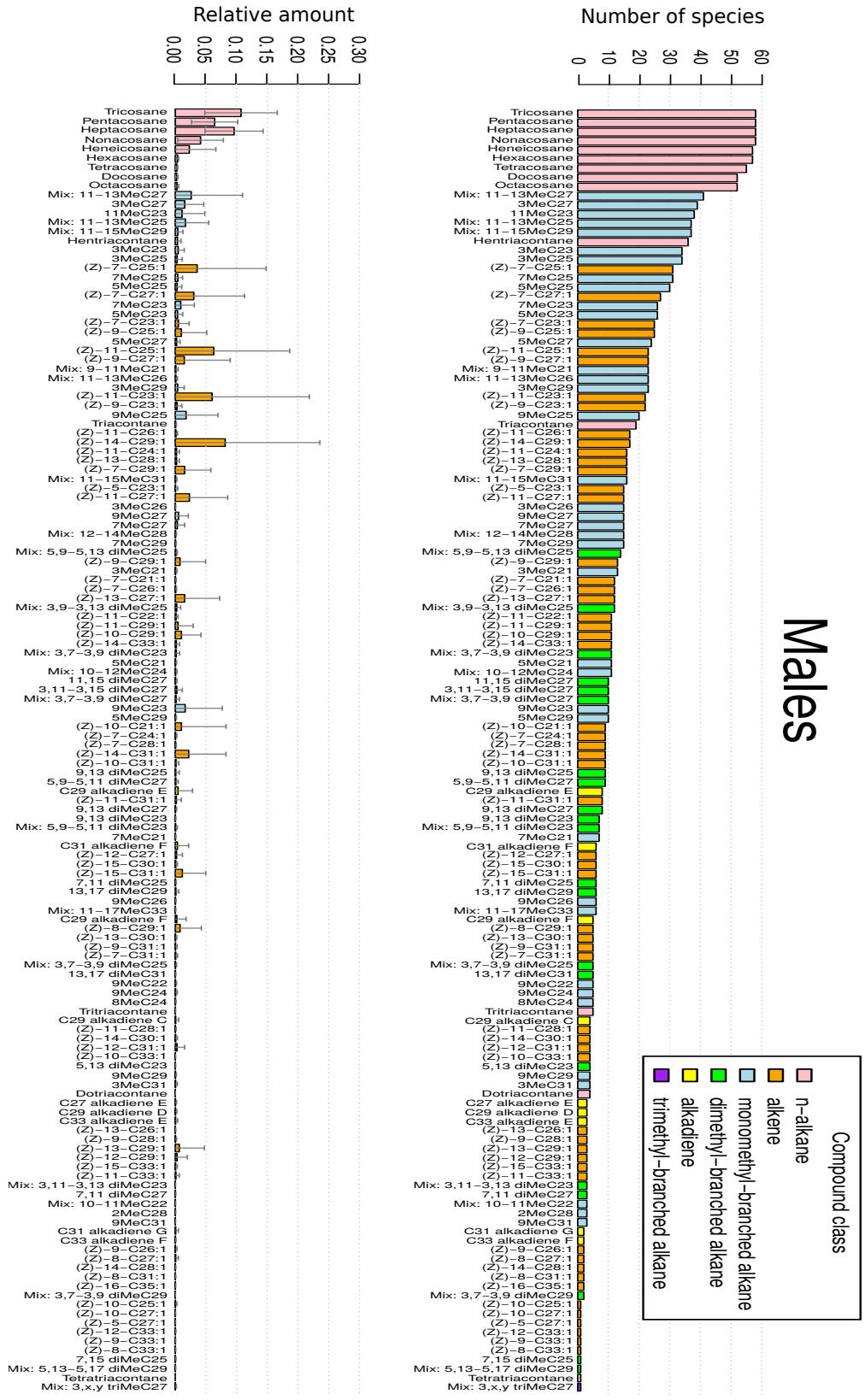

Figure 4.2.: Prevalence and average relative amount of cuticular hydrocarbons across all species in males. See legend of previous figure.

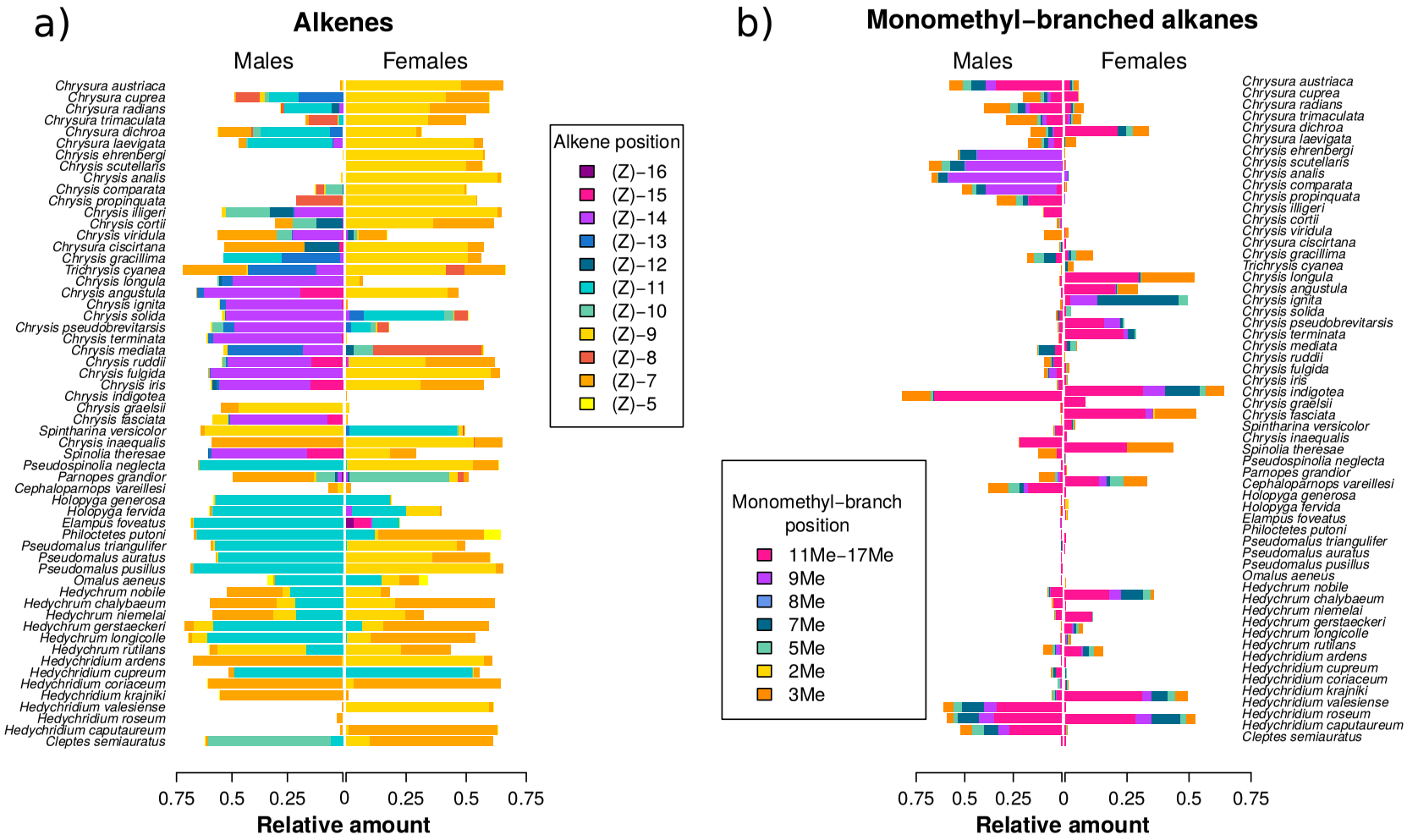

Figure 4.3.: a) Relative amount of alkenes coloured by the position of the double bonds in males (left bars) and in females (right bars) with species ordered according to their phylogenetic relationships (Chapter 3). Note the strong phylogenetic signal on the position of alkenes in males. Males are (majoritarily) dominated by alkenes with double bond position at the middle of the carbon chain (> 11) while females most often present alkenes with double bonds at the outer part of the carbon chain (9 and 7). b) Relative amount of monomethyl-branched alkanes coloured by the position where the methyl group is inserted.

compounds (Pearson's correlation: 0.44), and negatively correlated with the relative proportion of unsaturated compounds (Pearson's correlation: -0.36).

4.4.2.3. Relative abundance of CHC compounds

The three most abundant substance classes of CHC (linear alkanes, alkenes and monomethyl-branched alkanes) were present in all of the species analyzed (when missing in one of the sexes, they were present in the other), even if its relative contribution was small (*e.g.* alkenes in *Hedychridium roseum*, *Chrysis indigotea* and *C. scutellaris*). Although monomethyl-branched alkanes were the second most prevalent compound class in males, they contributed to the majority of the CHC profile only in few species, especially in bee-parasitizing cuckoo wasps. In general, alkenes dominated the profile of at least one of the sexes of all but three species (*Chrysis indidogea, Cephaloparnops vareillesi* and *Hedychridium coriaceum*, figure 4.5). The rest of the compound classes varied a lot depending on the species.

4.4.2.4. Mean chain length

The mean chain length ranged between 2258 (*Pseudomalus auratus* male) and 3013 (*Holopyga generosa* female). In species of *Hedychridium* and *Hedychrum*, the mean chain length was similar in both sexes and around 2500. In other species of Elampini, females have longer mean chain lengths than males (the difference between the sexes being larger in *Holopyga* and *Elampus*). In Parnopini and *Spinolia*, both sexes have relatively long mean chain lengths. In the rest of Chrysidini, mean chain length is longer in males than in females, but the difference between females and males is smaller in bee-parasitizing species than in other species of Chrysidini (Figure 4.6).

4.4.3. Sexual dimorphism of CHC profiles

Only three species (all of them in the genus *Hedychridium)* had a D index larger than 0.75 and can be defined as monomorphic (Figure 4.7c). A fourth species, *Omalus aeneus* also had a relatively large D value (0.7). In general, of the 58 species compared, only seven species (all belonging to the tribe Elampini) have a D value above 0.5. Thus, the great majority of species can be defined as dimorphic or relative dimorphic. The other index of sexual dimorphism (SDI) used (based on Alves *et al.*, 2010) showed similar results. In this case, however, smaller values indicate monomorphism, and the three *Hedychridium* species mentioned above had the smallest SDI among all. The calculation of this index, additionally allowed me to evaluate the contribution of the same (shared CHC) or different (unique in each sex) CHC compounds to the total difference between sexes. Quantitative differences seem to be more preponderant in Elampini while qualitative differences predominate in Chrysidini (Figure 4.7a). Thus, the percentage of different cuticular compounds over the total number of CHC compounds (in both sexes) appeared higher in the Chrysidini tribe (67%) than in all other species pooled (44%) as well as the contribution of qualitative differences to the sexual dimorphism index (in Chrysidini tribe, 56% and in all other species, 30%, figure 4.7a). However, these differences were not significant when corrected for phylogeny (phylogenetic ANOVA F: 44.27454, p value: 0.329).

The pairwise ANOSIM comparisons of CHC profiles between sexes revealed similar results. R values of the three monomorphic *Hedychridium* species are lower than

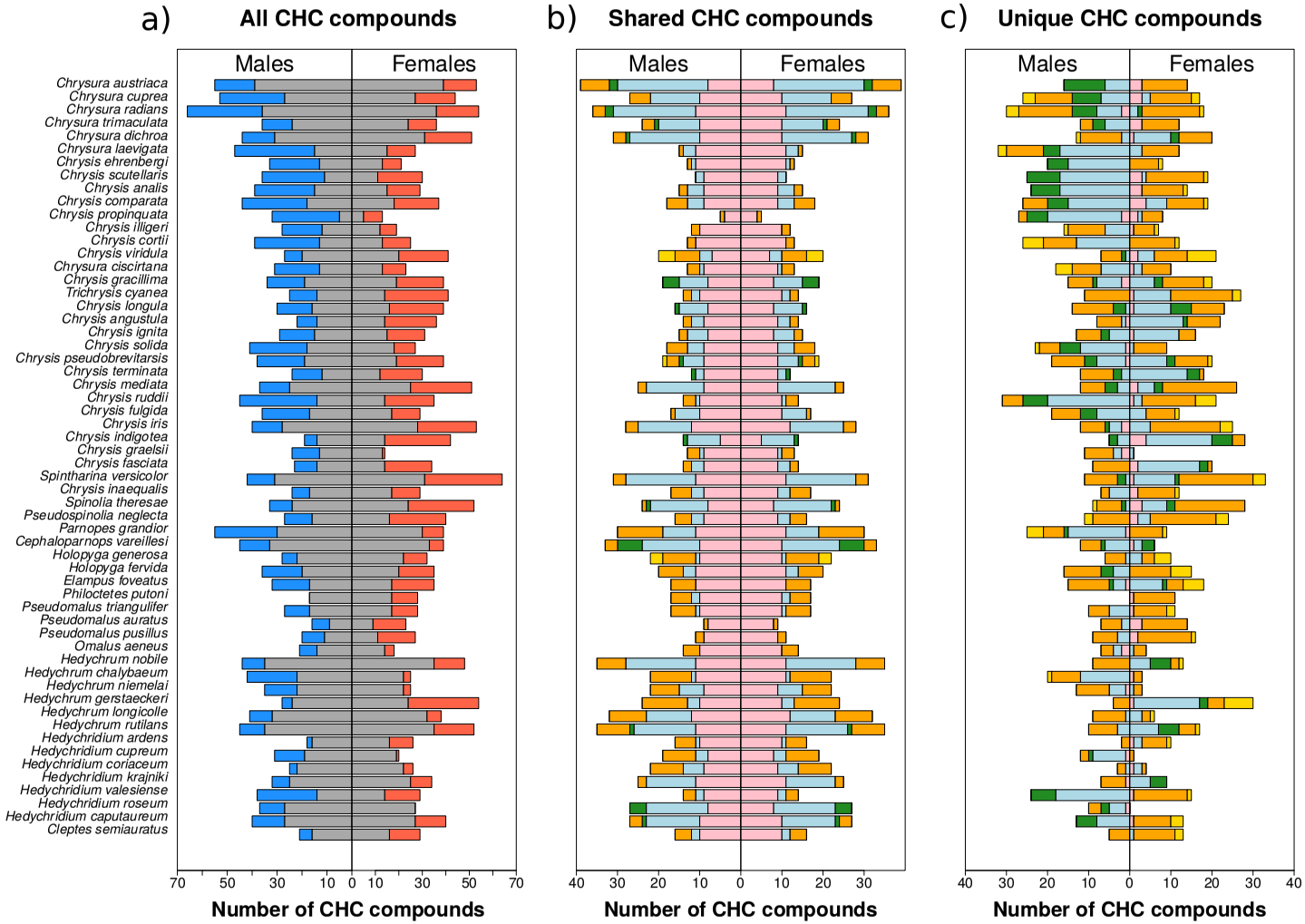

Figure 4.4.: Total number of CHC compounds per species and sex. a) Mean number of CHC compounds in males (left bars) and females (right bars) of cuckoo wasps. Shared CHC in both sexes are coloured in gray, and sex-specific CHC in blue and right, respectively for males and females, b) Shared number of compounds by compound class. Compounds are the same in females and males, c) Mean number of sex-specific CHC compounds by compound class in males (left bars) and females (right bars). Barplots colored according to compound class as in Figure 1 and 2 (alkanes: pink, monomethyl-branched alkanes: light blue, dimethyl-branched alkanes: green, alkenes: orange, alkadienes: yellow). Species ordered as in figure 4.3.

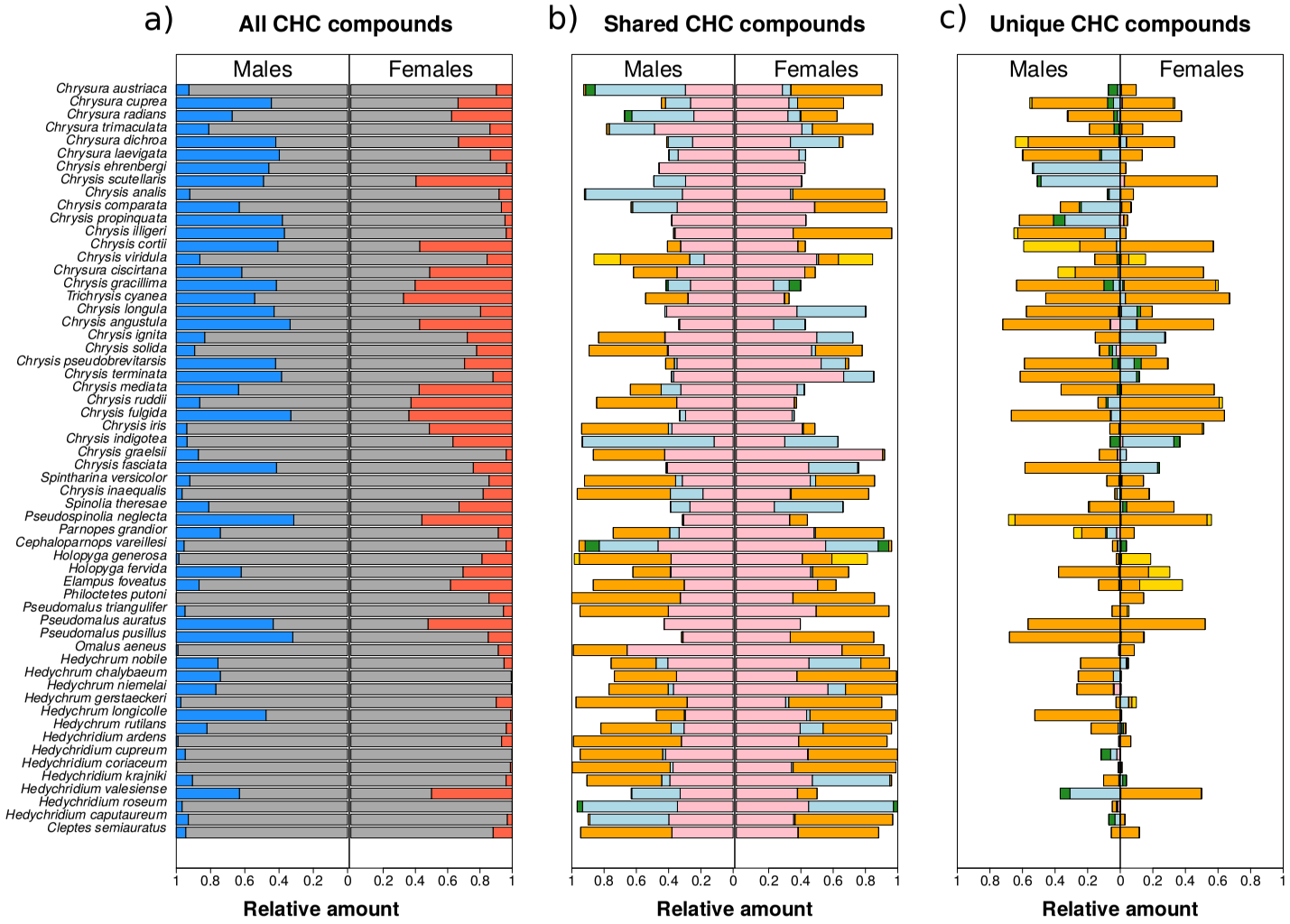

Figure 4.5.: Relative proportion of CHC compounds per species and sex. a) Mean relative proportion of shared (gray) and sex-specific CHC compounds (blue and red) in male (left bars) and female (right bars) cuckoo wasps, b) Relative proportion of shared CHC compounds by compound class in males (left bars) and females (right bars). Shared CHC compounds are the same for males and females but their relative contribution varies in each sex, c) Relative proportion of sex-specific CHC compounds by compound class. Colors indicate different compound classes and species ordered as in previous graphs.

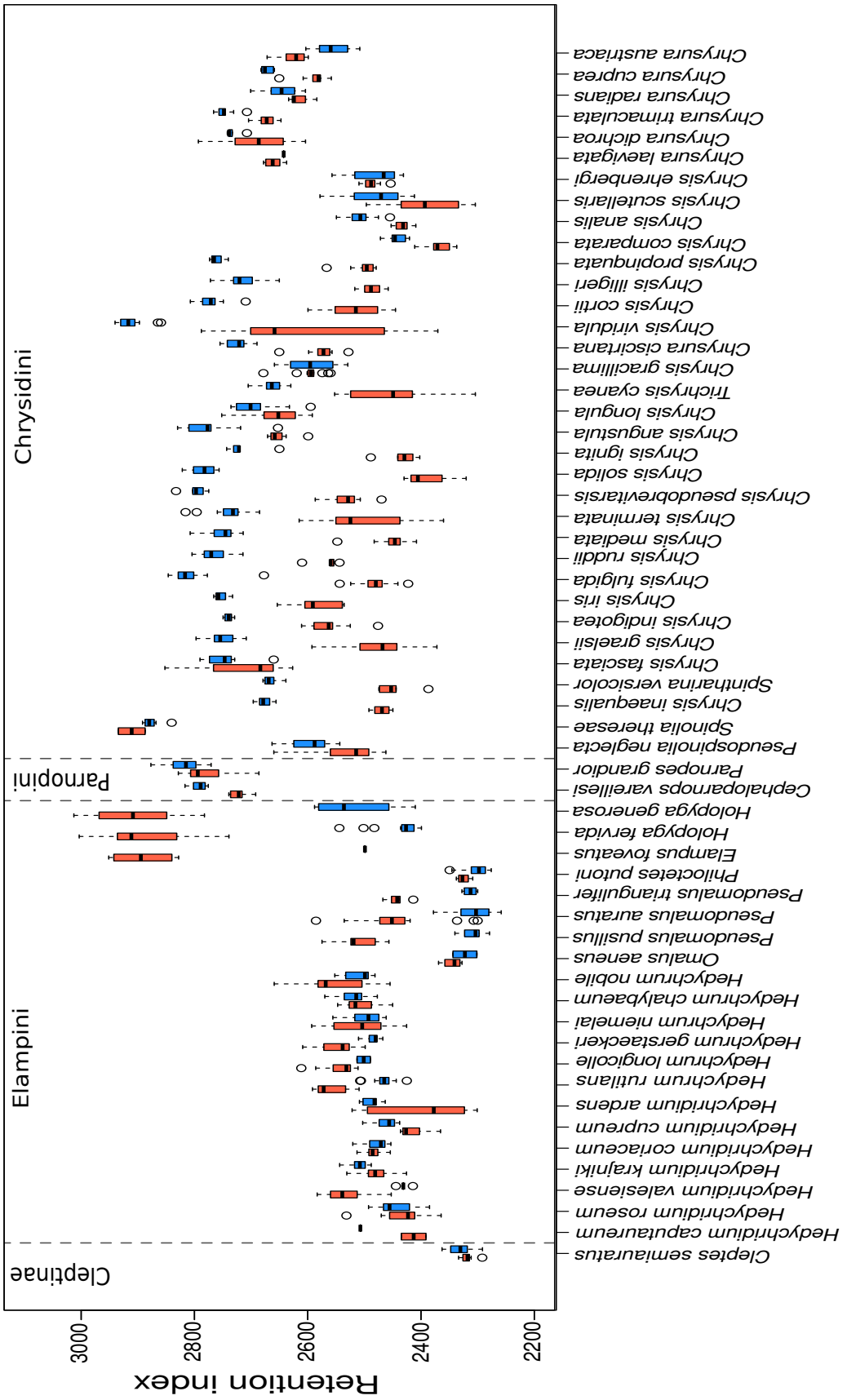

Figure 4.6.: Mean chain length variation per species and sex. A larger value of mean chain length indicates that most of the large peaks in the profile are long-chain CHC or occur at larger retention indexes. Values with shorter mean chain lengths suggesst the presence of short-chain CHC of large relative proportions. Red boxes depict values of mean chain length of females while blue boxes are used for showing values of males. Species ordered as in the phylogenetic tree of figure 4.3

0.35, while R was 0.64 for *Omalus aeneus*. On the other hand, 47 species exhibited R values above 0.97 indicating strong separation between female and male CHC profiles (Figure 4.7b, Appendix).

4.4.4. Sex-specificity and sex differences

The number of CHC compounds that contributed to at least 75% of the differences between the sexes in each species varied between 2 (in *Cleptes semiauratus* and in *Philoctetes putoni*) and 16 (*Chrysura radians*) (average 7.5 ± 2.85). There were 30 alkenes, 18 monomethyl-branched alkanes, 8 alkadienes, 6 n-alkanes, and 5 dimethyl-branched compounds (67 CHC) contributing to 75% of the differences between sexes in all species (Appendix). Alkenes were often the main CHC compound class contributing to differences between the sexes. This compound class accounted for more than 50% (211 of 413 CHC in the comparison of 55 species, Appendix) of the total CHC compounds that were selected contributing a minimum of the 75% of differences between the sexes in all species (see Methods). Monomethyl-branched compounds accounted for 19% while n-alkanes for 26%. Alkadienes and dimethyl-branched compounds were less often accounting for differences between sexes ($< 3\%$ of the cases).

In five species the first CHC compound differing between sexes was an odd-numbered alkane (C21–C27), while monomethyl-branched alkanes were the first CHC compound accounting for differences in only two species. In the rest of the species (48 species), an alkene was the first CHC compound contributing to the intersexual differences (Figure 4.8). The contribution of the first compound to the total dissimilarity between sexes varied between a minimum of 15% (*Chrysura cuprea*) to almost 45 % (*Philoctetes putoni*). There was an interesting pattern in the contribution of alkenes to the differences between sexes. In total, 14 alkenes were selected as the first CHC compound contributing to differences in 48 species. Of these, 7 alkenes with double bonds at internal positions in the chain (≥ 11) contributed to differences in 26 species because they were much more abundant in males than in females. On the other hand, 3 alkenes with the double bond at position 9 contributed to differences in 16 species, mostly because they were more abundant in females (see Figure 4.8, and Appendix). Interestingly, the combination of the first three selected CHC contributing to the differences in one species is often species-specific (Appendix).

4.5. Discussion

I compared the CHC profiles of males and females of the family Chrysididae with two general objectives: to describe and evaluate the strength and prevalence of chemical sexual dimorphism, and to provide some general answers to understand how CHC profiles diversify in Chrysididae.

To my knowledge, this constitutes the first effort to compare CHC profiles of males and females in a large number of species of an hymenopteran family.

4.5.1. Chemical diversity and complexity of CHC compounds

All major compound classes of CHC (*e.g.*, linear alkanes, olefins and methyl-branched compounds) were present in Chrysididae and within all taxonomic lineages investigated, suggesting that all compound classes were already present in the ancestor of

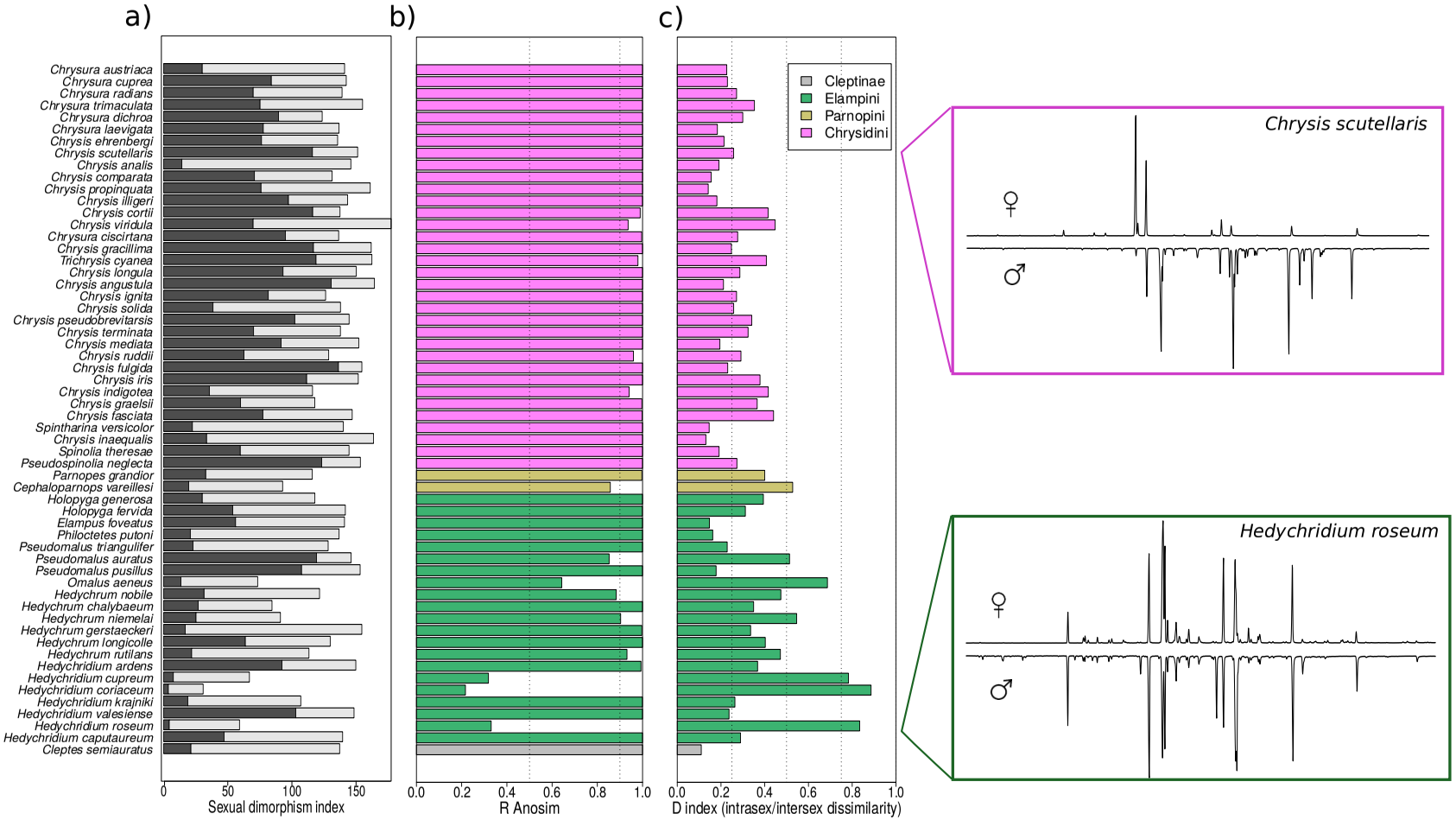

Figure 4.7.: Sexual dimorphism. a) Sexual dimorphism index (sensu Alves *et al.* 2010; *i.e.*, sum of absolute differences between mean female and male CHC profiles per species), percentage of the sexual dimorphism index due to sex-specific (black) or shared CHC compounds (gray, produced by the two sexes), b) Results of one way ANOSIM analysis conducted between sexes for each species. Values close to 1 indicate good separation between sexes, c) Sexual dimorphism (sensu Okamoto *et al.*, 2013). Values close to 1 represent monomorphic species, values close to 0, dimorphic species. Three species in the clade *Hedychridium* have values above 0.75 in the uppermost quartile, suggesting monomorphism in chemical profiles. The graphs at the right of the barplots show typical chromatogram profiles of a female (top) and a male (below) individual of a monomorphic (*Hedychridium roseum*) and a dimorphic species (*Chrysis scutellaris*). Note however that in both cases there are some CHC compounds exclusively produced by one of the sexes. In the case of *H. roseum*, most of the CHC differences are quantitative (variations in the relative amount of the same CHC compound), whereas in *C. scutellaris*, the majority of CHC compounds are not shared and the differences between both sexes are also qualitative. Colors in b) and c) indicate the taxonomic classification of cuckoo wasp into tribe or subfamily.

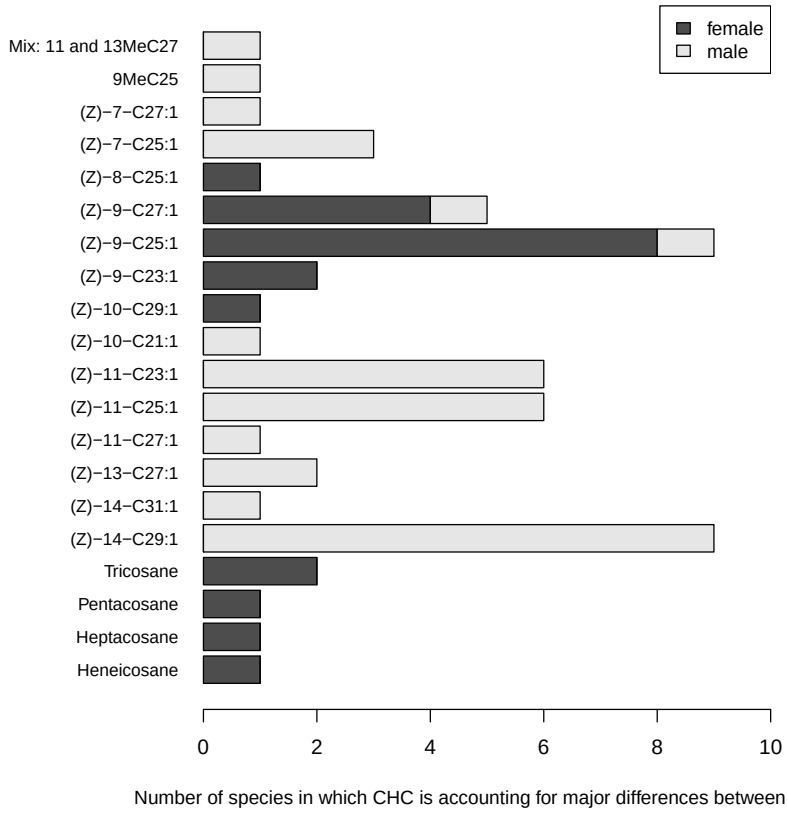

Figure 4.8.: Number of species in which the mentioned CHC compound is the major compound accounting for the main difference between sexes. If it is black it indicates the compound was more abundant (or exclusively present) in females than in males. Gray indicates the opposite was true in males. For example, (Z)-14-C29:1 was causing the major difference in 9 species because it was more abundant in males (mainly species of *Chrysis ignita* group).

cuckoo wasps. Moreover, when one of these three compound classes was not present in one of the sexes, it was produced by the other sex, indicating that the biosynthetic pathways to produce these CHC must have already evolved in the common ancestor. Martin and collaborators (Martin & Drifhout, 2009; Kather & Martin, 2015) have shown that ancestral families in Hymenoptera produced all types of olefins and methyl-branched alkanes. The most complex classes (*e.g.*, methyl-branched alkenes) were found only in Aculeata, but they probably evolved in basal clades. In fact, one species of Bethylidae, a sister family of Chrysididae, produces methyl-branched alkenes in their profiles (Howard & Infante, 1996).

The most structurally complex compounds (those having more than one double bond or methyl group) occurred less frequently and contributed to more than 5% of the CHC profile in only few species. They occurred only when the structurally simpler compounds occurred (no alkadienes were present without alkenes, or no polymethyl-branched compounds occurred without monomethyl-branched compounds), a pattern that has been also observed previously (Kather & Martin, 2015; Menzel *et al.*, 2017a), which is probably linked to constraints in the biosynthetic pathways.

A relative low number of CHC compounds (180) was found in this study of 58 species of Chrysididae in comparison to the much larger number of CHC compounds reported for 78 species of ants (almost 1000 CHC compounds, Martin & Drifhout, 2009). The main reason for this difference in the diversity of compounds may arise from a larger diversity of life histories and phylogenetic diversity in the ants of the previous mentioned study. Moreover, ants are eusocial species with different castes (workers, queens, etc), the comparison of which also increases the diversity of CHC and CHC compound classes. In any case, the minimum and maximum number of CHC compounds present on the cuticle of Chrysididae (13-66 CHC within the range C21-C35) falls in the range of CHC compounds found in the profiles of other insects (*e.g.*, between 11 and 34 CHC were identified in a study of CHC in orchid bees, Pokorny *et al.*, 2015, or up to 36 CHC in the profiles of closely related species of Diptera, de Oliveira *et al.*, 2011, and between 24 and 55 CHC in four species of a closely related family of solitary parasitoids, Bethylidae, Howard *et al.*, 1992, Howard & Infante, 1996; Howard & Perez-Lachaud, 2002).

A relative larger number of methyl-branched compounds and homolog series occurred in bee-parasitizing species. Males of these species are dominated by methyl-branched compounds while their conspecific females are mainly dominated by unsaturated compounds. Females of cuckoo wasps parasitizing bees may have been selected to produce CHC profiles largely dominated by alkenes as their hosts. Whereas I have not studied CHC profiles of hosts of these species, CHC profiles of solitary and social bee species are often dominated by olefins, occasionally presenting some methylated compounds (Kather & Martin, 2015). CHC compounds involved in intraspecific communication in bees (*e.g.*, for nestmate recognition, as sex or queen pheromones) are usually olefins (Kather & Martin, 2015). In fact, the dominance of olefins in CHC profiles of bees has been suggested to be a constraint because of a diet based on pollen for the developing larvae (in comparison to a protaineceous diet in wasps which may provide a sufficient supply of aminoacids necessary to produce methyl-branched alkanes, Kather & Martin, 2015). Whereas an adaptation to their hosts' profiles, may explain the diversification and dominance of alkenes in CHC profiles of female cuckoo wasps parasitizing bees, a diversification of methyl-branched compounds in males might have been selected for better species and sex recognition processes, both of which remain

to be tested.

4.5.2. Sexual dimorphism of CHC profiles

I found that sexual dimorphism of CHC in Chrysididae is very frequent, and that it is mainly caused by the production of different compounds by the two sexes. This was not an unexpected result because both sexes may be differently affected by natural and sexual selection given their parasitic lifestyle (see Chapter 6 and 7). Nonetheless, it was rather surprising to find that several CHC compounds were exclusively or dominantly produced by one sex only. The only review of sexual dimorphism of CHC profiles until now, already suggested that dimorphism in CHC profiles is rather widespread, though not universal among species (Thomas & Simmons, 2008). However, these authors did not provide any account on whether dimorphism between CHC profiles is more often due to quantitative or qualitative differences. Nevertheless, it has been more often shown that differences between sexes in different insect orders result from differences in the relative abundance of major CHC compounds that are shared between both sexes (*e.g.*, in *Laupala* crickets, Mullen *et al.*, 2007, ladybirds, Pattanayak *et al.*, 2014; in several species of *Drosophila*, Cobb & Jallon, 1990, Alves *et al.*, 2010, Jackson *et al.*, 2014; but also in solitary and social Hymenoptera: in Bethylidae, Howard, 1992, Howard & Infante, 1996, Howard & Perez-Lachaud, 2002, Philanthinae wasps, Chapter 7, in *Polistes* Layton, 1994). Cases of sexual dimorphism resulting from both sexes of the same species producing rather different CHC compounds have been less often described (tse-tse flies, Nelson and Carlson, 1986; some species of Drosophilids, Alves *et al.*, 2010; some species of *Euglossa* bees, Pokorny *et al.*, 2015).

In fact, maybe due to this potential erroneous assumption that the same major CHC compounds are present in both sexes, a recent study on the evolution of CHC compounds of around 250 species of Hymenoptera (Kather & Martin, 2015), did not mention which sex of the species was used in the meta-analysis. Although over 2/3 of the species used in this analysis were social (in which case, CHC of workers were explicitly mentioned to be chosen), there was a large number of solitary species for which one CHC profile (presumably that of females) is assumed to represent the species' CHC. As already shown in the previous lines, this may be adequate for species in which differences arise from quantitative variation in major compounds, but the analysis of CHC profiles in cuckoo wasps has shown that females and males possess almost always qualitatively different CHC profiles. Thus, a reader may erroneously conclude that each species can be represented by one CHC profile. Moreover, it is possible that CHC profiles of one sex retain more phylogenetic signal than the other being more suitable for explaining patterns of evolution. For example, CHC profiles of males of cuckoo wasps and of *Nasonia* species retain more phylogenetic signal than those of females (Chapter 6, Buellesbach *et al.*, 2013, see below). The cladogram constructed with a binary matrix of CHC in the study of Hymenoptera indicated no clear phylogenetic signal as species of different lineages were dispersed throughout the entire cladogram (Kather & Martin, 2015). I am tempted to suggest that a rather different scenario, would have appeared (at least in the last mentioned group of species) if males' CHC would have been used. For example, the two *Hedychrum* species used in the study of Kather and Martin cluster with their hosts in distant clades, when using CHC profiles to relate them (Kather & Martin, 2015), although they are

genetically close. Most probably, this arises as a result of an evolutionary arms race to achieve better chemical mimicry (Strohm *et al.*, 2008, Chapter 7). However, males from these species are chemically closer to each other than to their hosts (Chapter 7). In the future, it would be important to acknowledge that CHC profiles can be qualitatively dimorphic, and that using CHC profiles of one or the other sex may lead to different interpretations.

I discuss elsewhere (Chapter 6), that the strong dimorphism in CHC profiles in the family Chrysididae, may be explained by their parasitic lifestyle, in which both sexes are affected differently by natural and sexual selection. The CHC profiles of females are under strong natural selection to chemically mimic the profile of their female hosts (*e.g.*, Strohm *et al.*, 2008; Wurdack *et al.*, 2015), leading to a pattern in which closely related species can have very divergent CHC profiles and, distantly related species can converge to similar CHC profiles due to parasitizing similar hosts (Chapter 6). CHC profiles of males, however, are largely unaffected by the CHC profiles of their hosts (*e.g.*, Chapter 7), and changes in their CHC profiles are more gradual and probably affected mainly by sexual selection, to render them easily recognized and attractive to a conspecific female. Thus, males' CHC profiles show more phylogenetic signal.

By having explored differences in a more detailed manner here through the study of the CHC composition of both females and males in the family Chrysididae, I add indirect evidence to the findings that sexual dimorphism of CHC profiles in cuckoo wasps is largely driven by natural selection on females (Chapter 6). The level of sexual dimorphism is stronger in the tribe Chrysidini than in the tribe Elampini, and correlates with an increase in the number of CHC compounds differently produced by the two sexes (Figure 4.7). In fact, the few cases of CHC monomorphism that we observed occurred in members of Elampini only. Chemical deception in cuckoo wasps has been only shown in few species (Strohm *et al.*, 2008; Kroiss *et al.*, 2009; Wurdack *et al.*, 2015; Chapter 7), but it has been suggested to be common in the family (Schmitt, unpub. data; Bandorf *et al.*, 2017), probably because most species have a high degree of host specialization (Pärn *et al.*, 2015). In some cases, however, females may not profit from chemical mimicry, and thus may not have been selected to evolve a different chemical profile. Examples of this abound in the tribe Elampini. Females of species that parasitize Hemiptera-hunting crabronid wasps do not need to enter their hosts nests, but use a "hitch-hiking" strategy to get their eggs into the nest. They oviposit directly into their hosts' prey, which act as "Trojan horses" in which the parasite eggs are inadvertently transported into the hosts' nests. This behavior has been reported already for species of *Pseudolopyga* (Carrillo & Caltagirone, 1970), *Holopyga* (Veenendaal, 2012), *Omalus* (Winterhagen, 2015), as well as in *Pseudomalus* (Veenendaal, 2011 and Paukunnen *et al.*, 2015), all of them in Elampini and members of one monophyletic clade (Pauli *et al.*, accepted, Chapter 3).

In this study, one species that may not profit from chemical mimicry was relatively monomorphic (*Omalus aeneus*). Although the biology of the species is not well described, it has been reported already that females oviposit into live aphids that their hosts take to the nests (Paukunnen *et al.*, 2015) in a much similar way to the behaviour observed in *O. biaccinctus* (Winterhagen, 2015). Nevertheless, CHC dimorphism was found in members of species that oviposit into other Hemiptera (*e.g.*, *Holopyga* spp., *Pseudomalus* spp.) which requires a better understanding of the biology and a comparison to their hosts' profiles. In species of *Holopyga*, females showed very long chain lengths, with the production of many sex-specific alkadienes and alkenes at long chain

lengths (> C31), while the chain length of males reflects phylogenetic signal. It is possible that these CHC compounds, which are less volatile, may only be detected by direct contact, and may not be recognized when attached to the prey of their hosts. Nevertheless, until comparisons of CHC profiles of females with those of their hosts and their hosts' preys, are not undertaken, I can not discard that chemical mimicry may not be playing a role in these species. It is also possible that a lesser perfect chemical mimicry (or match of only very abundant CHC compounds with those of aphids or of their hosts may need to be achieved), thus CHC dimorphism caused by changes in the female cuckoo waps's CHC profile may be less strong in these species of Elampini than in Chrysidini.

Three members of another monophyletic clade in Elampini, all belonging to the genus *Hedychridium* were sexually monomorphic. In these species, the major differences between sexes were due to variation in the relative amounts of shared compounds and not to the contribution of compounds produced exclusively in one or the other sex. Moreover, in two species (*H. roseum* and *H. cupreum*), sex-specific CHC compounds were produced majoritarily by males (although in low amounts, figures 4.4 and 4.7). This suggests that, in this case, males are the ones producing different compounds, and that other selection pressures act to keep female profiles similar to those of their conspecific males. From the few observations that have been published on the nesting biology of *Hedychridium*, females are not known to employ the prey of their hosts as "Trojan horses", but enter their hosts' nests to oviposit directly (Kurczewski, 1967). Unfortunately, very little is known about the biology of the species in this study to suggest why monomorphism of CHC profiles was relatively common in this genus. Nevertheless, it is worth mentioning that species of *Hedychridium*, as many other in the tribe Elampini, are kleptoparasites, not parasitoids. Their larvae consume the prey of their hosts, not necessarily killing directly the host larvae, as parasitoid species do. Thus, it is possible, that some host larvae may develop from a parasitized nest, provided enough prey was supplied. Whereas, this represents a fitness reduction for the host, some offspring may survive still. Recent observations on the nesting biology of another *Hedychridium* species, have shown that few individuals of the host (4 *Soleriella compedita*) hatched from three nests parasitized with *Hedychridium monochroum* (of which 12 individuals hatched, Martynova, 2017). Evolutionary adaptations and counteradaptations in an evolutionary arms-race may evolve faster when the strength of selection is stronger, and also depend on the relative benefits/cost of evolving them. It is therefore possible that the relative differences in the degree of sexual dimorphism of CHC profiles (which may have originated due to selection acting on females to chemically deceive their hosts) observed between Elampini and Chrysidini may be linked to the preponderance of kleptoparasitic behavior in the former and the preponderance of a parasitoid lifestyle in the latter. This remains however, as an interesting hypothesis to test when the host-parasite relationships are better established.

An interesting observation further suggests that females are probably the sex that evolves more differences and contributes to the sexual dimorphism in CHC. *Chrysis mediata* and *Pseudospinolia neglecta* parasitize two different chemotypes of *Odynerus spinipes*, and their cuticular profiles resemble those of their respective host chemotypes (Wurdack *et al.*, 2015). Females of *C. mediata* are dominated by alkenes with double bonds at the 8th position, just as its host (Wurdack *et al.*, 2015) while their conspecific males produce alkenes with double bonds at central parts of the chain (14 and 12), as

many other males of the *Chrysis ignita* group do (strong phylogenetic signal, figure 4.3). Interestingly, females of other closely related species of *C. mediata* also have double bonds at the 8th position, but the proportion of alkenes with double bonds at position 8th is much larger in *C. mediata*, a possible adaptation to match that of their host. By analyzing the CHC profiles of *C. mediata* and closely related species and those of their hosts, it could be tested whether the increase in the production of alkenes with double bonds at position 8, is a synapomorphy in this species arisen due to the selective pressure exerted by its host.

4.5.3. Sex specific signaling

There was a difference in the occurrence of CHC compound class by sex. Unsaturated compounds occurred more often and were more abundant in females, whereas methyl-branched compounds occurred more frequently in males. The double bond positions of alkadienes could not be determined, but those of all alkenes were. A consistent sex-specific difference was found in the production of alkenes by the position of the double bond. While females produce mainly alkenes with double bonds at the 9th position, males produce alkenes with double bonds at more internal regions of the backbone (11-16); and in the latter sex this position presented phylogenetic signal (Figure 4.3). In 42 species (75% of the species analyzed here), the major differences between sexes were caused by a difference in the relative production of alkenes at these positions (Figure 4.8).

A comparative analysis of the patterns of sexual dimorphism may be the starting point, and thus provide clues, for discovering candidates of sex pheromones (Bernier *et al.*, 1998; Buda *et al.*, 2003). Sex pheromones of species of Coleoptera, Diptera, Hemiptera or Lepidoptera have been more frequently studied than those of Hymenoptera, because many of its members are important pests for agriculture. The great majority of sex pheromones in species of those orders include a variety of different chemical compounds (*e.g.*, acetates, alcohols, aldehydes) which are more effective as long-distance attractants. Polyunsaturated and saturated hydrocarbons have been attributed a short-range pheromonal role not only in Lepidopteran (Millar, 2000; Ando *et al.*, 2004), Dipteran (*e.g.*, Nemoto *et al.*, 1994; Ferveur, 1997; Doi *et al.*, 1997; Jurenka, 2004) or Coleopteran (*e.g.*, Fukaya, 2003, Peschke & Metzler, 1987; Ginzel *et al.*, 2003b), but also in Hymenoptera (Keeling *et al.*, 2004). Parasitic solitary species of Hymenoptera (*e.g.* Braconidae, Ichneumonidae) have been shown to use alkadienes for species recognition and courting (Keeling *et al.*, 2004) while social Hymenoptera employ alkenes and methyl-branched compounds for nestmate recognition (*e.g.*, *Polistes* spp, Dani *et al.*, 2001, ants, Kleeberg *et al.*, 2017). Alkadienes have also been suggested as male attractants in the almond seed wasp (Krokos *et al.*, 2001) and alkenes as components of sex pheromones in solitary pollinating bees (*e.g.*, Paulmier *et al.*, 1999).

Interestingly, when looking at the double bond position of alkene components of known sex pheromones, there is a consistent pattern. Often, (Z)-9 alkenes (and to lesser extent (Z)-7) have been found to be major component of female contact sex pheromones, which enhanced mating behavior of males in many species ranging from flies (*e.g.*, (Z)-9-C23:1, Carlson *et al.*, 1971, Richter *et al.*, 1976; (Z)-9-C23:1, Uebel *et al.*, 1976) to beetles (*e.g.*, (Z)-9-C25:1 in *Megacyllene robiniae*, Ginzel *et al.*, 2003b; (Z)-9-C29:1 in the closely related species *M. caryea*, Ginzel *et al.*, 2006; (Z)-9-C23:1,

(Z)-9-C25:1, (Z)-9-C27:1, (Z)-7-C25:1, and (Z)-7-C27:1 in the Asian longhorned beetle, *Anoplophora glabripennis*, Zhang *et al.*, 2003) and hymenopterans (*e.g.*, (Z)-9-C29:1, (Z)-7-C27:1 and (Z)-7-C29:1 in the woodwasp *Sirex noctilio*, Boroczky *et al.*, 2009; several (Z)-7 alkenes in the bee *Colletes cunilarius*, Mant *et al.*, 2005, (Z)-9 and (Z)-7 alkenes, particularly of chain length C25 are more dominant in younger females than in older females or males, and have been suggested to act as sex pheromones, Paulmier *et al.*, 1999). This suggests that the moieties and the chemical structure of these substances may have some advantages to be selected as a signal that does not only helps identifiying the (in many cases) limiting sex (because females are usually mated only once), but that may also enhance mating behaviour. In fact, the attractivity of some of these alkenes may be so strong that these are major CHC components being mimicked by orchid species to be pollinated by deceived male bees, see Mant *et al.*, 2005).

Although male contact sex-pheromones have been less reported in the literature, some alkenes have been identified to stimulate female mating behaviour and receptivity (*e.g.*, (Z)-7-C23:1 in flies, Grillet *et al.*, 2006). Another function of these CHC compounds can be preventing or reducing male homosexual courtship (cited in Grillet *et al.*, 2006).

Interestingly, whenever alkenes were not produced (or produced in low amounts) within one sex of any species, methyl-branched alkanes were biosynthesized. Males of bee-parasitizing chrysidids (*Chrysis analis, C. ehrenbergi*, etc.) produced monomethyl-branched alkanes instead of alkenes, the majority of them 9- and 8-monomethyl-branched compounds, which resulted in indicator compounds for these species (Chapter 5). These saturated hydrocarbons also showed strong phylogenetic signal. Methyl-branched alkanes have also been found to be sex pheromones in a number of species of several orders (*e.g.*, Coleoptera, Ginzel *et al.*, 2003a, Lacey *et al.*, 2008, Rutledge *et al.*, 2009; Diptera, Carlson *et al.*, 1998a; Lepidoptera, Ando & Yamakawa, 2015; Hymenoptera, Kühbandner *et al.*, 2012b).

No behavioral assay has been done to confirm the role of alkenes (or of monomethyl-branched compounds) as putative female/male contact sex-pheromones in this study. However, having identified potential compounds that may act as such, allows for the testing of these hypotheses, especially using relative commonly encountered species of cuckoo wasps (*e.g.*, *Hedychrum* species) for which some aspects of their biology have also been studied (*e.g.*, chemical deception for evading host detection, Strohm *et al.*, 2008, Kroiss *et al.*, 2009a; and even indications of sex pheromones, Kroiss, 2008). Even, if behavioural assays would be difficult to conduct with solitary brood parasite species, it should be possible to explore whether the specific alkenes that were selected as causing major differences between sexes are bioactive by conducting electroantennogram experiments.

4.5.4. Conclusions

The examination of sexual dimorphism of CHC profiles in a relative large number of species within an hymenopteran family has shown that CHC profiles may not only differ in the relative amounts of major compounds but that each sex may produce different CHC compounds. All major compound classes were occurring in Chrysididae, but there was a sex-specific difference in the frequency of occurrence of unsaturated compounds and methyl-branched compounds. While the former were more frequent

in females and the latter in males, unsaturated compounds (especially alkenes) often comprised the most abundant compounds in both sexes. There was a subtle increase in the number of CHC compounds, especially when grouping them by homolog series, when comparing species of the tribe Elampini with that of Chrysidini in males but not in females. Also, sex-specific compounds were more prevalent in the Chrysidini tribe with respect to Elampini, which resulted in more species showing sexual dimorphism in the latter. In general, the detailed comparison of CHC profiles supports that in these brood parasites, sexual dimorphism may have arisen due to strong selection acting on females to chemically deceive its host to inadvertently oviposit in their nests. Finally, a comparison of the compounds responsible for the major differences between sexes, allowed discovering a sex-specific consistent pattern in alkenes, with females exhibiting majoritarily alkenes with double bond position at the 9th carbon and males presenting double bonds at more internal locations (> 11). This sets the basis for the possible search of putative contact sex pheromones in the family Chrysididae.

5. Species-specific patterns of CHC in cuckoo wasps

5.1. Abstract

Cuticular hydrocarbons (CHC) are used by insects as a protective barrier against desiccation and as intra- and interspecific signals in communication. While insects utilize a wide array of chemicals for different processes (*e.g.*, defenses, signaling alarm pheromones, sex pheromones, etc.), CHC are usually regarded as one of the main means for intraspecific recognition in both social and solitary species. Recognition of members of the same species, should thus select for the evolution of species specific signals. Insects are by far the most-species rich class of animals in the world with many undescribed species. The identification and delimitation of species via morphology, thus, usually requires a high degree of expertise by specialists (taxonomists), whose numbers are decreasing worldwide. Genetic and genomic approaches to delimit species are then most commonly being used nowadays, but are nevertheless sometimes not helpful, especially with sibling species at early stages of divergence/speciation. I therefore explore the use of CHC in delimiting species by comparing CHC profiles of 59 species of cuckoo wasps (Hymenoptera: Chrysididae). In this family of parasitoid and kleptoparasitic wasps, I demonstrate that CHC are species- and sex-specific. Moreover, I evaluate the reliability of CHC as markers by comparing CHC profiles of different populations of five species of one genus of this family (*Hedychrum*) across geographic ranges. I found that although CHC profiles are intraspecifically (and intrasexually) variable, CHC are stable across populations and species are correctly delimited via CHCs. Overall, these results emphasize the usefulness of CHC as a complementary approach in taxonomy, where closely related species that are morphologically or genetically indistinguishable show very divergent chemical profiles.

5.2. Introduction

Species constitute the fundamental unit of biological diversity (de Queiroz, 2005b). Irrespective of the field and scope of study, species are then central in any biological study. In contrast to abiotic entities, species are composed of living organisms, thus apt to enormous variation and evolution. For this reason, their definition and delimitation impose several constraints, both of which have been subject of continuous debate (Mayden, 1997; de Queiroz, 2005a, 2007). Traditionally, species have been delimited by using (exclusively) morphological characters, which are meticulously scrutinized to differentiate between closely related species (Sites & Marshall, 2003). Unfortunately, the number of specialists able to describe and provide means for identifying species based on morphological characters, known as taxonomists, are dwindling in time (Hopkins & Freckleton, 2002; Krell, 2002; Kim & Byrne, 2006;

Werner, 2006; Bacher, 2012). Simultaneously, the revolution of molecular techniques (*e.g.*, amplifying DNA sequences via polymerase chain reaction) opened up new possibilities. As a result, the traditional morphological approach to both identify described species and discover new ones, has given rise to the molecular approach (*e.g.*, DNA barcoding using cytochrome oxidase subunit I, Hebert *et al.*, 2003). This approach has proven useful (Waugh, 2007; Schindle & Miller, 2005; Hajibabaei *et al.*, 2007; Packer *et al.*, 2009), and in many cases, it is the fastest and most reliable method to delimit and identify species, especially those that are morphologically challenging (*e.g.*, Bhadury *et al.*, 2006; Begerow *et al.*, 2010). Nevertheless, despite its broad benefits, and the reduction of the sequencing costs due to technological advances, there are still a number of potential problems associated with its use (*e.g.*, misconceptions and shortcomings in experimental design and analytical procedures summarized in Collins and Cruickshank, 2013), which need to be considered. In addition, although it may be useful for most of the species, it does not always provide conclusive results for species with too low intergenetic distance (*e.g.*, Soon *et al.*, 2014). Furthermore, it must be taken into account that whereas current major genomic and computational extensions are being applied (Coissac et al, 2016; Yang & Rannala, 2017), it will still take some time for them to be regularly used. Given the disagreements that might arise due to using one or another approach, combining information from different disciplines to use a multisource approach is being promoted more recently. This new "integrative taxonomy" "uses a large number of characters including DNA and many other types of data, to delimit, discover and identify meaningful, natural species and taxa at all levels" (Will *et al.*, 2005) avoiding the use of one single character system but integrating evidence from many others (Dayrat, 2005, Schlick-Steiner *et al.*, 2010; Padial *et al.*, 2010; Yeates *et al.*, 2011).

In insects, by far the most species-rich class of animals (Zhang *et al.*, 2013), an alternative source of evidence is possible and has been suggested to be helpful for delimiting species (Lockey, 1991, Bagneres & Wicker-Thomas, 2010; Kather & Martin, 2012). Insects possess on the external layer of their cuticle, a variable number of hydrophobic compounds, collectively known as cuticular hydrocarbons (CHC). These substances serve two important functions in an insect's life. They protect it from desiccation and they are used as chemical signals (and cues) in intra- and interspecific communication (Blomquist & Bagneres, 2010b). Chemical communication is the most important mode of communication in insects (Steiger *et al.*, 2011) and CHC are used as short-range contact pheromones in species and mate recognition processes (Singer, 1998). Species vary not only in the number of different hydrocarbons they possess on their cuticle but also in the amount each of these compounds are produced (Blomquist & Bagneres, 2010b). As such, they have been suggested to be species-specific (Singer, 1998; Blomquist & Bagneres, 2010b). Often however, the same species may possess more than one CHC profile that differs not only quantitatively but also qualitatively (*e.g.*, each of the castes of social species such as ants and bees, van Zweden & d'Etorre, 2010, or sometimes both sexes of the same species, chapter 4) and even within an individual's lifetime (*e.g.*, age, mating status, dominance status, Polerstock *et al.*, 2002, Liebig, 2010, Kuo *et al.*, 2012, Vanickova *et al.*, 2012). Despite these variations in CHC composition within a species, it should be expected that CHC should not vary much among individuals (belonging to the same sex) to avoid intraspecific recognition errors that could lead to wasting energy and resources mating with the wrong species. Whereas there have been several studies showing the usefulness of CHC in delimiting

closely related species, they have usually compared few species (< 12, Bartelt *et al.*, 1986, Page *et al.*, 1997, Berville *et al.*, 2013, Pokorny *et al.*, 2014, but see Lockey & Metcalfe, 1988, who compared CHC of 22 closely related species of tenebrionid beetles or Pokorny *et al.*, 2015, who compared CHC profiles of males of 35 species of orchid bees). Moreover, in order to be used as reliable markers for delimiting species, CHC composition needs to be relatively stable over geographical ranges. Few studies have compared CHC profiles of several related species of insects across large geographical ranges (*e.g.*, in ants, Martin *et al.*, 2008b; Berville *et al.*, 2013, Guillem *et al.*, 2016, in wasps, Bonelli *et al.*, 2015), showing that CHC are stable and appropriate to delimit species. However, these studies were all done on social species, having compared CHC profiles belonging to only one of the castes (*e.g.*, workers in the ants, or females of *Polistes biglumis*), and it would be interesting to study how CHC profiles vary across geographic ranges in (solitary) species possessing rather dimorphic CHC profiles. In addition, CHC profiles could be used in combination with other approaches to better resolve cases of species with little morphological differentiation. Indeed, whereas they have successfully been applied as a complementary approach in integrative taxonomy (*e.g.*, Schlick-Steiner *et al.*, 2006, Seppä *et al.*, 2011, Wachter *et al.*, 2015), their use is not so widespread.

Here, I study the CHC profiles of 59 species of parasitic wasps to demonstrate the usefulness of CHC in delimiting species. Cuckoo wasps constitute a large and diverse family of parasitoid and kleptoparasitic solitary aculeate hymenopterans (Kimsey and Bohart, 1991). At least 2500 species have been described worldwide (Aguiar *et al.*, 2013), but there are many species especially in the tropics and subtropics that are not yet, or have just recently been described (Kimsey and Bohart, 1991; Kimsey, 2012; Rosa *et al.*, 2016b; Lucena, 2018). In Europe, the majority of species present bright metallic coloration, making them an attractive group for insect collectors and entomologists for several centuries. Nevertheless, they are still considered a difficult group to identify based on morphological external characters (Kimsey and Bohart, 1991; Paukkunnen *et al.*, 2015). Members of the family Chrysididae in Europe belong to two species-rich subfamilies (Chrysidinae and Cleptinae) and they are all kleptoparasites and parasitoids of other solitary wasps and bees (Kimsey and Bohart, 1991). Furthermore, the species-rich subfamily Chrysidinae is subdivided into five tribes, three of which have representatives in the Paleartic region, and are used in this study (Elampini, Parnopini and Chrysidini). With few exceptions, the biology of most species remains little studied, but cuckoo wasps have a high degree of specialization on their hosts (Pärn *et al.*, 2015). Females of cuckoo wasps need to find the adequate host and enter their host's nest without being detected by the female host. Otherwise, the latter could eject the parasitic egg or abandon its nest before ovipositing, either of which might signify a fitness cost for the cuckoo wasp (Strohm *et al.*, 2008). It has been shown that some species of cuckoo wasps use some sort of chemical deception to overcome their host's first line of defense (*e.g.*, Strohm *et al.*, 2008, Wurdack *et al.*, 2015). In at least three species, chemical mimicry of the CHC profile of the host female by the parasitic female has been demonstrated (Strohm *et al.*, 2008; Wurdack *et al.*, 2015; Chapter 7) and unpublished evidence suggests that chemical mimicry may occur in more species (Bandorf, 2017). This also hints to an extreme degree of specialization by cuckoo wasps.

In cuckoo wasps, CHC profiles of females and males tend to differ not only quantitatively but also qualitatively with only few exceptions (Chapter 4). Thus, cuckoo

wasps provide a good model to evaluate the usefulness of CHC in species and sex specificity. Moreover, the most species-rich genus, *Chrysis,* is polyphyletic, defined by a number of non-exclusive characters, and with many species of unresolved taxonomy (Soon *et al.*, 2014). This heterogeneous genus was divided into many species groups by Linsenmaier (1951), of which *Chrysis ignita*, the largest species group, is still one of the most difficult ones containing many species morphologically alike (Soon & Sarma, 2011). Some attempts have been done to delimit species in this species group using morphological and molecular characters, and information on their hosts (Soon *et al.*, 2014, Orlovskyté *et al.*, 2016). Whereas the results of phylogenetic analyses have supported the taxonomic status of many of the species, they have also pointed out to the existence of cryptic species and shown that the genetic distance between some species is too low to correctly use barcoding for species delimitation in these cases (Soon *et al.*, 2014). It remains to be seen how chemically different, species of the *Chrysis ignita* group are among each other, especially among species that are genetically close. Additionally, I look at other genera containing closely related species and evaluate whether a comparison of their CHC would help for species delimitation in these groups. Five species of the kleptoparasitic genus *Hedychrum*, which are commonly found in several localities of Germany (one of the species also collected in Italy), are used to test how stable CHC are across geographic locations. Adaptations in the CHC composition of these species have been studied in the context of an evolutionary arms race with their digger wasps hosts (Chapter 7).

The overall aim of this study is to demonstrate that CHC can be used as a complementary approach in species delimitation. Thus, I attempt to answer the following questions: 1) Do cuckoo wasp species have species-specific profiles in both sexes? 2) Can CHC help differentiating morphologically difficult to separate species (*e.g. Chrysis ignita* species group)? 3) Are CHC profiles of species of *Hedychrum* stable over geographical ranges? 4) are certain CHC compounds useful for distinguishing and characterising groups of closely related species? 5) can CHC reflect phylogenetic relatedness? Finally, in any system, it is important to compare the level of between versus within species variation to correctly establish whether species form separate entities, therefore we compare variability in CHC profiles and ask 6) whether the between species variation is larger than the within species variation.

5.3. Material and Methods

5.3.1. Collection of samples

Insects were collected by netting between June 2005 and October 2014 in different locations of Europe and North Africa. A total of 1585 individuals belonging to females and males of 59 species were used in the different analyses conducted in this study. The number of insects used per species, sex and location of collection are summarized in the Appendix. After collection, each specimen was placed in a glass vial, transported to the lab, killed by freezing and stored at -20°C until the CHC extraction was conducted. After CHC extraction, all specimens were identified to species level by Oliver Niehuis.

5.3.2. GC/MS analysis

To extract CHC from insects, n-hexane was used as solvent and added to each glass vial. The extraction time was 10 minutes, after which the insect was stored in 100% ethanol and the CHC extract was concentrated to a volume of ~80 mL, by evaporating it under a gentle CO_2 stream. The extract was subsequently analyzed with a gas chromatograph coupled to a mass selective detector (GC/MS).

Chemical analyses were conducted on either a HP 6890 GC coupled with a HP 5973 MS (Hewlett Packard, Waldbronn, Germany) or on an Agilent 7890/5975 GCMS System. The GC (split/splitless injector in splitless mode for 1 min, injected volume: 1 µL at 300°C injector temperature) was equipped with a DB-5 Fused Silica capillary column (30 m x 0.25 mm ID, df = 0.25 µm, J&W Scientic, Folsom, USA). Helium was used as carrier gas with a constant flow of 1 mL/min. Both GC/MS were run with the same temperature program: start temperature at 60°C, with an increase of 5°C/min until 300°C were reached, then and isotherm at 300°C for 10 min. An ionization voltage of 70 eV (source temperature: 230°C) was set for the acquisition of the mass spectra by electron ionization (EI-MS).

Dimethyl disulfide (DMDS) derivatives were prepared for each sex and species following the protocol of Carlson and colleagues (Carlson *et al.*, 1989). When the relative amount of unsaturated compounds (CHC peak area) was low, up to five individuals were pooled. DMDS derivatives enable the determination of the double pond position of unsaturated compounds. Since the double bond position of alkadienes was not determined, they were grouped according to their retention indices.

5.3.3. Characterization of cuticular hydrocarbons

The large diversity of CHC compounds can be classified into three major groups depending on the presence of special features: alkanes are straight chains composed of carbon and hydrogen atoms, unsaturated compounds possess one or more double bonds inserted along the chain, and methyl-branched compounds possess one or more methyl groups along the chain. The existence of double bonds and methyl groups in the same compound is also possible but much rare (*e.g.*, methyl-branched alkenes, Menzel *et al.*, 2008, Martin & Drifjhout, 2009a; Kather & Martin, 2015). The insertion of double bonds and methyl groups into the chain confer the CHC compound a "pseudobent conformation" (Dani *et al.*, 2001) which can be detected and distinguished easier by insects than straight alkane chains (Dani *et al.*, 2001, Dani *et al.*, 2005). For this reason, unsaturated and methyl-branched compounds are hypothesized to be preferably used in a communication context whereas alkanes may be more involved in antidesiccation primarily (Gibbs, 1998, Dani *et al.*, 2001; Chung & Carroll, 2015). Given these differences in the functions of compound classes, compounds are grouped and evaluated separately whenever indicated.

I ran batch jobs in AMDIS (Automated Mass Spectral Deconvolution and Identification System, http://chemdata.nist.gov/mass- spc/amdis/), and processed them using custom scripts in R version 3.0.2 (R Core Team, 2013). AMDIS requires a mass spectral library and may also use retention indices to select target peaks. I generated a mass spectral library that contains more than 900 identified mass spectra of common hydrocarbons and their retention indices (see Chapter 8). Retention indices were used to correctly identify methyl-branched alkanes in this library (following Carlson *et*

al., 1998b,, Chapter 9). The parameters used in AMDIS were as follows: component width = 22, adjacent peak subtraction = 2, resolution = medium, sensitivity = low, shape requirements = medium). Refer to Chapter 8 for further explanations of the procedure applied in AMDIS to identify and quantify target peaks suitable for CHC analyses. Non-hydrocarbon compounds (*e.g.*, acetates, alcohols, esters) were excluded from all analysis. Note that even when AMDIS can separate coeluting compounds, a complete separation is impossible if the retention time of elution is too similar. Therefore, some CHC compounds were grouped together and are referred as mixes (*e.g.*, 11-13-15 monomethyl-branched compounds).

5.3.4. Statistical analysis

5.3.4.1. Species specificity and comparison among all species

For comparing data among all species, a mean CHC profile per species and sex was calculated. To obtain a representative CHC profile per group, I removed CHC compounds that were infrequent (present in less than 50% of the individuals) or whose relative abundance was negligible (the mean relative abundance fell below 0.1% of the total ion count of the chromatogram) in any group (each sex and each species considered separately). Afterwards, the mean of each of those representative CHC compounds in each group was calculated. Thus, the final dataset contained 180 CHC compounds or mixes of them between C21 and C35 (Appendix).

In the comparison among all species, CHC profiles of 1221 wasp specimens (622 females, 599 males) were used. The number of samples per species and sex varied. In some cases, only one individual was available in one of the sexes (*e.g.*, *Hedychridium caputaureum, Chrysis propinquata, Elampus foveatus*). One species lacked representatives of one sex (*Elampus panzeri*). Otherwise up to a maximum of 15 individuals were used to calculate the mean CHC profile of a group.

To assess the variation of cuticular profiles among individual samples and species, I used non-metric multidimensional analysis (NMDS) based on Bray-Curtis dissimilarity indices, which is generally preferred in ecology because it only considers shared entities (*e.g.*, species, or in this case, CHC compounds) between sites (here, species) in the calculation of the similarity (Kindt & Coe, 2005). Thus, shared absence (zero values) of compounds do not influence the measurement of similarity. NMDS allows the visualization of similarity among individuals in a two- (or three-) dimensional space (Kruskal, 1964a; 1964b). The closer individuals are depicted in the reduced space, the more chemically similar they are. In NMDS, the goodness of fit between the spatial representation and the dissimilarity matrix used to infer the graph is reflected by the stress value. Values below 0.05 are considered a good fit, whereas values above 0.20 indicate unreliable representations (Kruskal, 1964a). Additionally, I used ANOSIM (ANalysis Of SIMilarity, Clarke, 1993) with Bray-Curtis dissimilarities to test whether the observed differences in cuticular profiles could be attributed to predefined groups: each species and sex separately, or considering sex separately. When ANOSIM was done on all species included together, 999 permutations were conducted. NMDS, Bray-Curtis dissimilarities and ANOSIM values were calculated in R (version 3.0.2) using the package vegan (Oksanen *et al.,* 2013). In all the above analyses, particular attention was given to two groups of closely related species: 1) the *Chrysis ignita* species group, composed of many morphologically similar species, and appar-

ently some cryptic species (Soon & Sarma, 2011), and for which 11 representative species are included, 2) species of *Hedychridium*, some of them are morphologically so similar that were thought to belong to one species (Niehuis, 2001). For these last mentioned groups of related species, I conducted additional NMDS analyses which differ from the others in that they include both sexes of the species belonging to each group on a single analysis.

5.3.4.2. Stability of CHC across geographic locations

For the comparison of CHC of *Hedychrum* species across geographic ranges, 522 insects collected from 24 localities were used (see Appendix for collection information). Geographic separation among localities varied between a few to several hundred kilometers. Some species are more commonly encountered than others, and therefore, the number of localities per group (each sex of each species) varied between three (males of *H. chalybaeum*) and nine (males of *H. gerstaeckeri*). On average, each group was collected from 5.6 different localities, and no difference was observed in the number of localities per sex (5.2 in females, 6 in males; $t(8) = -0.59$, $p = 0.572$). Although the number of males was almost four times larger than that of females (110 females vs. 412 males), the number of insects collected per locality did not vary per sex ($t(16)=-1.27$, $p = 0.223$). However, female individuals were less often caught than conspecific males in at least three species (*H. gerstaeckeri, H. nobile* and *H. rutilans*), with some localities showing a number of males an order of magnitude larger than that of females. All CHC compounds detected between C18 and C35 in all specimens (522 wasps) accounted for a total of 186 CHC or mixes of CHC. Compounds below C21 and 19 rare hydrocarbons (*i.e.*, occurring in less than 5 out 522 individuals) were discarded. In the end, 159 CHC or mixes of CHC between C21 and C33 (retention index of 3350) were used in the analyses. Note that in this case, a larger number of CHC compounds are reported and used in the analyses of species of *Hedychrum* in comparison to other studies (Chapters 7 or the analysis including all species). This is because CHC compounds occurring in less than 50% of the individuals in each group were not discarded due to the low number of individuals sampled at some localities (1-3). The majority of these compounds are of scarce abundance (monomethyl-branched compounds with methyl groups at even positions and dimethyl-branched compounds). Two procedures were used to select a representative CHC profile per species, sex and locality. First, an average mean profile per species, sex and locality was calculated. Second, a representative sample was selected for each group by choosing that sample having the shortest distance to the centroid of an NMDS plot (calculated as explained in the section above). The aim was to see if the different groups would correctly aggregate together (by sex and species), irrespective of the locality of collection, thus variation introduced by geographic factors would not influence delimitation of species. I conducted hierarchical cluster analyses using the Ward's method, which has been applied to analyses of CHC (*e.g.*, Martin *et al.*, 2008; Bonelli *et al.*, 2015). All analyses were calculated for each sex separately. First, I compared the effect of using relative amounts or only the presence/absence of CHC compounds. Then, since different compound classes may have different communicative roles, I tested whether the inclusion of n-alkanes allowed for a better separation of species by removing them from the dataset before the calculation of the dissimilarity matrix. Similarly, I tested the effect of alkenes and methyl-branched alkanes. Furthermore, I also looked at the influence

of scarce compounds on the analyses, by removing all those CHC that accounted for less than 0.5% of the total ion count in all species. This reduced the dataset consisting of 159 CHC to only 47 CHC, and allowed me to test the effect of having used a larger number of CHC in the analyses. The effect of using two different distance metrics (Euclidean distance, or by default Bray-Curtis dissimilarity) and different clustering methods was also evaluated (average, complete, single and Ward).

Additionally, I tested the association between geographical and chemical distances for each species and sex by conducting a correlation Mantel Test using 9999 permutations. I used distances calculated among individuals and not among mean CHC profiles for each locality in the pairwise comparisons. To calculate geographical distances, I used a function created by Scott Chamberlain and available via github (https://gist.github.com/sckott/931445) which calculates a matrix of pairwise geographic distances between localities. Chemical distances (dissimilarity index) were calculated in all cases using the function vegdist of the R package vegan (Oksanen *et al.*, 2013). Other functions used for plotting were taken from the R package geosphere (Hijmans *et al.*, 2017). Cluster analyses were done using the function hclust in R (version 3.02, RCore Team, 2013).

5.3.4.3. Indicator Analysis

Some cuticular compounds can be indicative of a certain species or a group of species. In order to identify these indicator compounds, I performed an indicator value analysis (IndVal, Dufrene and Legendre, 1997), an approach that is commonly used in community analyses (*i.e.*, in environmental biomonitoring to identify indicator species of environmental disturbances). The indicator analysis can help detecting significant indicator compounds which can be used as diagnostic for species or species groups. The analysis uses relative abundances of compounds together with their frequency of occurrence in the species to produce an indicator value for every compound in the dataset. This indicator value is the product of two types of information: i) compound specificity (*i.e.*, the percentage of individuals within a group that produces the compound) and ii) compound fidelity (*i.e.*, the percentage of other groups which may also produce that compound). An indicator value of 1 (maximum) indicates that the compound appears exclusively within a group and that each individual within that group presents the compound, whereas an indicator value of < 1 suggests that the compound may not be present in all individuals of that group or that it is also present in other groups. In IndVal analysis, it is necessary to define groups *a priori*. This can be done via a hierarchical or non-hierarchical classification method, which is independent of the IndVal analysis. Additionally, clusters can also be defined based on any other information. I conducted two types of analyses. In the first one, I selected the number of clusters rendering the largest summation of significant indicator values following a methodology explained in Hetherington-Rauth and Ramírez (2016). Indicator values for varying k-means partitioning values (1–20) were calculated in each dataset (females and males separately), and k values producing the maximum summation of significant indicator values ($p < 0.05$) were chosen. Thus, $k = 10$ was used when analyzing the dataset comprising the CHC profile information of females and $k = 13$ when males were analyzed. In the second analysis, the selection of groups was based on the phylogenetic relationships, and the number of clusters used was the same for both sexes ($k = 14$). In addition, I ran an Indval analysis not only for the

mean CHC values per species but also using the total number of specimens available for each species and sex. In this case, I selected the number of clusters using the first approach mentioned above (k in females = 16 and k = 20 in males). The aim of this last analysis was to test how individuals belonging to different species would group. Indval analyses were carried out using the indval function in package labdsv v1.6.1 (Roberts, 2013) in R.

5.3.4.4. Estimating phylogenetic relationships among species using hydrocarbons

Cluster analyses are commonly employed to explore and visualize the relatedness of CHC profiles (*e.g.*, Nelson *et al.*, 2008, Martin *et al.*, 2008a). I first utilized a clustering method (Ward, as performed in the analyses of *Hedychrum* species). Procedures applied to molecular sequence data have only rarely been used for hydrocarbons (parsimony analyses: Kaib, 1991, Page *et al.*, 1997; Page *et al.*, 2002; Neighbor Joining (NJ): Bonelli *et al.*, 2015). Therefore, and as a comparison, I also employed an improved version of the NJ algorithm (Saitou & Nei, 1987), which is one of the most popular and fast methods for reconstructing phylogenetic trees from a matrix of pairwise evolutionary distances. This improved version (BIONJ) outperforms the simpler NJ, especially when substitution rates are expected to be high and very variable among lineages (Gascuel, 1997). Since CHC compounds probably evolve fast (especially in females, see Chapter 6), the choice of BIONJ over NJ seems appropiate. Another advantage of applying this approach is that it was possible to root the tree with the CHC profile of *Cleptes semiauratus*, ancestral to all other Chrysidinae in this study (see Chapter 3). The matrix of pairwise distances in both procedures were calculated in the same way (Bray-Curtis dissimilarity using the vegdist function in R package vegan, Oksanen *et al.*, 2013). Analyses were conducted separately for each sex, since cuckoo wasps have been shown to exhibit highly dimorphic CHC profiles (Chapter 4). To build these trees, two types of data were used: a matrix containing mean CHC profiles per species and sex, and a matrix containing one individual CHC profile for each species and sex. The CHC profile selected in the latter case was one showing the shortest distance to the centroid in an NMDS calculated with Bray-Curtis dissimilarities (as explained in the previous section). These phylochemical trees were built to assess how good the match between a phylogenetic tree based on molecular data (Chapter 3) and a chemical-based tree is, and evaluate the usefulness of CHC in chemosystematics. To conduct all analyses and plotting of the trees packages vegan (Oksanen *et al.*, 201), ape (Paradis *et al.*, 2004), phytools (Revell, 2012) were used in R version 3.02.

5.4. Results

5.4.1. Species specificity and intraspecific variability

The species and sex-specific composition of CHC has been described in Chapter 4. In total, 180 unique or coeluting CHC (15 n-alkanes, 72 alkenes, 26 alkadienes, 41 monomethyl-branched alkanes, 25 dimethyl-branched alkanes and one trimethyl-branched alkane) were used in the analyses (Appendix). The overlap in chemical space of all species was visualized with NMDS. However, plotting all species and

sexes together in a two-dimensional plot resulted in a difficult to visualize separation of groups. Nevertheless, when plotting females and males separately, it was possible to observe that within species variability was lower in males than in females, and that the CHC profiles of males showed less overlap among species than those of females, both within the tribe Chrysidini and the tribe Elampini (Figures 5.1 and 5.2). A three-dimensional NMDS plot shows that all species and sexes are separated in the chemical space with only some little overlap among closely related species (Appendix). The ANOSIM analysis revealed that all groups are separated (R: 0.971, p = 0.001). As in the NMDS, this separation is stronger in males (R: 0.986, p = 0.001) than in females (R: 0.909, p = 0.001). Calculations of intraspecific variation based on Bray-Curtis dissimilarities also showed larger intraspecific variation in males than in females (Appendix).

Chemical similarity among closely related species varied depending on the clade and on the sex (Figures 5.1 and 5.2). Females of the genus *Chrysura* (excluding *C. ciscirtana*) are chemically more similar than males. However, males of the closely related species of the *Chrysis ignita* group (with the exception of *C. mediata*) cluster all together while females are more chemically different among each other showing less overlap in their chemical space (Figure 5.3a). Nonetheless, females of closely related sister species appear next to each other in the chemical space (*e.g., Chrysis iris* and *C. fulgida, C. longula* and *C. angustula*). Distantly related species of basal Chrysidini do not overlap in chemical space. Within Elampini, *Holopyga* and *Elampus* are more chemically apart than other closely related species both in females and males. However, species of *Hedychridium* and *Hedychrum* cluster differently in chemical space between both sexes. Males cluster chemically in a way that resembles their phylogenetic relatedness while females do not (*e.g.*, *Hedychrum niemelai, H. chalybaeum* and *H. nobile*). Particularly interesting is the case of *Hedychridium*. Females of *Hedychridium roseum, H. caputaureum* and *H. valesiense* are all chemically different. However, males possess chemically similar profiles with those of *H. valesiense* being undistinguishable with those of *H. roseum* (see Figure 5.3b). Overall, males cluster chemically more similarly to the phylogeny than the NMDS of the females (Mantel Test of females: r=0.07151, p = 0.116; males: r=0.3463, p < 0.0001). For example, species of Elampini are all grouping on one side of the NMDS in males, whereas females tend to be more dispersed in the chemical space (Figures 5.1 and 5.2).

5.4.2. Stability of CHC across geographic regions

Among the 159 CHC compounds used in the comparison of *Hedychrum* across geographic regions, there were 21 alkadienes, 38 alkenes, 27 dimethyl-branched alkanes, 60 monomethyl-branched alkanes and 13 n-alkanes (Appendix). The cluster analysis showed that species could be correctly delimited in most of the cases, but that in neither males nor females all species could be unequivocally assigned to a separate cluster irrespective of the locality of collection (Figure 5.4). For example, in females, one locality of *H. nobile* clustered with *H. rutilans* and another with *H. niemelai* (Figure 5.4a). In the case of males, individuals of *Hedychrum niemelai* collected in three localities grouped with its closely related species *H. chalybaeum* while *H. niemelai* of three other localities clustered with the other closely related species *H. nobile* (Figure 5.4b). When looking at the effect of different compound classes in the delimitation of species, the use of only unsaturated compounds, did not improve the clustering.

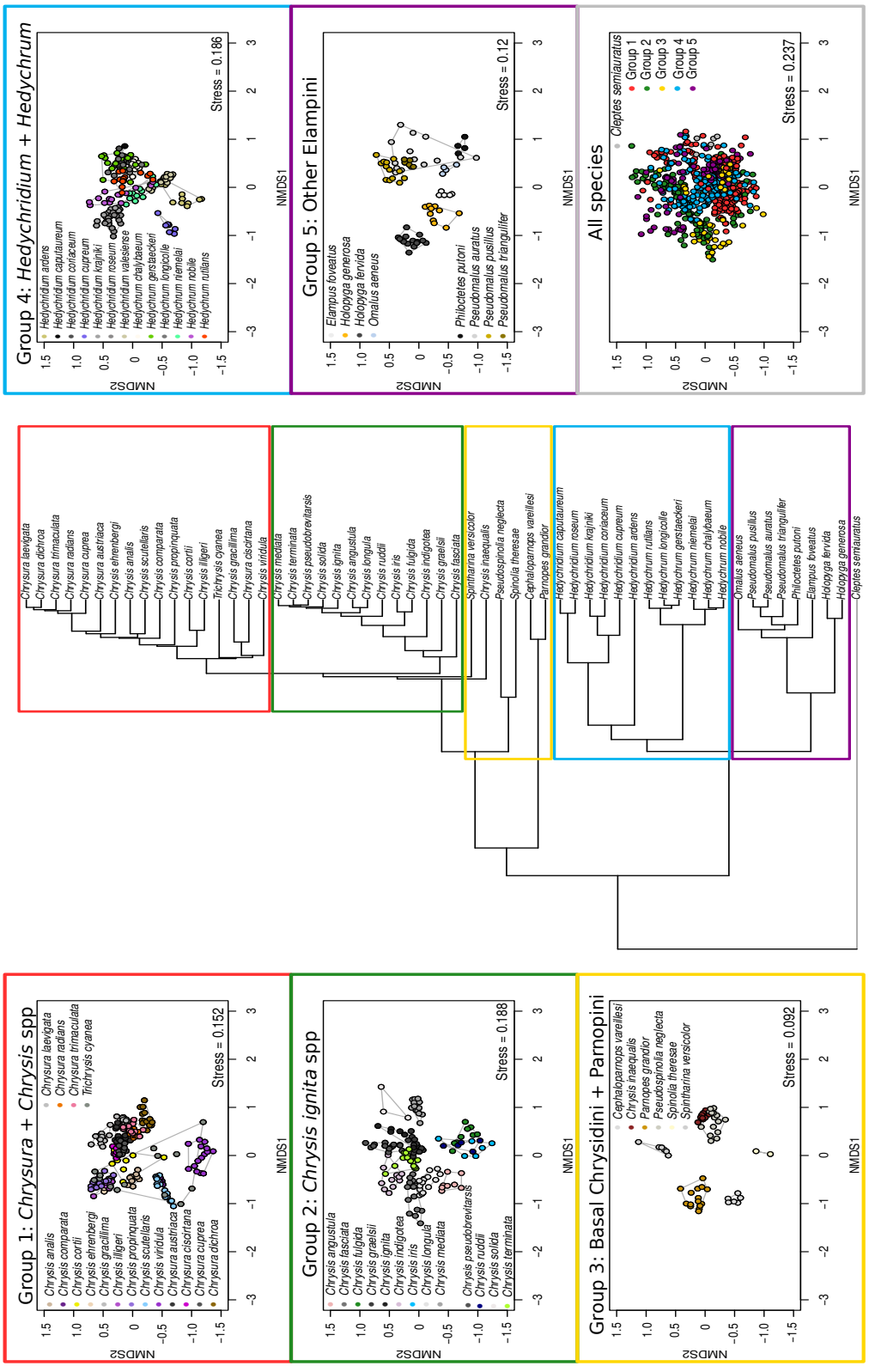

Figure 5.1.: Non-metric multidimensional scaling (NMDS) on a two dimensional scale using Bray-Curtis dissimilarity index of cuticular hydrocarbons in females. Species have been separated into five groups according to phylogenetic relatedness indicated by the different colors of the clades and boxes in the graphs. Each NMDS is plotted at the same scale for easeness of comparison. The sixth NMDS at the bottom right shows the total dataset for a) females and b) males together and colored according to the above defined clades. Stress values are also provided in each graph. Female individuals are indicated with a circle. Note that *Hedychridium valesiense* (sister species of *H. roseum*) is not included in the phylogenetic tree.

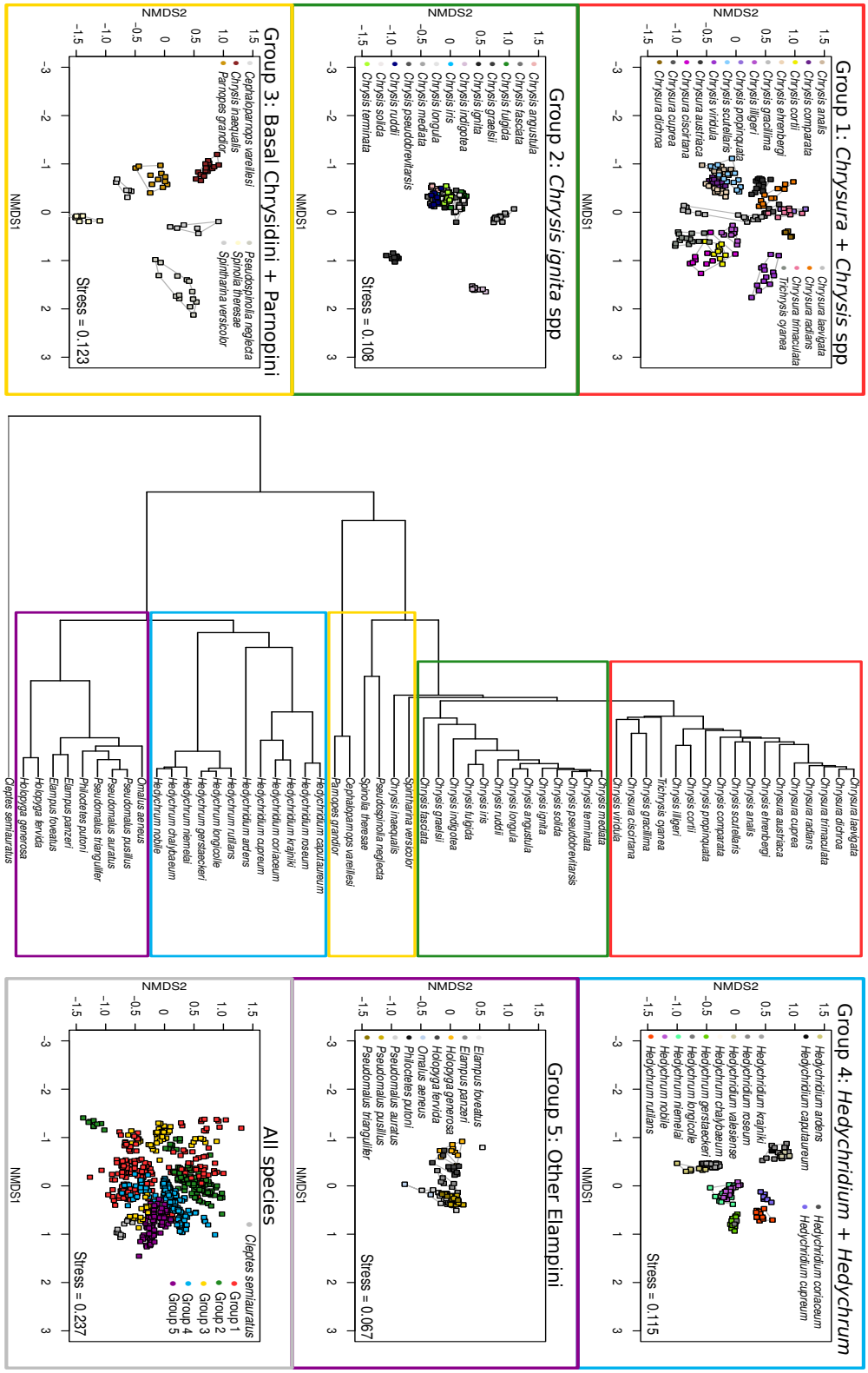

Figure 5.2: Non-metric multidimensional scaling (NMDS) on a two dimensional scale using Bray-Curtis dissimilarity index of cuticular hydrocarbons in males. Legend as in previous graph. Here, males are indicated as square symbols.

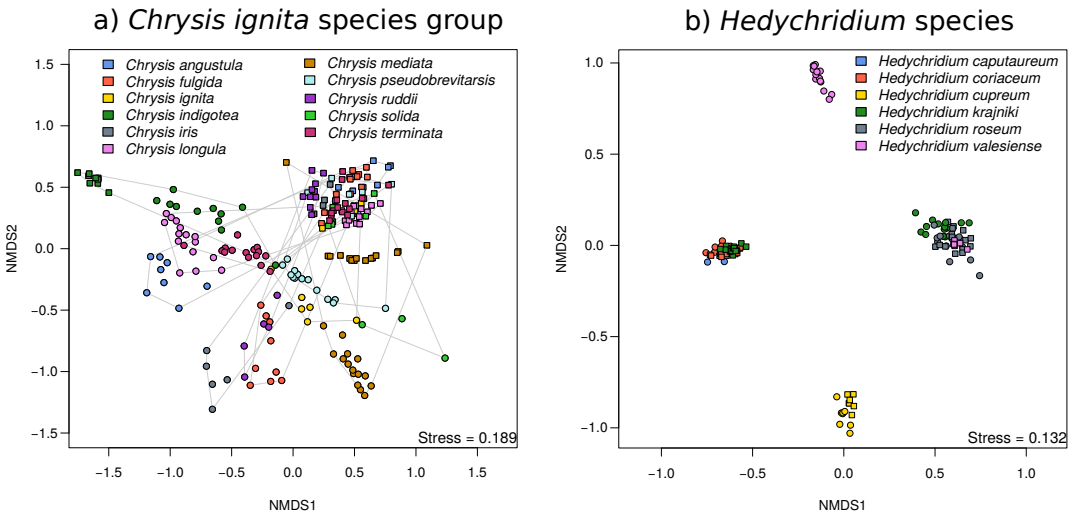

Figure 5.3.: a) NMDS on a two-dimensional scale using Bray-Curtis dissimilarity index of CHC in closely related species that are morphologically difficult to distinguish. a) *Chrysis ignita* species group, b) *Hedychridium* species. Females and males are plotted together, female individuals are indicated by a circle and males by a square symbol.

However, excluding the linear alkanes from analysis, provided a better delimitation of species in both females and males (Appendix). Reducing the number of compounds used in the cluster analysis (by removing many of the scarce CHC compounds, most of them methyl-branched compounds), did not have any effect, suggesting that the observed cluster patterns are due to CHC compounds that occur in relatively high amounts. On the contrary, reducing the matrix to one of presence/absence had negative effects because species did not cluster separately anymore (Appendix). Using a sample with the shorter distance to the centroid in an NMDS, instead of a mean profile, did not improve the clustering of females, but did improve the clustering pattern in males, especially for the closely related species *H. niemelai* and *H. nobile*, with only one population of *H. niemelai* remaining clustered within *H. nobile* (Appendix). Of the hierarchical clustering methods, Ward's and average performed best.

Moreover, no pattern emerged with respect to the geographic distance of the populations and their chemical similarity. Geographically close localities did not appear together in the clusters. However, there was a general pattern in which females showed stronger positive correlation between geographic distance and chemical distance, while males did not. Nevertheless, these results might be interpreted with care due to the low number of individuals available for some of the localities. In fact, the only species in which chemical distances among females were not correlated with geographic distances was *H. rutilans*, and this may have resulted because of the low number of individuals in the only geographically separate locality where females of this species were caught (Figure 5.5).

5.4.3. Indicator Compound analysis

Indicator compounds were identified in females and in males separately. The IndVal analysis which did not take into account the phylogeny, grouped species into 13 groups in males and 10 groups in females. In this analysis, many more CHC were significant

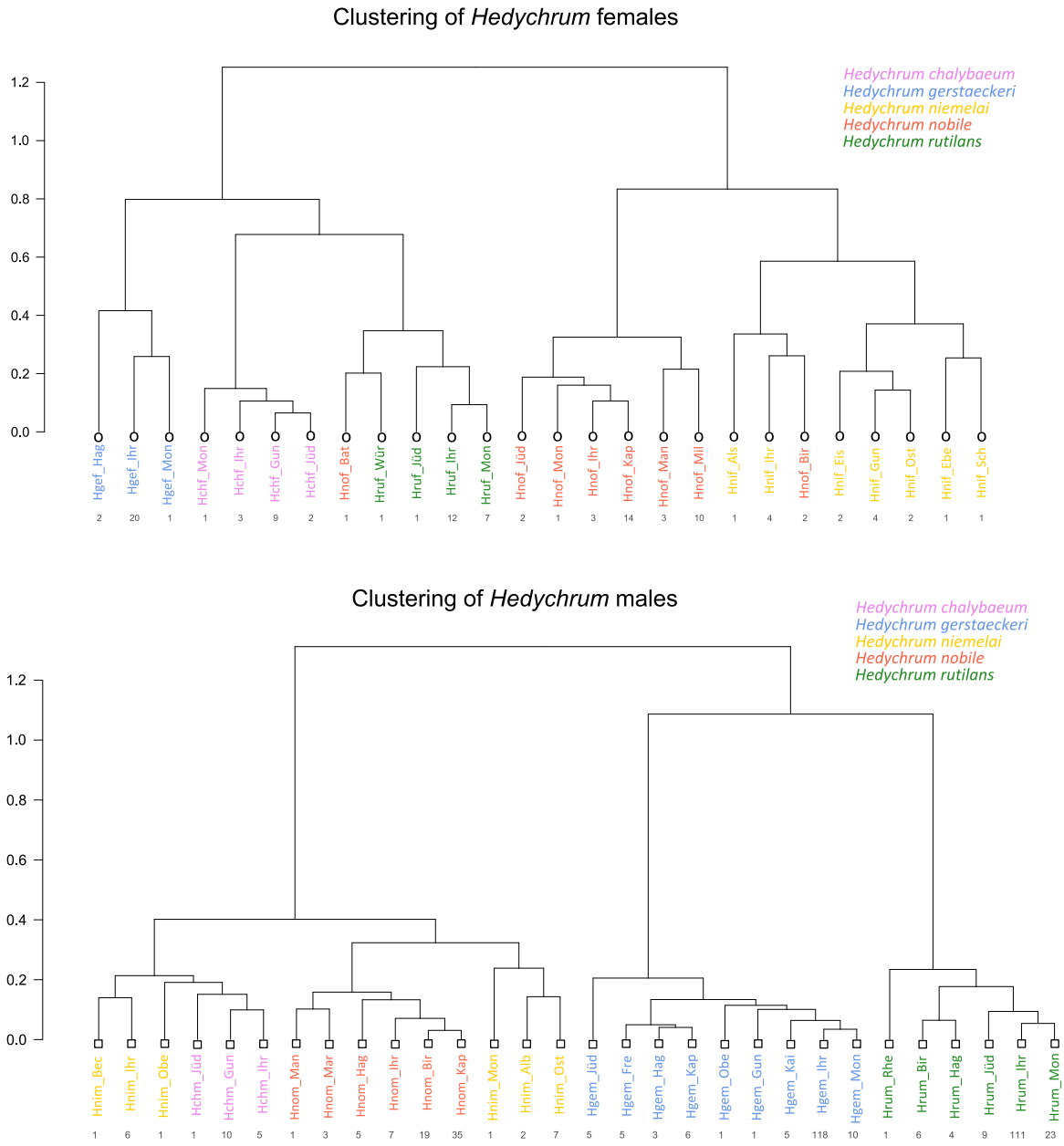

Figure 5.4.: Dendrograms based on hierarchical cluster analysis (Ward's Method on Bray-Curtis dissimilarity matrices) of CHC profiles in a) females, b) males of *Hedychrum* species. Species are indicated by different coloring and by the first three letters of the labels. The locality code is found in the Appendix and follows the hyphen. Numbers below the labels indicate the number of specimens used to calculate a mean CHC profile for each group (specimens sampled at the same locality).

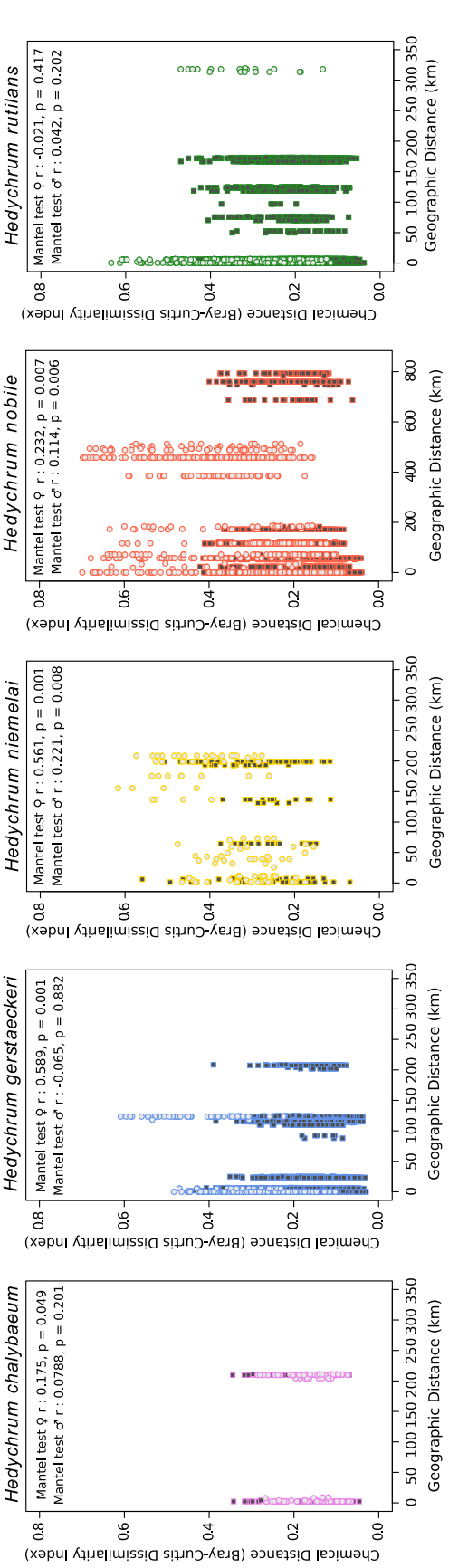

Figure 5.5.: Geographic distance vs. chemical distance of *Hedychrum* species. Colors used as in the previous figure. Clear-colored circles indicate pairwise chemical/geographic distances in female individuals, while dark-colored squares indicate distances between males. Note the different x-axis scale used in *H. nobile*, which was sampled across a larger geographic range.

(70 CHC in females and 81 CHC in males) than when using the phylogeny to define the clustering groups (24 CHC in females and 29 CHC in males, Appendix). In the former analysis, the association of species according to shared similarity of their compounds was more congruent with the phylogeny in males than in females (Appendix). When instead the phylogeny was used to define the clusters of species, the number of significant groups reduced to 8 in males and 5 in females (out of the original 14, tables 5.1 and 5.2). As a general pattern, CHC compounds belonging to the same homolog series (only varying in chain length, but sharing the same position at which methyl groups or double bonds are inserted) were significant indicator values in many of the species associations in both type of analyses. For example, long carbon chain alkenes (C28-C31) with double bonds at internal positions (13-14), seemed to define male species belonging to *Chrysis ignita* species group. On the other hand, 9-monomethyl of C22-C27 were good indicator compounds of males of *Chrysis ehrenbergi, C. scutellaris, C. analis* and *C. comparata*. Long chain alkenes (C29-C33) with double bond position at the 11 carbon and long chain alkadienes were good indicator values of females of *Holopyga fervida, H. generosa* and *Elampus* species, whereas (Z)-11 alkenes of C31 and C33 were good indicator compounds for males of the same latter species.

When using the total number of individuals in each species (both sexes separately) a larger number of groups were selected (16 in females and 20 in males). Interestingly, in this IndVal analysis male individuals belonging to the same species were grouped correctly together with only one exception (*Elampus panzeri*). In females, however, individuals belonging to 19 species were separated into different groups, because their CHC profiles were similar to those of other species, and there tends to be larger intrasexual variability in females of all species (Appendix). In general, this IndVal analysis conducted with all samples rendered a very large number of significant compounds because p values increased due to the large number of samples used (around 600 in each case). Nevertheless, the fact that the compound is significant, does not indicate that it is good. In general, the higher the indicative value, the better (see Methods).

5.4.4. Estimating phylogenetic relationships from CHC profiles

The cluster analysis of CHC profiles across species in females showed very little phylogenetic signal. Only in few cases, did closely related species group together, but almost always there were species of different clades intermixed. One exception was the genus *Chrysura* which grouped separately (except for one species, figure 5.6a). The cluster analysis of males showed, however, better agreement with the phylogenetic tree, but still important differences appeared. Elampini species group correctly together, although the clade containing *Hedychridium roseum* and closely related species grouped with other species of Chrysidini, particularly some parasitizing bees (Figure 5.6b). Due to the similarities of the CHC profiles of the *Chrysis ignita* species group, males of most species clustered separately from all other species. However, males of *Spinolia theresae* were also included in this group. Males of many *Chrysura* species and related species also clustered together (Figure 5.6b).

The trees calculated using the Neighbor-Joining approach, yielded similar results, showing that the relationships obtained by using CHC profiles of males were more congruent with the phylogenetic tree calculated with molecular sequence than those calculated with CHC profiles of females (Figure 5.7). One particular similarity was the main splitting of the species into three large clades: one containing almost all Elampini

Table 5.1.: IndVal analysis of CHC profiles of female cuckoo wasps. 24 CHC compounds can be used as indicators of 6 groups of related species. Clustering groups defined based on the phylogenetic relatedness. Frequency refers to the number of species in which the CHC compound also appears. The closer the value is to 1, the more exclusive the indicator compound is and the better it defines the group. If it is 1, it only occurs in the taxa that define the group.

Group	Taxa	Indicator value	P value	Frequency	CHC compound
1	*Chrysura spp.*	0,372	0,031	35	(Z)-9-C29:1
		0,342	0,038	48	(Z)-9-C27:1
2	*Chrysis analis, C. comparata, C. ehrenbergi, C. scutellaris*	0,689	0,034	8	(Z)-9-C22:1
		0,524	0,018	43	(Z)-9-C23:1
6	*Chrysis fasciata, C. graelsii, C. indigotea*	0,406	0,048	35	Mix: 11 and 13MeC27
9	*Cephaloparnops vareillesi, Parnopes grandior*	0,503	0,039	24	5MeC27
		0,436	0,046	29	5MeC25
		0,352	0,016	35	Triacontane
		0,207	0,014	58	Heptacosane
		0,204	0,025	57	Hexacosane
		0,165	0,047	58	Nonacosane
13	*Elampus foveatus, Holopyga fervida, H. generosa*	1,000	0,001	3	(Z)-11-C33:1
		0,992	0,001	4	(Z)-11-C29:1
		0,963	0,001	5	(Z)-11-C31:1
		0,667	0,046	2	C32 alkadiene C
		0,667	0,027	2	C33 alkadiene C
		0,667	0,026	2	C33 alkadiene D
		0,667	0,023	2	C33 alkadiene E
		0,667	0,025	2	C35 alkadiene B
		0,667	0,029	2	(Z)-11-C30:1
		0,599	0,035	4	C31 alkadiene D
14	*Cleptes semiauratus*	0,841	0,011	8	(Z)-7-C22:1
		0,658	0,011	43	(Z)-7-C23:1
		0,359	0,024	57	Heneicosane

Table 5.2.: IndVal analysis of CHC profiles of male cuckoo wasps. 29 CHC compounds can be used as indicators of 9 groups of related species. Same as in legend of 5.1.

Group	Taxa	Indicator value	P value	Frequency	CHC compound
2	*Chrysis analis, Chrysis comparata, Chrysis ehrenbergi, Chrysis scutellaris*	0,998	0,001	5	8MeC24
		0,997	0,001	5	9MeC24
		0,986	0,001	10	9MeC23
		0,984	0,001	5	9MeC22
		0,972	0,001	6	9MeC26
		0,754	0,001	20	9MeC25
		0,744	0,006	6	7,11 diMeC25
		0,707	0,007	15	9MeC27
		0,409	0,035	31	7MeC25
5	*Chrysis iris, C. fulgida, C. ruddii, C. mediata, C. terminata, C. pseudobrevitarsis, C. solida, C. ignita, C. angustula, C. longula*	0,548	0,016	9	(Z)-14-C31:1
		0,364	0,015	17	(Z)-14-C29:1
6	*Chrysis fasciata, C. graelsii, C. indigotea*	0,607	0,047	5	(Z)-9-C31:1
9	*Cephaloparnops vareillesi, Parnopes grandior*	0,833	0,004	10	5MeC29
		0,709	0,031	10	Mix: 3,7 and 3,9 diMeC27
		0,556	0,050	15	7MeC29
		0,555	0,030	19	Triacontane
		0,473	0,047	24	5MeC27
		0,384	0,019	37	Hentriacontane
		0,287	0,013	53	Octacosane
		0,250	0,019	59	Nonacosane
10	*Hedychridium* spp.	0,658	0,013	32	(Z)-7-C25:1
11	*Hedychrum* spp.	0,861	0,001	25	(Z)-9-C25:1
		0,733	0,002	22	(Z)-9-C23:1
12	*Omalus aeneus, Philoctetes putoni, Pseudomalus* spp.	0,595	0,001	23	(Z)-11-C23:1
13	*Elampus panzeri, E. foveatus, Holopyga fervida, H. generosa*	1,000	0,001	4	(Z)-11-C33:1
		0,943	0,001	9	(Z)-11-C31:1
14	*Cleptes semiauratus*	0,987	0,010	10	(Z)-10-C21:1
		0,773	0,023	13	(Z)-7-C21:1
		0,388	0,035	58	Heneicosane

species plus *Pseudospinolia neglecta*, another one containing most of the species of the *Chrysis ignita* species group and species of basal Chrysidini and Parnopini, and a last clade grouping all bee-parasitizing species plus three closely related species of *Hedychridium*, which apparently share with males of bee parasitoids a very similar methyl-branched enriched CHC profile (Chapter 4).

5.5. Discussion

5.5.1. Species specific signaling and the use of CHC in chemotaxonomy

Males and females of cuckoo wasp species showed a distinctive chemical profile. This high degree of species and sex specificity in CHC of cuckoo wasps suggests CHC may mediate species recognition in these wasps. In several Hymenoptera, CHC are used for nestmate and for species (mate) recognition in social and solitary species (*e.g.*, Bruschini *et al.*, 2011; Sturgis & Gordon, 2012; Pradella et al, 2015; Weiss *et al.*, 2015). However, in some species, CHC alone may not be sufficient to allow species recognition (Weiss *et al.*, 2015; Buellesbach *et al.*, 2018) and other chemical or behavioural cues may be necessary to correctly elicit sexual mating behavior in co-occurring related (solitary) species (*e.g.*, Weiss *et al.*, 2015; Buellesbach *et al.*, 2018). Nevertheless, a unique species (and in some species, even gender) CHC signal would be necessary if CHC would be the only cue mediating species and mate recognition. Despite the high degree of species specificity, some few species in this study showed indistinguishable chemical profiles (*e.g.*, CHC profiles of males of closely related species *Hedychridium roseum* and *H. valesiense* or of some species of *Chrysis ignita* species group). In these cases, the overlap of CHC profiles was apparent in one of the sexes only (males) and it may be explained by the relatively short genetic differentiation in these closely related species (see below).

In other cases, however, there was a partial overlap among CHC profiles of different species. In spite of this overlap, CHC could still be used to signal species identity to conspecifics. First, species' profiles may overlap due to many commonly occurring CHC compounds (*e.g.*, n-alkanes or some commonly occuring alkenes), which may not necessarily be used as recognition signals in the species. Depending on the species, CHC shown to be species specific are different isomers of unsaturated and/or (poly) methyl-branched compounds (Martin *et al.*, 2008b; Martin *et al.*, 2017). Second, I have taken a conservative approach while analyzing CHC compounds. Several coeluting CHC compounds were grouped in several species, even if qualitative differences existed (*e.g.*, 11Me, 13Me and 15Me were grouped together and treated as a compound mix in species A, B and C, irrespective of whether species A only produced 11Me, species B produced 11 and 13Me and species C all of them). This was done to simplify analysis. In any case, a further separation of these CHC compounds may have increased the level of species specificity. Third, even if two or more species seemed to overlap in our analyses (i.e. NMDS of Figures 5.1 and 5.2), not all of them co-occur both in space and time. For instance, two allopatric species may show very similar CHC profiles and still they would not interbreed because of their geographic separation. Prezygotic isolation is known to be stronger in sympatric than allopatric species (Coyne & Orr, 2004). CHC profiles of sympatrically occurring species may be

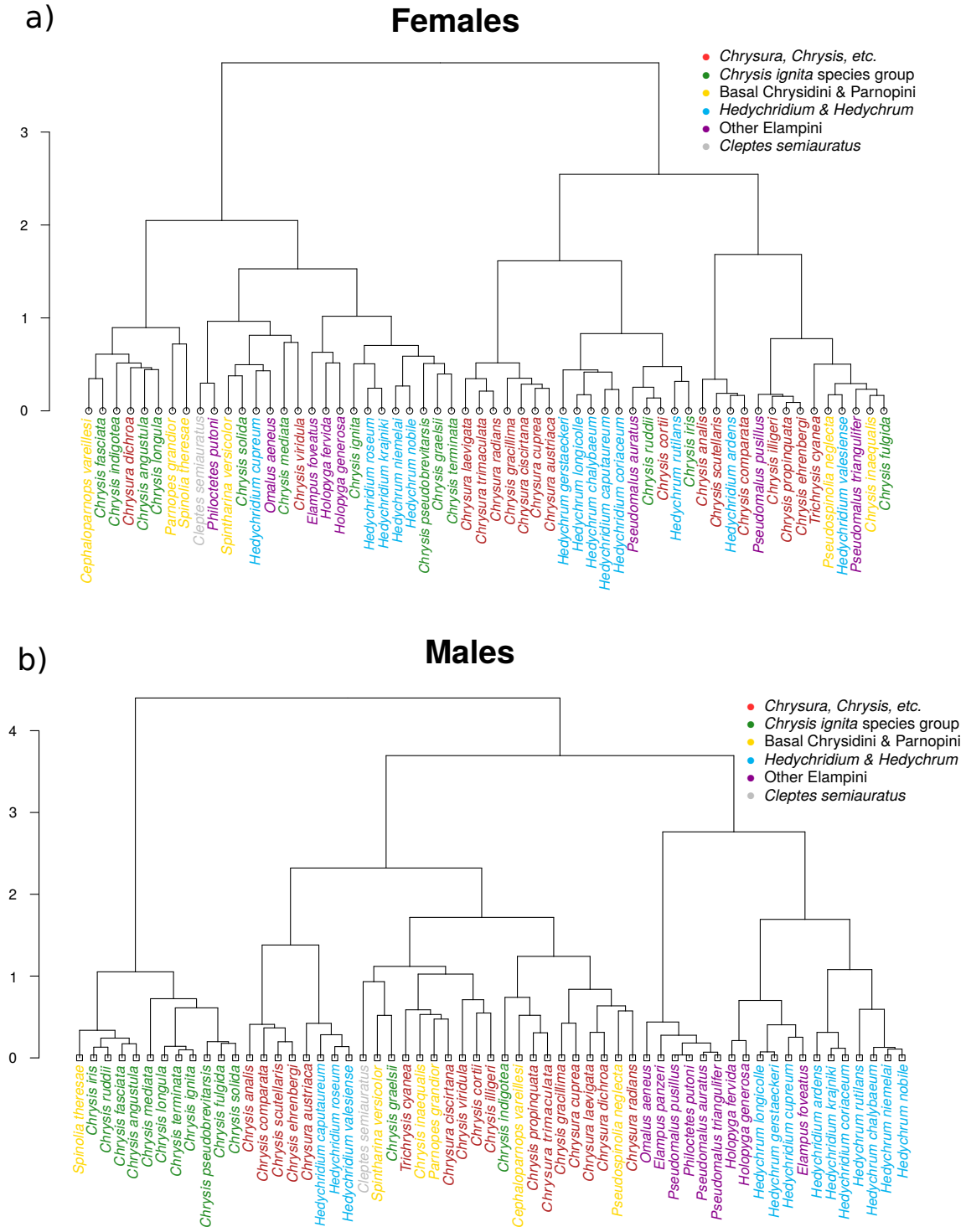

Figure 5.6.: Phylogenetic relationships inferred by CHC profiles in a) females, b) males using a hierarchical clustering approach (Ward's method on Bray-Curtis dissimilarity matrix). Species are colored according to how they were grouped by clades in the phylogenetic tree of Figure 5.1. Note the better separation of species belonging to the tribe Elampini in males than in females.

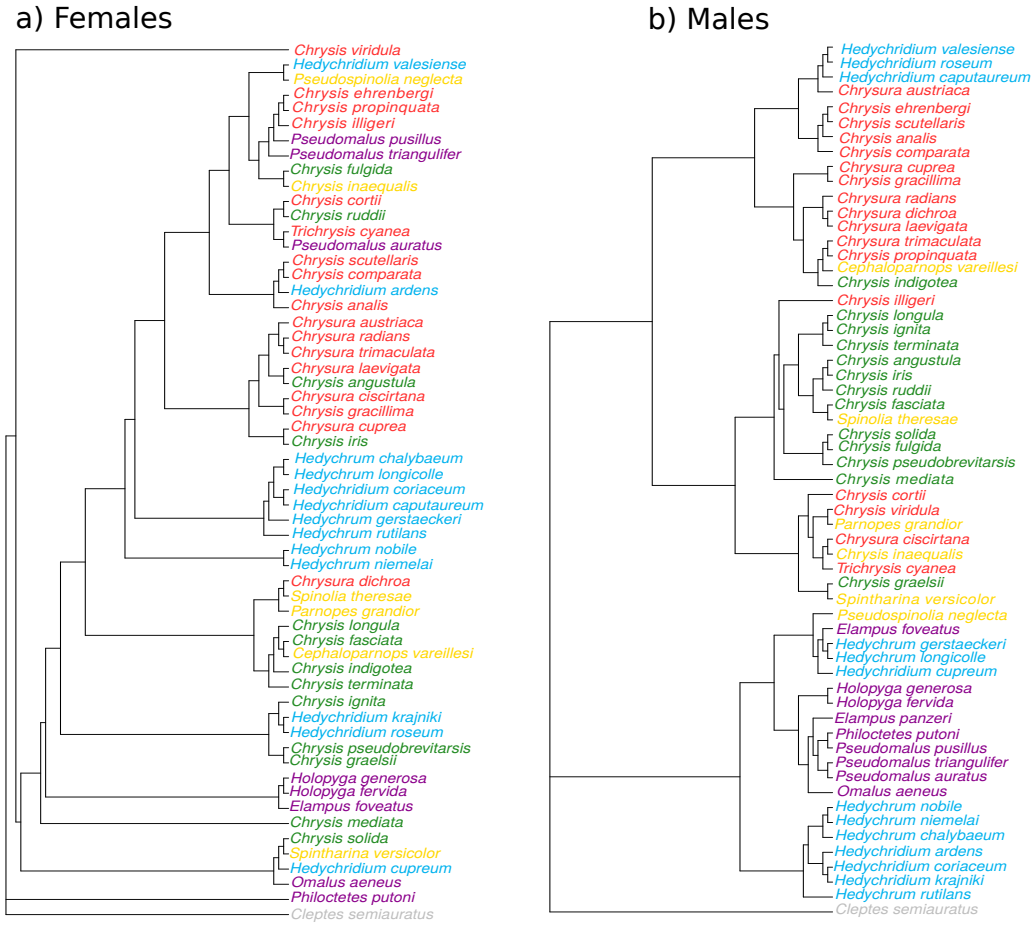

Figure 5.7.: Phylogenetic relationships inferred by CHC profiles in a) females, b) males applying a Neighbor-Joining algorithm. *Cleptes semiauratus* was used as an outgroup to Chrysidinae to root the tree. Note the better congruence of the recovered phylogenetic relationships in the tree of males.

expected to show more species-specific signaling and diverge faster than allopatrically occurring species due to reinforcement contributing to accelerate species differentiation to reduce fitness costs of hibridization (Noor, 1999). For example, allopatric populations of *Drosophila serrata* rapidly evolved CHC differences when subject to experimental sympatry with *D. birchii*. Furthermore, these evolved CHC profiles resembled those of field populations of *D. serrata* co-occurring with *D. birchii* (Higgie *et al.*, 2000).

In general, CHC were found to be species- (and sex-) specific, allowing to differentiate almost always closely related species that are difficult to separate by morphology or genetic analyses. Two particular examples merit some discussion here since they illustrate how CHC may help in species delimitation. The *Chrysis ignita* species group is one of the most challenging groups to separate via morphological characters (Soon & Sarma, 2011). Species in this species-rich group share a number of similar morphological, ecological and behavioural characteristics with many species parasitizing Eumeninae wasps and co-occurring with each other. Kunz (1994) failed to separate species based on analyses of morphological characters, uniting many of the species into groups. More recently, however, molecular approaches were used to help differentiate among species of this group and confirmed many of the original separations by Linsenmaier (1951) suggesting the existence of a number of cryptic species in this recently diverged and diverse group (Soon *et al.*, 2014). The analysis of CHC profiles revealed that females of all species could be very well distinguished from their conspecific males and among each other. Males of some of these species were nonetheless chemically similar (*e.g.*, *Chrysis ignita, C. longula* and *C. terminata*) and difficult to separate (Figure 5.3a). Interestingly, males and females of two species which show very short genetic distances, and are thus, difficult to separate using mitochondrial or nuclear genes or morphology (*e.g.*, *Chrysis mediata* and *C. solida,* Soon *et al.*, 2014, Orlovskyté *et al.*, 2016) were chemically distinct. Furthermore, a recent study has shown that these species were hardly differentiated by molecular phylogeny reconstruction methods but were clearly divergent by their host selection (Orlovskyte *et al.*, 2016). The other example refers to two closely related species in the genus *Hedychridium*. The morphological distinction between *H. valesiense* and *H. roseum* is so slight that the former species (described by Linsenmaier in 1959) was not reported from other regions for a long time and was synonymized with *H. roseum* (Niehuis, 2001). Males have a metallic coloration, otherwise are very similar to males of *H. roseum*. However females were never encountered by Niehuis (2001) in Germany and he then suggested the metallic and the non-metallic morphs were two different color morphs of the same species *H. roseum*. Years later, Arens (2004) analyzed some specimens from Greece and found that *H. valesiense* is a different species with sexual dimorphic coloration with its non-metallic females sharing extreme similarities with females of *H. roseum*. CHC profiles confirm the specific status of these species. Females of *H. roseum* and *H. valesiense* are chemically very different, but males of both species are chemically very similar. Thus, in both examples, the analysis of CHC profiles results helpful and suitable to be used as a complementary approach within the frame of an integrative approach (Schlick-Steiner *et al.*, 2010). CHC differences are indeed expected to be observed at the very first stages of divergence between species before other trait differences evolve. Being involved in intraspecific recognition, hydrocarbons are subject to strong selection to diversify, especially in closely related species that occur sympatrically, which may quickly evolve disparate CHC profiles (see above,

Coyne & Orr, 2004).

The fact that sibling species strongly differ in CHC composition has been also shown in other insects (*e.g.*, in ants, Martin *et al.*, 2008b, Morrison & Witte, 2011; Guillem et al, 2016, Menzel *et al.*, 2017a; orchid bees, Pokorny *et al.*, 2015), but this study additionally shows that *strong* chemical differentiation can occur in one of the sexes only. More conservative CHC profiles of male cuckoo wasps coincide with their conservative morphology, since they are usually very difficult to differentiate, at least in the *Chrysis ignita* species group (Niehuis, 2000, Soon *et al.*, 2014).

A number of advantages promote the use of CHC as an additional tool in chemotaxonomy. First, extraction of CHC is a nondestructive technique (Kather & Martin, 2012). After washing the specimen in a solvent, the insect can be either used for DNA analyses or pinned and further used for morphological analyses. Second, some special extraction techniques (*e.g.*, SPME, solid-phase microextraction) even allow the obtention of CHC without needing to kill the animal, enabling the use of these specimens for other analyses (*e.g.*, measuring CHC again after some time or using them in behavioral experiments). Third, CHC can be even extracted and recovered from museum specimens that have been stored for years (*e.g.*, Martin *et al.*, 2009). Moreover, the implementation of computer programs that help in a more rapid data processing (automatic alignment, *e.g.* flagme, Robinson, 2010, eRah, Domingo-Almenara *et al.*, 2016), a rapid identification of complex CHC compounds (*e.g.*, Chapter 9) shall allow an increased utilization of CHC as a complementary taxonomical tool. Eventually, CHC (online) databases could also aid in identification purposes, as it was proposed over 25 years ago (Lockey, 1991) and in the field of forensic entomology more recently (Moore & Drifhout, 2015).

5.5.2. Stability of CHC across geographic regions

The CHC profiles of co-occurring species of *Hedychrum* in several localities were compared to evaluate how variable they could be across geographic regions. One requisite for CHC to aid in delimitation of species, is that the CHC profile should be relatively stable across geographic regions and habitats (Martin *et al.*, 2008). The analysis showed that in most cases individuals of the same species collected at different localities clustered together in both females and males. There were two exceptions, however. The mismatching observed in males of *H. nobile* and *H. niemelai* reflects the relatively close genetic distance between these species, which have nevertheless very distinct CHC profiles in the females (Chapter 7). In fact, females of *H. niemelai* and *H. nobile* have CHC profiles predominantly composed of methyl-branched compounds, probably because of an ongoing evolutionary arms race that selects for a better chemical mimicry of their hosts (Chapter 7). Moreover, mean CHC profiles of the females of *H. nobile* from localities that clustered with other species were calculated from very few specimens (1 or 2). Whereas females of *H. nobile* and *H. niemelai* are morphologically similar (Kunz, 1994), explaining an unlikely but not impossible mistaken identification of the individuals clustering within *H. niemelai*, females of *H. rutilans* and *H. nobile* are morphologically different and difficult to erroneously identify. Therefore, it is more probable that the inclusion of one female individual of *H. nobile* collected at Battenberg with females of *H. rutilans* (which also present a large diversity of methyl-branched hydrocarbons) may be the result of this individual exhibiting a rather variable profile rather than a mistaken identification. This

emphasizes the need to include several specimens to calculate a mean CHC profile. The results showed that the clustering pattern was highly influenced by the most abundant and representative CHC compounds. CHC compounds that were not very abundant and that were therefore excluded from the analyses did not alter the results. However, cluster analysis conducted with only presence/absence data was not sufficient to correctly recover species as separate groups. Indeed, variation in quantitative composition of CHC compounds across species may result from differences in ecology, physiology and particular adaptations, and thus provide biologically relevant information that is used by the species (Menzel *et al.*, 2017a).

One limitation of this study was the relatively short geographic distance among the sampled populations/localities. Only in *Hedychrum nobile*, individuals collected at localities differing by 600-800 km from each other were available. Nevertheless, there seems to be a trend showing that in general, CHC profiles are relatively stable, especially in males. It is not clear yet why female individuals would vary their CHC profile across geographic ranges more than males. One possibility though could be that female individuals show more local variation and adaptation to host's populations, being more variable with geographic differentiation.

5.5.3. Intraspecific variation of CHC profiles

Different results showed that intraspecific variability of CHC profiles is larger in females than in males. This difference in intraspecific variability between sexes was also observed in the different dataset used for analysing *Hedychrum* species of different localities, in which chemical distances among female individuals were larger than among males in all species (Figure 5.5). There are several non-exclusive hypotheses explaining this difference.

The first reason for the larger intraspecific variation in CHC profiles of females is their parasitic lifestyle. In cuckoo wasps, it is only the female sex the one engaging in an evolutionary arms race with the female host (Chapter 7). Several species of cuckoo wasps are known to follow some type of chemical deception (mainly chemical mimicry) with the aim of reducing or avoiding detection of their presence and oviposition in the host's nest (*e.g.*, Strohm *et al.*, 2008, Chapter 2). Many species are known to have a preferred host but use related species as secondary hosts as well (*e.g.*, *Trichrysis cyanea*, Pärn *et al.*, 2015, Chapter 3). Therefore, the observed CHC profile variation among females may arise from a higher genetic variability that permits individuals of one species to accomodate to one or the other host. In fact, as a result of strong selection pressure from their parasites, females of the hosts may have variable CHC profiles, and this may also induce an increase in the variability of CHC profiles of their brood parasites (Chapter 3).

Another explanation for the larger intraspecific variability in females is that CHC profiles of females may be subject to variation after mating (Thomas, 2011). The chemical odour of females of several species varies after mating, becoming less attractive to males (Table 1 in Thomas, 2011). In insects, the chemical odour used by males to discriminate mated versus unmated mates, may include non-hydrocarbon volatiles (an eusocial bee, Kukuk, 1985, flies, Mair & Blackwell, 1988), hydrocarbons (*e.g.*, solitary bee, Simmons *et al.*, 2003, flies, Polerstock *et al.*, 2002), or changes in both non-hydrocarbons and hydrocarbons (*e.g.*, in an eusocial bee, Ayasse *et al.*, 1999, flies, Everaerts *et al.*, 2010). In all these cases, changes in odour are generated

by an increase or decrease in production by the female or are transferred by the male during copulation (Thomas, 2011). Since no assessment of the mating condition of cuckoo wasps was done, it is impossible to know if an specimen was mated or virgin at the moment of collection. However, it is possible that both types were collected for most of the species, since several sampling dates and locality collections were necessary to obtain enough individuals for these otherwise rarely occurring solitary species. A related hypothesis for this larger intraspecific variation is also a physiological explanation. Ageing can alter the CHC profiles of some species (Everaerts *et al.*, 2010, Kuo *et al.*, 2010, Vanickova *et al.*, 2012). Females are able to survive longer and this could have an effect on the CHC variation of a population. Males are usually flying for a shorter time than females in the field and they may have all been collected at a similar age range. Thus, although it is impossible to determine whether one or both of these factors has influenced the higher within species variability of females, they could indeed be explaining part of this variation.

In addition, there could be a natural lower variability of CHC profiles in males. Male CHC composition may be subject to strong stabilizing and directional sexual selection driven by female choice (*e.g.*, Steiger *et al.*, 2013, Lane *et al.*, 2016). Theory predicts that a trait under strong selection should show reduced genotypic variance (Lande, 1975). A meta-analysis testing this prediction using empirical studies of acoustic courtship traits of insects and amphibians found support for a reduction of intraspecific phenotypic variation in male traits (Reinhold, 2011). Although it has not been studied, sexual selection is expected to be stronger in males than in females in cuckoo wasps, because males court and search for females while females only mate once, like many solitary hymenopterans (ONeill, 2001). Indirect evidence suggests that the difference in intraspecific variability in CHC profiles between sexes is particularly stronger in methyl-branched compounds, several of which may be acting as sex and species-specific signals (Appendix). Thus, it remains to be tested whether the lower variability of CHC compounds (especially of methyl-branched compounds) could be the result of sexual selection on males.

5.5.4. Use of CHC in chemosystematics

Reconstructions of the evolutionary history of cuckoo wasps based on CHC composition were in general not congruent with reconstructions based on nuclear genes (Pauli *et al.*, accepted, Chapter 3). Nevertheless, reconstructions of certain clades could be correctly inferred using CHC composition of males (*e.g.*, *Pseudomalus, Omalus, Philoctetes*). This congruence is remarkable given that relatively few characters were used in this reconstruction (180 CHC compounds, in comparison to ~5000 base pairs, Chapter 3). CHC profiles of males of chemically dimorphic *Nasonia* species have been shown to correlated with the evolutionary history of the species better than those of their conspecific females (Buellesbach *et al.*, 2013). However, in the previously mentioned study, only four closely related species were compared, whereas a much larger number of species having a longer history of divergence has been used here. It is possible that reconstruction at the level of related species or genus can be correctly recovered using CHC profiles of males, but when looking at larger levels of divergence, CHC profiles of males are not anymore informative.

Overall and despite their suitable application for chemotaxonomy, CHC are shown not to be useful for establishing phylogenetic relationships across species, and caution

should be applied when trying to use hydrocarbons to infer past history, especially because we often do not know what selection pressures have acted on those species (Morrison & Witte, 2011). Past attempts (Martin & Drifjhout, 2009a; Kather & Martin, 2015) using different hymenopteran species show no phylogenetic signal in the reconstructions. Here, this is confirmed at the level of families. In order to be useful to unravel phylogenetic relationships, chemical characters should not be subject to strong selection pressure to signal species-specificity and thus, changes in the compounds are expected to go in accordance with speciation events (Hefetz, 1993). In comparison to other chemical signals (*e.g.*, allelochemicals in plants, exocrine defensive secretions in insects (*e.g.*, Alvarenga *et al.*, 2001; Raspotnig *et al.*, 2017), CHC are less useful in reconstructing the phylogenetic history of related species.

6. Natural selection and sexual dimorphism of CHC

6.1. Abstract

Explaining the origin and evolution of intersexual differences has been a common subject of study. Although few studies have shown that sexual dimorphism can arise due to natural selection influencing change on the female phenotype, the origin of sexual dimorphism is commonly attributed to sexual selection on males: males evolve traits that increase their fitness in female mate choice or in intra-sexual competition. Here, I show that natural selection has played an important role in the evolution of sexual dimorphism of chemical profiles of cuckoo wasps (Hymenoptera: Chrysididae). Cuticular hydrocarbons (CHC) are hydrophobic molecules in the outer layer of the insect cuticles with (at least) a dual function: they are desiccation barriers and they play a role as signals in intra- and interspecific communication. Using comparative phylogenetic analyses of CHC profiles of males and females of 57 species of cuckoo wasps, I explore the evolutionary changes that have given origin to chemical sexual dimorphism. Cuckoo wasps are solitary parasitoids and cleptoparasites of other solitary wasps and bees and represent a good example to study how natural selection has contributed to the origin of sexual dimorphism because several cuckoo wasps take advantage of chemical mimicry to remain undetected after having oviposited in their hosts nests. According to the results, the mode by which CHC profiles evolved in cuckoo wasps differs strongly between both sexes, with males but not females exhibiting a strong correlation between chemical (Bray-Curtis dissimilarity of CHC profiles) and phylogenetic distances. Disparity through time plots and a comparison of the cuckoo wasps' phylochemospace suggests that females diverged significantly more than males and that this divergence was likely driven by natural selection on females for mimicking the CHC profiles of their hosts. I suggest that in cuckoo wasps, natural selection acting on females has played a more important role than sexual selection acting in cuckoo wasp males in generating sexual dimorphism.

6.2. Introduction

Sexual dimorphism is ubiquitous in the animal kingdom. Intersexual differences in size, coloration, morphology and other traits, which can all occur simultaneously, are frequently encountered in many species (*e.g.*, Lindenfors *et al.*, 2007; Székely *et al.*, 2007; Bell & Zamudio, 2012; Ficetola *et al.*, 2013; Streinzer *et al.*, 2013). Explaining the origin and evolution of these widespread differences between the two sexes has been a common subject of study (*e.g.*, Price, 1984; Hedrick & Temeles, 1989; Emerson & Voris, 1992; Kratochvil & Frynta, 2002; Fairbairn *et al.*, 2007 and references therein; Williams & Carroll, 2009; Ficetola *et al.*, 2013). Darwin proposed

that sexual dimorphism arises as a result of sexual selection, when traits in one sex (primarily the males) are selected because of conferring an advantage in mate choice or in intrasexual competition. In contrast, Wallace thought that natural selection could be implicated in the evolution of sexual dimorphism, as for example in driving the coloration of the less conspicuously colored sex as a result of a differential natural selection pressure on it (cryptic coloration of females to avoid predation) (Kottler, 1980).

Although both natural and sexual selection could theoretically explain the evolution of sexual dimorphism (Lande, 1980; Slatkin, 1984; Lande & Arnold, 1985), sexual selection has been traditionally accepted as the main and often only selective force in explaining the evolution of sex differences (Andersson, 1994; Allen *et al.*, 2011). Reasons for this are that the traits involved in sex differences could easily be attributed to be used as secondary sexual traits and the observation that sexual dimorphism was more exaggerated in polygynous species than in monogamic species (*e.g.*, Dunn *et al.* 2001), in which the strength of sexual selection is weaker. Moreover, many studies have provided support to sexual dimorphism having originated due to sexual selection (*e.g.*, Price, 1984; Anderson & Vitt, 1990; Moore, 1990; Emerson & Voris, 1992; Dunn *et al.*, 2001; Kratochvil & Frynta, 2002). Finally, because of the difficulties of testing hypotheses of an ecological origin for sexual dimorphism (Shine, 1989), natural selection has been less often implicated in its evolution.

Nevertheless, a growing number of studies is showing that at least in some instances sexual selection alone seems insufficient to explain the evolution and origin of sexual dimorphism (*e.g.*, Martin *et al.*, 1996; Götmark *et al.*, 1997; Temeles *et al.*, 2000; Butler & Losos, 2002; Kunte, 2008; Cooper *et al.*, 2016). For example, differential predation in females has been shown to cause sexual dimorphism in plumage coloration in birds (Martin *et al.*, 1996; Götmark *et al.*, 1997), the evolution of female limited mimetic coloration in Lepidoptera (Kunte, 2008), and the evolution of morphological defensive traits in female sticklebacks that inhabit more open water environments than their conspecific males (Reimchen & Nosil, 2004). Also, ecological causes seem to have been involved in the origin of sexual size dimorphism in pinnipeds prior to the appearance of polygyny in the group (Krüger *et al.*, 2014).

Kunte (2008) has tested the ideas proposed by Wallace on sexual dimorphism (see above) by comparing the coloration patterns of *Papilio* butterflies, whose females use Batesian mimicry to gain protection from predators. He found that the males' wing color patterns represent the ancestral state and that sexual dimorphism has evolved due to the deviation in coloration patterns of the females' wings as a result of selection pressure for Batesian mimicry. Thus, his results have given support to the idea of sexual dimorphism having evolved by natural selection. However, not only predation could be causing different selection pressures on the sexes. Brood parasitism may as well drive the evolution of sexual dimorphism. Krüger and colleagues (Krüger *et al.*, 2007) tested whether sexual dimorphism in size and coloration patterns of common cuckoos could be explained by sexual or by natural selection (exerted on females to circumvent host defenses). Their results indicated that the evolution of sexual dimorphism in cuckoos, both in body size and plumage coloration, is more likely explained by natural selection (coevolution between hosts and parasites).

The above studies on *Papilio* and on cuckoos have contributed to the notion that the differences observed between the sexes in a certain trait may not be only evolving due to sexual selection and, that we need to consider also the influence of natural

selection, especially when we deal with species in which predation or parasitism could be differentially affecting females. It is reasonable to assume that both types of selection frequently contribute to the evolution of sexual dimorphism, but it is still not clear when and how both selection forces interact. For instance, the influence of natural vs. sexual selection may differ depending on the location of the signal on the body. For example, it has been suggested that natural selection constrains the evolution of sexual dichromatism in ornamentation patterns of agamid lizards only on body regions that are exposed to visual predators whereas sexual selection enhances it on body regions that are concealed from predators (Stuart-Fox & Ord, 2004).

The aim here is to show that natural selection has played an important role in the evolution of sexual dimorphism of chemical profiles in parasitoid and cleptoparasitic wasps of the family Chrysididae. Cuticular hydrocarbons (CHC) are hydrophobic molecules in the outer layer of the insects' cuticle, with a dual function: they are desiccation barriers and they play a role as signals and cues for intra- and interspecific communication. Thus, they can be affected by natural and sexual selection (Chung & Carroll, 2015). Whereas the influence of natural selection (in the form of environmental conditions) on CHCs has been acknowledged first (Gibbs *et al.*, 1998; Frentiu & Chenoweth, 2010), the role of sexual selection in the evolution of chemical signals, such as CHC, has long remained unappreciated and is only recently being demonstrated (Thomas & Simmons, 2009; Steiger *et al.*, 2013; Ingleby *et al.*, 2014; Steiger & Stökl, 2014, Ingleby, 2015 , Lane *et al.*, 2016).

Chrysidid wasps, commonly known as cuckoo wasps, are a diverse and cosmopolitan group of often brilliantly coloured solitary wasps. They are parasitoids and kleptoparasites (subsequently referred as brood parasites) of other insects, primarily solitary wasps and bees (Bohart & Kimsey, 1991). Their common name refers to their behaviour of laying eggs inside their host nests. Although it has not yet been tested widely, many of the cuckoo wasps species use different types of chemical deception (mimicry, crypsis and/or chemical insignificance) to avoid being chemically detected by their hosts (Strohm *et al.*, 2008; Wurdack *et al.*, 2015). Thus, different types of selection are possibly acting on the two sexes of cuckoo wasps which are sexually dimorphic in most of the species (Chapter 4). Female cuckoo wasps may gain fitness advantages by either producing a CHC profile that matches that of its host or by simplifying its CHC profile so that it cannot be detected by their host. Males, however, may only gain fitness by finding and recognizing appropriate mating partners. It is possible that both natural and sexual selection act on both sexes but with different strengths. I hypothesize that in the case of cuckoo wasps, whose species are brood parasites, natural selection acting on females plays a dominant role in directing the evolution of sexual dimorphism.

Here, I investigate whether CHC evolution in cuckoo wasps is being primarily driven by natural selection on females. I am specifically interested in 1) comparing the mode of evolution of CHC profiles in the two sexes, 2) exploring the evolutionary changes of the CHC profiles in order to better understand whether females or males have changed more, and 3) investigating whether different compound classes (*e.g.*, unsaturated compounds, methyl-branched alkanes, and linear alkanes, which may play different roles) differ in their mode of evolution as well.

To achieve this, I first relate chemical distances to phylogenetic distances in extant species and compare how their correlation differs between sexes. I then investigate the divergence pattern of CHC profiles in both sexes separately through the morphological

(in this case chemical) diversity index (MDI, Harmon *et al.*, 2003). A positive MDI suggests that CHC profiles of closely related species are very different (diversity is partitioned within subclades) whereas a negative MDI indicates closely related species are chemically very similar to each other (diversity is partitioned among subclades). I expect the divergence pattern of CHC profiles to be largely partitioned within subclades in the CHC profiles of females, but not in those of males, because adaptation of the CHC profiles of females to those of their hosts may have led closely related species to differ significantly. On the other hand, sexual selection acting on males, may also drive divergence of males' CHC profiles. However, this divergence may not be across all hydrocarbon compounds and I still expect to find more phylogenetic signal in the CHC profiles of males in comparison to females.

6.3. Materials and Methods

6.3.1. Collection of wasps

Insect specimens were collected at several localities in Europe and Israel. For details on the origin of the samples, refer to the Appendix. Live specimens were placed in glass vials and killed by freezing. Subsequently, CHC were extracted with hexane, in which wasps were submersed for 10 minutes. CHC extracts were stored at -20°C, and the washed insects were transferred to vials with pure ethanol for long-term preservation. All specimens were identified by Oliver Niehuis and are stored at the Zoological Research Museum Alexander Koenig in Bonn (Germany).

6.3.2. Gas Chromatography/Mass Spectrometry

Following CHC extraction, the extracts were analyzed with a gas chromatograph (HP 6890, GC) coupled to a mass selective detector (HP 5973, MS) or with an integrated Agilent 7890/5975 GC/MS system. The GC (split/splitless injector in splitless mode for 1 min, injected volume: 1 µl at 300°C injector temperature) was equipped with a DB-5 Fused Silica capillary column (30 m x 0.25 mm ID, df = 0.25 µm, J&W Scientific, Folsom, USA). Helium was used as carrier gas with a constant flow of 1 ml/min. The temperature program used was: start temperature at 60°C, with an increase of 5 °C/min until 300°C and isotherm at 300°C for 10 min. An ionization voltage of 70 eV (source temperature: 230°C) was set for the acquisition of the mass spectra by electron ionization (EI-MS).

6.3.3. Chemical characterization of CHC profiles

To analyze the composition of the CHC extracts, I used a semi-automatic procedure that consisted on running batch jobs in AMDIS (Automated Mass Spectral Deconvolution and Identification System, http://chemdata.nist.gov/mass-spc/amdis/) and processing them using built-in scripts in R (see Chapter 8). The parameter setting used was: component width = 22, adjacent peak subtraction = 2, resolution = medium, sensitivity = low, and shape requirements = medium.

CHC are classified into three main substance classes: n-alkanes (simple straight chains of C and H), methyl-branched compounds, that possess one or more methyl-groups along the chain, and unsaturated compounds, with one (*i.e.*, alkenes) or more

(*i.e.*, alkadienes, alkatrienes) double bonds along the chain. N-alkanes are easily identified by their diagnostic ions. In contrast, unsaturated and methyl-branched compounds are much more diverse and their identification is comparatively more complex (see Chapter 9). Retention indices were calculated and used to confirm the identification of methyl-branched alkanes (Carlson *et al.*, 1998). The double bond positions of alkenes were identified after a dimethyl disulfide (DMDS) derivatization following the protocol of Carlson and colleagues (Carlson *et al.*, 1989). The positions of the double bonds of alkadienes were not determined, but they were grouped according to their retention indices.

All CHC profiles are composed of a variable number of hydrocarbons of different structural groups and chain lengths. The absolute total ion count of each representative CHC compound was converted to relative percentages. A compound was considered representative of each species and sex when it occurred in at least 50% of the specimens and it accounted for at least 0.1% of the total ion count of the CHC profile. The relative proportion of each representative compound across samples that belonged to the same group was averaged, obtaining a mean CHC profile characteristic of each species and sex (relative abundance matrix). In addition, a binary matrix (presence/absence) was created by neglecting the abundance information. In this case, all CHC compounds are equally weighted. CHC information was summarized into several variables. First, individual compounds were grouped by compound classes (*e.g.*, linear alkanes, alkadienes, alkenes, the two last groups together as unsaturated compounds, monomethyl-branched and dimethyl-branched compounds or including them together as methyl-branched compounds) and by homologous series of hydrocarbons. An homologous hydrocarbon series includes hydrocarbons that possess the same structure (*e.g.*, the same position at which the methyl group inserts or the double bond occurs), but varies in chain length only (*e.g.*, all 5Me: 5MeC21, 5MeC23, 5MeC25, etc.). Homologous series are thought to be produced by the same enzimatic machinery (Chung & Carroll, 2015), are often correlated (*e.g.*, Martin *et al.*, 2008a) and may even convey the same information (*e.g.*, van Wilgenburg *et al.*, 2011). A second group of variables summarized the number of compounds present per species, sex, compound classes or major homologous series. Finally, a mean chain length, an indirect measure of how long or short the CHC profile may be, was estimated for each species and sex. Each hydrocarbon can be characterized by the number of carbon atoms in its backbone. The mean chain length is calculated as the mean value of the chain length of all hydrocarbons present in the profile weighted by their relative abundance.

Whenever pairwise chemical distances were calculated, Bray-Curtis dissimilarity indices were used. These were estimated with the function vegdist in the vegan package (Oksanen *et al.*, 2013) in R version 3.0.2 (R Core Team, 2013). The Bray-Curtis dissimilarity is usually preferred in ecology over other metrics because it is not affected by joint absences: it considers only shared compounds between individuals to calculate distances.

6.3.4. Calculation of chemical dimorphism

The degree of chemical dimorphism in each species was measured by counting the number of CHC compounds that differ between females and males and dividing this number by the total number of CHC compounds present in both sexes. This value

varies between 0 (all characters are shared between females and males, monomorphism) and 1 (no CHC are shared between the sexes, dimorphism).

6.3.5. Molecular phylogeny of Chrysididae

A molecular phylogeny inferred by Pauli and colleagues (Chapter 3) which contains over 180 species of Chrysididae was pruned for including the species for which chemical profiles were available (56). Cuckoo wasps are currently subdivided into four subfamilies, two of which are not very well known and occur mainly in the tropics. The subfamily Chrysidinae is the most species-rich and the majority of extant species of Chrysididae in Europe belong to this subfamily (Kimsey & Bohart, 1991). Cleptinae are also common but the subfamily is less species-rich than Chrysidinae. Its species served in the analyses for outgroup comparison. The subfamily Chrysidinae is subdivided into five tribes: Allocoelini, Elampini, Kimseyini (only known from a single species not included in the sampling, Antropov, 1995), Parnopini and Chrysidini, the latter being the most species-rich (Kimsey & Bohart, 1991). In this study, species of the tribes Elampini and Chrysidini (the two most species-rich tribes of the subfamily Chrysidinae) are compared because they do not only differ in morphology but also show differential behavioral adaptations that can have implications for hypotheses on the origin of sexual dimorphism. Note that *Chrysura* species do not form a monophyletic group (Chapter 3). For example, *Chrysura ciscirtana* is more closely related to *Chrysis gracillima* than to the other *Chrysura*. When I refer to *Chrysura* species in the present study, I refer to the monophyletic group of *Chrysura* species that are brood parasites of bees and not to *C. ciscirtana*.

6.3.6. Calculation of phylogenetic signal

Several methods were used to estimate the degree of phylogenetic signal in each of the CHC compounds in the datasets and in a number of summarizing variables (see above). Felsensein (1985b) warned about the statistical non-independence of species traits due to phylogenetic relatedness more than 30 years ago, and since then, several indices have been proposed to measure and test for this phylogenetic signal, defined as "the tendency for related species to resemble each other more than they resemble species drawn at random from the tree" (Blomberg & Garland, 2002). To assess the phylogenetic signal using the relative abundances of each CHC, three common metrics were used: Pagel's λ (Pagel, 1999), Blomberg's K (Blomberg *et al.*, 2003) and Abouheif C mean (Abouheif, 1999). They all follow different approaches to calculate phylogenetic signal, the two first assume a Brownian motion (BM) model of evolution (Munkemüller *et al.*, 2012). When sample sizes are not too small (> 30), both K and λ should perform well (Kamilar & Cooper, 2013). Brownian motion is the most common model of evolution for continuous traits in which trait evolution follows a random walk through the trait space. Because of its simplicity, it is commonly proposed as a null model of evolution. Pagel's λ is a branch length transformation method tested by likelihood-ratio test that provides the best fit of the trait data to a BM model ($\lambda = 1$, traits follow a BM model). It is generally tested against the null hypothesis that traits are randomly distributed along the phylogeny ($\lambda = 0$, no signal). Blomberg's K quantifies phylogenetic signal by comparing the amount of observed variance in a trait relative to its expected variance under BM. K varies from 0 (no phylogenetic

signal) to infinity. K values of 1 indicates strong phylogenetic signal and that the trait has evolved under BM whereas values larger than 1 suggests that close relatives are more similar than expected under BM (Kamilar & Cooper, 2013). On the other hand, Abouheif C is based on Moran's I spatial autocorrelation index and does not depend on a model of evolution (Pavoine *et al.*, 2008). It is tested by randomized permutations and stronger deviations from 0 suggest strong phylogenetic signal. In contrast to λ, Abouheif C mean values cannot be compared between different phylogenetic trees (Munkemüller *et al.*, 2012). In addition to Pagel's λ and Abouheif C, I used multivariate Bloomberg's K to estimate the degree of phylogenetic signal for the complete chemical dataset (multivariate approach). Kmult is a multivariate generalization of the K statistic of Blomberg and colleagues (Blomberg *et al.*, 2003) and as such, it is also based on a BM model of evolution. A Kmult value of 1 indicates evolution of traits by Brownian motion. Finally, the phylogenetic signal of the presence/absence of the same CHC was measured using the D statistic metric (Fritz & Purvis, 2010), especially suitable for estimating phylogenetic signal of binary traits. The D metric was calculated using the function phylo.d in the package caper (Orme *et al.*, 2013). Abouheif C mean was calculated using the function abouheif.moran in package adephylo (Jombart & Dray, 2010) and Pagel's λ was estimated with the function fitContinuous in the package geiger (Harmon *et al.*, 2008). Kmult was computed with the R code provided in Adams (2014b).

6.3.7. Tempo and mode of CHC evolution

In order to investigate whether or not, and how the tempo and mode of CHC evolution differed between females and males, I applied four approaches.

First, chemical distances were plotted against phylogenetic distances between all possible species pair comparisons using relative abundance and presence/absence data. I then assessed the correlation between the pairwise phylogenetic distances and chemical distances using Mantel tests. Note that in those cases, in which I calculated chemical distances using compound classes separately, relative amounts were recalculated so that the sum of all individual compounds constituting a compound class was the unity.

Second, I compared rates at which CHC profiles evolved in males and females using the multidimensional approach proposed by Adams (2014a). Whereas most previous methods were able to calculate evolutionary rates for univariate traits or a short number of variables that reduce information (*e.g.*, principal component scores), the method by Adams is able to quantify phylogenetic evolutionary rates for high-dimensional data, and it has been successfully applied in multivariate phenotypic traits such as shape (Gómez *et al.*, 2015) and scents (Weber *et al.*, 2016). Evolutionary rates are estimated based on distances rather than covariance and the main assumption of the method is that interspecific variation over time evolves under a Brownian Model of evolution (Adams, 2014a). Here, I used both the complete matrix of relative abundances and a four-dimensional NMDS scores (Nonmetric Multidimensional Scaling calculated from the matrix of relative abundances using Bray-Curtis dissimilarities and four dimensions) to compare rates of evolution. Whether the absolute values of the rates of evolution might differ depending on the use of the complete dataset (Euclidean distances are calculated by default) and the NMDS scores (reduced via Bray-Curtis dissimilarity), I am interested in the differences I observed using the same

source of data. Using these two datasets, I compared rates of evolution between the two sexes and also between clades within each sex.

Third, I investigated the pattern of diversification of CHC profiles with the Morphological Diversity Index (MDI), which quantifies the overall difference in trait variation among and within subclades in a phylogeny with respect to the expectations under Brownian motion model of evolution (Harmon *et al.*, 2003). A negative MDI statistic indicates a pattern that is explained if diversity is partitioned among subclades (in this case, it would mean that closely related species are very similar in their chemical profiles) and a positive value reflects the opposite, a pattern in which diversity is largely partitioned within subclades (closely related species differ considerably in their chemical phenotypes). To visualize these patterns, I used Disparity Through Time (DTT) plots following Harmon et al (2003) on an ultrametricized phylogenetic tree. To render the phylogenetic tree ultrametric I used the function chronos (package ape, in R), which estimates divergence times using the penalized maximum likelihood method created by Sanderson (2002). Disparity is measured as the average pairwise distance between species for each subclade in the phylogeny. Relative disparity is estimated from the root to the tips of the tree and measured at each node as the average of the relative disparities of all subclades whose ancestral lineages were present at that time (Harmon *et al.*, 2003). I conducted 1,000 simulations to test whether disparity differed from expectations if the evolution of the characters was according to a Brownian motion model. Since the data are multivariate and the dissimilarity index of Bray-Curtis is the most appropriate one when analysing CHC profiles, I used a modified source code of the publicly available dtt function in the package geiger kindly provided by Luke Harmon via Tamara Pokorny (Harmon *et al.*, 2008).

Finally, I projected phylogenies onto the chemical morphospace (a two-dimensional NMDS calculated with Bray-Curtis dissimilarity metric on the chemical profiles) based on the phylomorphospace of Sidlauskas (2008) and implemented in the package phytools (Revell, 2012) with the function phylomorphospace in R. Sidlauskas (2008) suggested an approach to distinguish two different scenarios that may lead to unequal morphological (in this case, chemical) diversification among clades in a phylogenetic context. Either a clade may experience more changes per phylogenetic branch (an increase in magnitude) which may have contributed to their diversification in comparison to another clade, or it may have experienced a higher efficiency in the exploration of novel morphospaces, irrespective of the number of changes per phylogenetic branch (Sidlauskas, 2008).

6.4. Results

6.4.1. General patterns of CHCs in females and males of Chrysididae

Major differences and similarities among related species are observed in the relative amount and the number of CHC grouped by compound class (Figure 6.1). For example, species of the genera *Holopyga* and *Elampus* are characterized by a large proportion of alkadienes in females (but not in males), whereas males of the *Chrysis comparata* species group (*Chrysis comparata*, *C. analis, C. scutellaris*) and of *C. ehrenbergi* are mainly composed of methyl-branched alkanes with unsaturated com-

pounds contributing very little to the CHC profile. In contrast, in their conspecific females, alkenes make up at least half of the total amount of CHC. The number of CHC compounds per sex and species varied between 13 (females of *Chrysis propinquata*) and 66 (males of *Chrysura radians*). When taking into account all CHC compounds of a species (both sexes considered together), *Omalus aeneus* and *Chrysis graelsii* are the species with the lowest number of CHC compounds: 25 compounds in total. On the other hand, *Chrysura radians* exhibit with 86 CHC compounds the most diverse CHC profile. Linear alkanes constitute the only compound class that is invariably present in all CHC profiles. Odd-numbered alkanes are more abundant than even-numbered alkanes, and with few exceptions, they make up on average between 35% and 41% of the total CHC profile in males and females, respectively. Linear alkanes contribute less than 12% to the CHC profiles of males of *Chrysis indigotea,* whereas they make up more than 90% of the CHC profile of females of *Chrysis graelsii*. A total 72 alkenes were identified, with species possessing between three (*Chrysis indigotea*) and 30 (*Chrysura radians*) different alkenes. Alkenes comprise up to 71% of the CHC profile of some species (*e.g.*, *Trichrysis cyanea*). Mono- and dimethyl-branched alkanes are numerous (the latter ones more in males) in the majority of species but are abundant in relatively few species, whereas alkadienes contributed to more than 10% of the profiles in only six species (Figure 6.1). Mean chain length varied between ~ 2300 and ~2900, in both females and males (the shortest mean chain length of females belonged to the species *Cleptes semiauratus* while the longest was observed *Pseudospinolia neglecta*, while in males the shortest was observed in *Philoctetes putoni* and the longest in *Chrysis viridula* (Figure 6.1d).

6.4.2. Chemical dimorphism

Chemical dimorphism was measured as the proportion of the number of compounds that are different between the two sexes. Overall, I found moderate to high levels of qualitative chemical dimorphism. Chemical dimorphism in cuckoo wasps varies between 0.14 in *Hedychridium coriaceum* (in which CHC profiles are very similar between females and males with some few scarce compounds that account for differences, Chapter 4) and 0.78 in *Chrysis propinquata* (in which males are dominated by methyl-branched compounds whereas females are dominated by alkenes, Figure 6.1e). It is important to note, however, that this index does not consider relative amounts and this may add a second dimension to dimorphism. No single species had a value of 1, because linear alkanes occurred in both sexes (on average ten compounds per sex) and the main difference between the sexes is quantitative. In general, the index of dimorphism is larger in species belonging to the tribe Chrysidini clade than in species of the Elampini tribe (0.49 vs. 0.31, t33: 5.368, $p < 0.001$). However, this is only significant when phylogenetic relatedness is not taken into account.

6.4.3. Phylogenetic signal

The relative amounts of several CHC showed significant phylogenetic signal (75 CHC compounds in the males and 48 in the females, Appendix). When using the presence/absence dataset, the number of CHC compounds with phylogenetic signal was 69 in males and 32 in females (Appendix). All indices calculated phylogenetic signal in a different manner and not always the same CHC compound was selected, never-

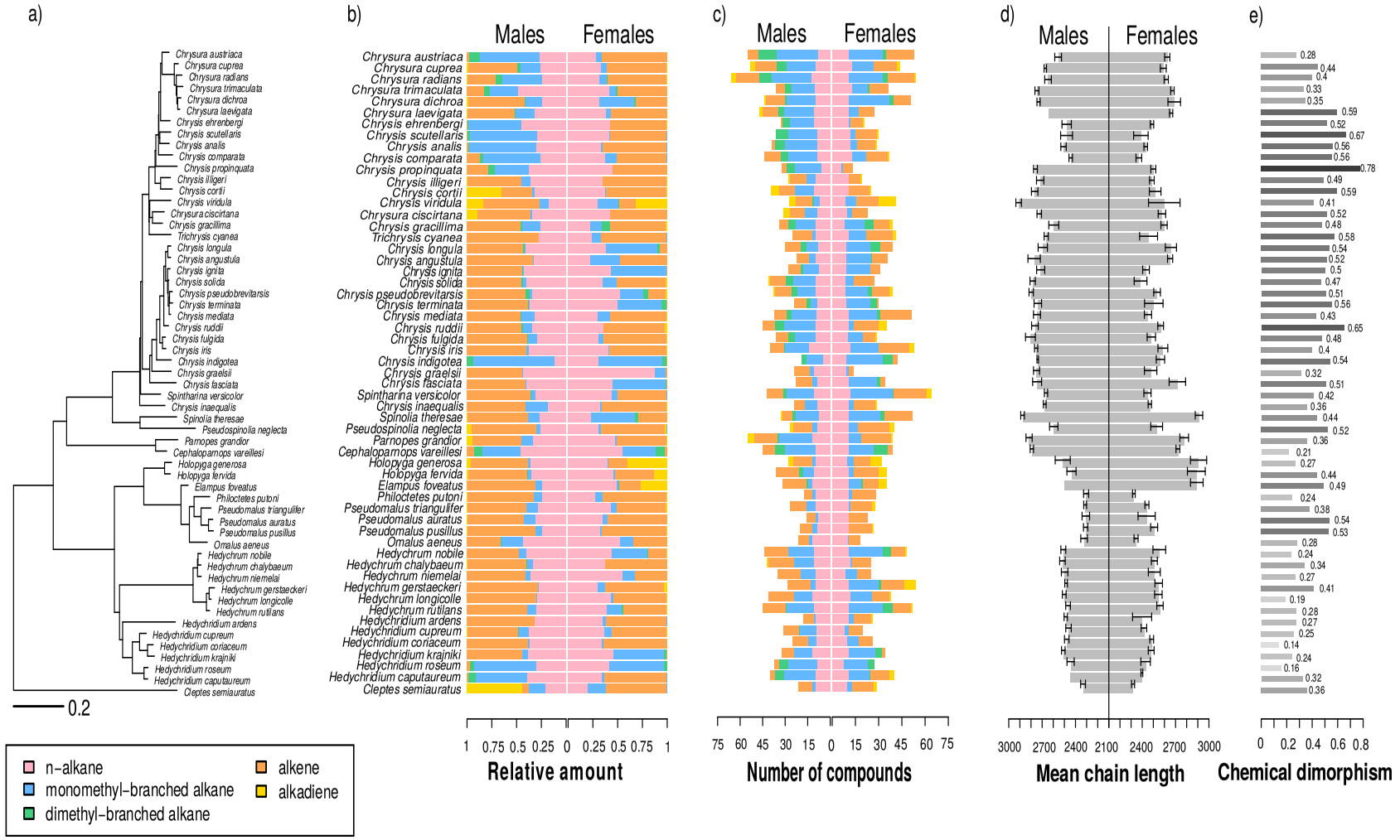

Figure 6.1.: a) Molecular phylogeny of species of Chrysididae used in this study, b) Mean relative amount of CHC by compound class in males and females, c) Mean number of CHC compounds by compound class in males and females. Colors indicate compound classes: n-alkanes (pink), monomethyl-branched alkanes (blue), dimethyl-branched alkanes (green), alkenes (orange) and alkadienes (yellow), d) mean chain length in females and males with bars indicating standard deviation, e) Chemical dimorphism index with values at the right side. The gray scale depicts chemical dimorphism, with light gray values showing species in which chemical dimorphism is low and darker shades indicating greater differences in CHC compounds of both sexes.

theless the result was consistent in showing in all cases more phylogenetic signal in males than in females. The multivariate method also indicated this, as Kmult was larger in males than in females (0.212 vs. 0.12). When looking at the summarizing variables, the result was the same. Of the 25 variables that summarized CHC profile information (including relative abundances per compound class, per homologous series of hydrocarbons, number of compounds and mean chain length), only 3 at the most were selected as having phylogenetic signal in females (the relative amount of alkadienes, the relative amount of alkenes with double bond position at 9, and mean chain length). On the contrary, the number of summarizing variables with phylogenetic signal varied between 8 (Pagel's λ) and 18 (Cmean) in males (Appendix). Figure 6.2 plots phylogenetic signal using one of the most commonly used index of phylogenetic signal (Blomberg's K) in males and females. Note that more compounds and variables show significant phylogenetic signal in males and the signal is also stronger in males than in females. Moreover, two CHC compounds: (Z)-10-C21 and (Z)-7-C21) show a K value larger than 3 (Figure 6.2).

6.4.4. Tempo and mode of evolution

6.4.4.1. Phylogenetic versus chemical distances

The mode of evolution differed strikingly between males and females. Males evolve gradually with phylogenetically distant species showing larger chemical distances, which results in a strong significant Mantel correlation between CHC profiles and phylogenetic distances in males (Mantel r: 0.3424, $p< 0.001$). Female chemical distances, on the other hand, showed no clear relationship with the molecular distance: two phylogenetically distant species can have a similar CHC composition (Mantel Test r 0.0715, $p = 0.131$, Figure 6.3). This pattern was not only observable when using information on relative abundances of the CHC compounds but also when using presence/absence data (males Mantel r 0.221, $p < 0.001$; females Mantel r -0.0022, $p = 0.475$, Figure 6.3). The same results were also obtained when plotting all pairwise distances (calculated with relative amounts) between all individuals of different species (almost 180000 pairwise comparisons using about 600 individuals for each sex (Appendix) rather than comparing average values per species (Figure 6.3). There were different patterns of divergence between tribes. Phylogenetic signal is stronger in males, especially in the tribe Elampini as reflected by the Mantel Test (Elampini r: 0.714, $p < 0.001$ vs. Chrysidini r 0.3058, $p < 0.001$). There was a significant correlation between chemical distances and phylogenetic distances when the analysis included only females of the tribe Elampini (Mantel r 0.230, $p < 0.001$).

The differences in the mode of evolution observed between males and females remain even if Bray-Curtis dissimilarities are calculated selecting compounds belonging to different classes of hydrocarbons (Figure 6.4). Linear alkanes may have little informative value in comparison to other substance classes (*e.g.*, Dani et. al., 2005) and represent a relatively constant proportion of a CHC profile, with the same compounds appearing in relatively similar amounts across species. I conducted Mantel tests on CHC separated by compound classes. As expected, chemical differences between species due to alkanes were more constant and very similar between the sexes in comparison to differences calculated using all other substance classes. However, no correlation between chemical distances and phylogenetic distances calculated with separate compound classes was found in females (Mantel test r only alkanes = 0.0816,

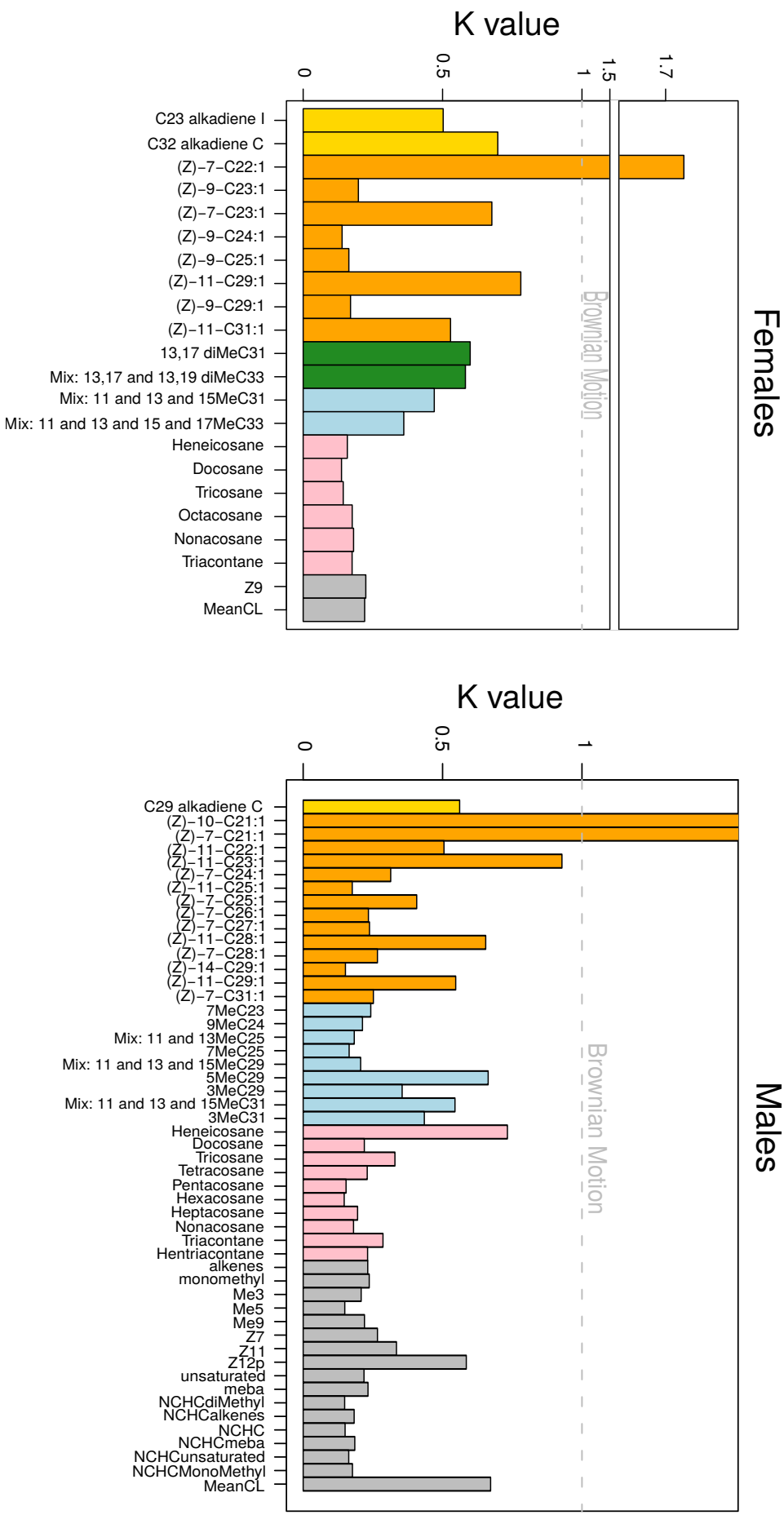

Figure 6.2.: Phylogenetic signal using Blomberg 's K index for females (left) and males (right). Note that a value of K = 1 indicates evolution under a Brownian motion model. Values larger than 1 indicate that closely related species are much more similar than expected under BM, whereas values of 0 indicate no phylogenetic pattern. Colors used for the CHC compounds indicate compound class (yellow = alkadienes, orange = alkenes, green = dimethyl-branched compounds, light blue = monomethyl-branched compounds, pink = linear alkanes). Gray has been used for summarizing variables. The Y axis of both graphs have been kept equal for comparison.

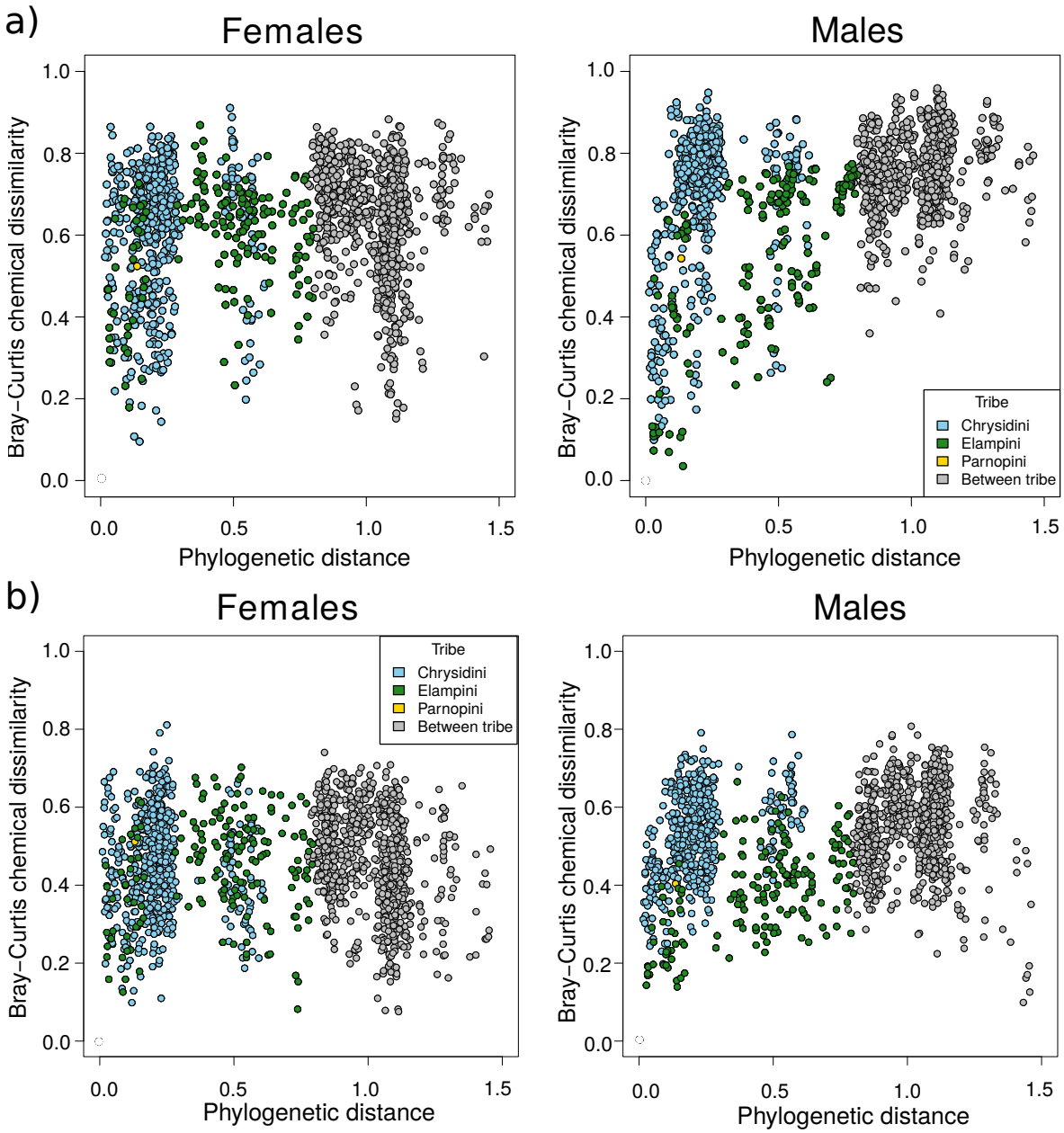

Figure 6.3.: Chemical distances vs. phylogenetic distances. a) using Bray-Curtis dissimilarities on the relative amounts of CHCs. b) using binary (presence/absence) data from which distances are calculated. Each point represents a between species comparison. Colors identify species pair comparisons within the same tribe. Gray colors depict species pairs between different tribes.

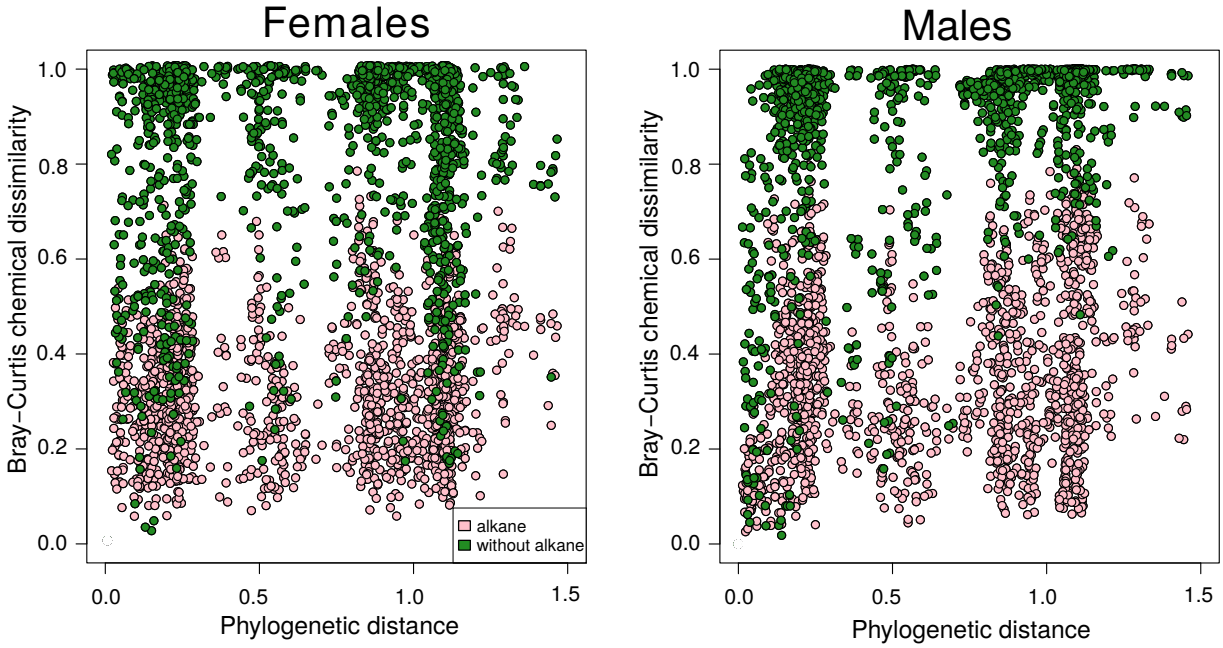

Figure 6.4.: Chemical vs. phylogenetic distances calculated from different classes of compounds. A matrix containing only alkanes was used to calculate Bray-Curtis dissimilarities (pink) between species pairs whereas the remaining compounds were then used in a second analysis and plotted on top.

p = 0.097 and all other substances r: 0.0574, p = 0.155). In contrast, there was a weak but significant correlation between chemical distances calculated with alkanes (Mantel r only alkanes: 0.192, p = 0.006) and a stronger correlation with all other substance classes (Mantel r: 0.380, p < 0.001) in males (Figure 6.4).

6.4.4.2. Rates of chemical evolution

There was more phylogenetic signal in males (more CHC compounds and stronger values), one possibility being that females's phylogenetic signal erodes faster. The multivariate calculation of rates of evolution (using Adams' method; Adams, 2014a) confirmed the expectation that females are evolving faster than males.

The comparison of rates of evolution using both the NMDS scores and the matrix of relative abundance of CHC traits yielded similar results, despite the absolute values of sigma being different (σ values based on the NMDS scores are 1.32 and 0.88 in females and males, respectively; when using the matrix of relative abundance, σ values are 0.006 and 0.004 in females and males, respectively). In general, the rates of evolution of the CHC profiles of females are seemingly larger than those of males, and species of the tribe Chrysidini seem to have evolved significantly faster than species of other lineages irrespective of the analyzed sex (but in males Chrysidini evolved almost five times faster than non-Chrysidini, in females the rate of evolution in Chrysidini was about two-fold larger than non-Chrysidini).

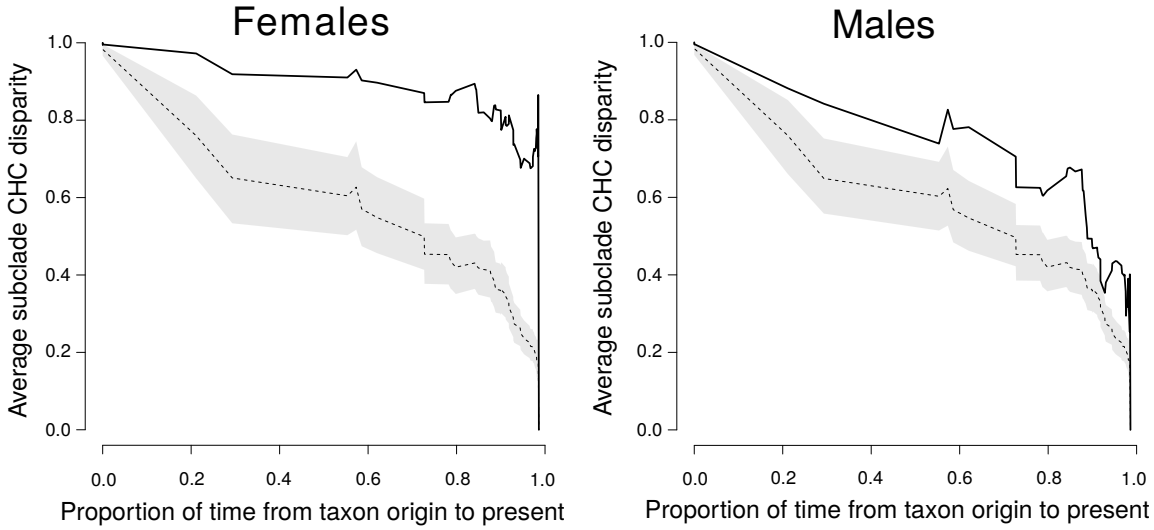

Figure 6.5.: Disparity through time plots (DTT) of female and male CHC profiles. Observed disparity is depicted with a solid black line, whereas the dash line indicates the expected mean disparity under 1000 simulations of character evolution under Brownian model. The grey shaded area represents 95% confidence intervals of the simulated data.

6.4.4.3. Chemical disparity

The chemical disparity through time analysis revealed that chemical profiles of females and males have a positive Morphological Disparity Index (MDI) suggesting that disparity of CHC profiles is higher than expected under BM model of evolution (Figure 6.5). However, there were some differences to note. Females experienced a relative high disparity along their entire history, indicating that there has always been CHC diversification in females. Males on the other hand have an MDI (0.153) that is about half as large as that of their females counterparts (0.292). In this sense, although closely related species may differ in their chemical phenotypes, this difference is not so strong as in females (Figure 6.5).

Disparity through time plots (DTT) conducted separately for substance classes produced also intriguing results. The DTT plot of n-alkanes did not deviate from a BM model in the males, but in females it showed an increase in disparity in the most recent history (Figure 6.6). Alkenes showed a relative larger disparity only for certain periods of time in males but not in females, which showed a larger disparity all along their evolutionary history (Figure 6.7). Both sexes show a large disparity of methyl-branched alkanes, but the magnitude of the MDI was larger in females than in males (0.282 vs. 0.2, figure 6.8).

6.4.4.4. Phylochemospaces

The projection of the phylogeny onto the chemical space revealed differences in the patterns of chemical diversification between females and males. In general, both sexes have explored a similar area of chemical space (Figure 6.9). However, clades have diversified differently in both sexes. For example, the *Chrysis ignita* species group shows not only more phenotypic changes (CHC compounds) per phylogenetic branch but also a larger exploration of the chemospace in females than in males. In

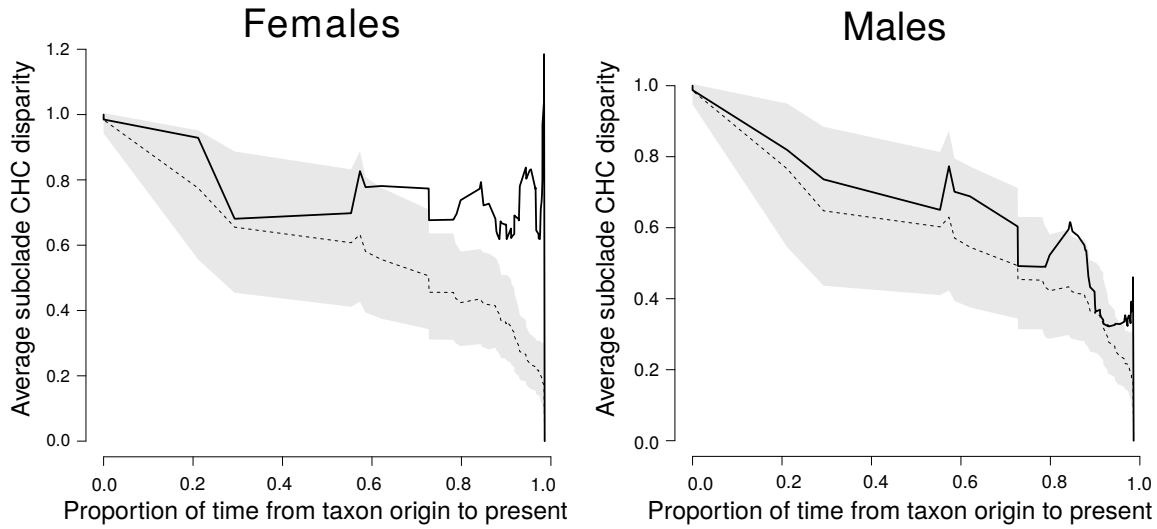

Figure 6.6.: DTT graphs per compound class. These disparity through time plots were calculated using only n-alkanes. Legend as in figure 6.5.

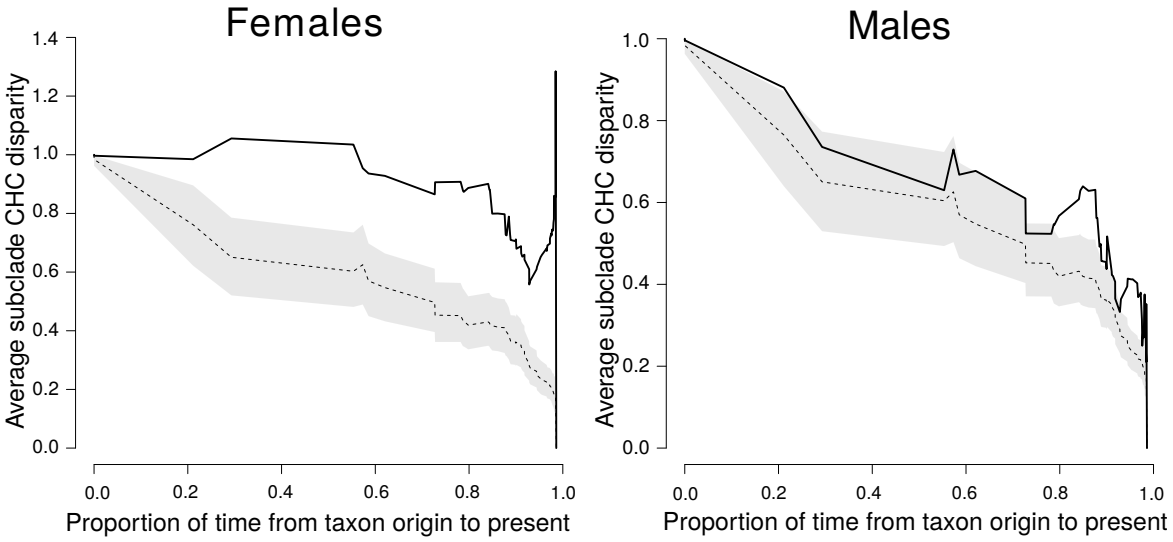

Figure 6.7.: DTT graphs per compound class. These disparity through time plots were calculated using only unsaturated compounds. Legend as in figure 6.5.

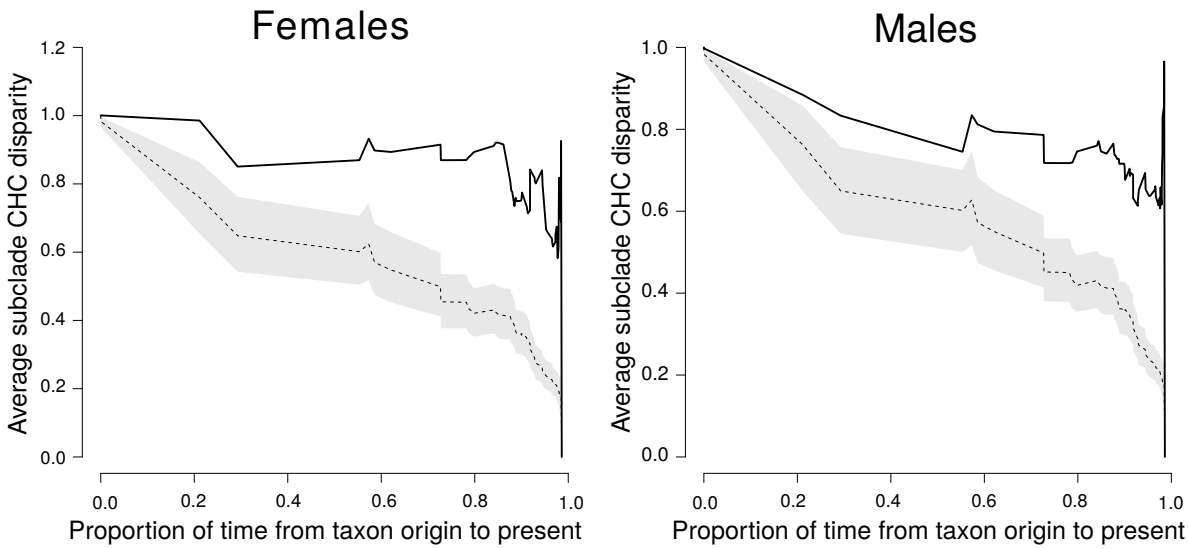

Figure 6.8.: DTT graphs per compound class. These disparity through time plots were calculated using only methyl-branched compounds. Legend as in figure 6.5.

general, males of closely related species have explored less area of the chemospace, but in total they have diversified the most. In contrast, only females of the closely related *Chrysura* explored a smaller region of the chemical space than their conspecific males (Figure 6.9).

6.5. Discussion

Traits showing dimorphism between sexes have traditionally been attributed to be the outcome of sexual selection acting on males, to render them more attractive to their female mates. Using a combination of different phylogenetic comparative methods, I investigated the tempo and mode of evolution of CHC profiles in female and male

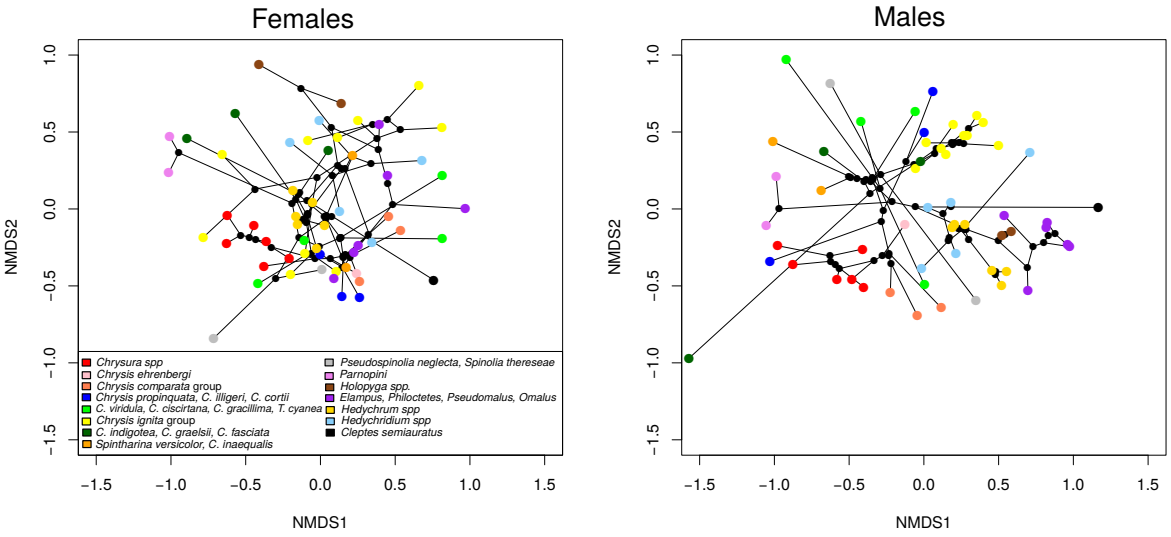

Figure 6.9.: Phylochemospace of females and males. Species are colored according to phylogenetic relatedness.

cuckoo wasps in order to explore the effect of natural and sexual selection in the CHC dimorphism of cuckoo wasps. The results suggest a general pattern: CHC profiles of females diversified faster, have not evolved gradually, and as a result, showed a larger disparity among closely related species than CHC profiles of males. In the following, I discuss these results in light of the biology of cuckoo wasps and advocate that natural selection has had an important role in driving the origin of chemical sexual dimorphism in cuckoo wasps.

6.5.1. Stronger phylogenetic signal in males than in females

A high relative disparity is indicative of a greater variation within subclades and a larger overlap among subclades (Harmon *et al.*, 2003), suggesting that distantly related species have evolved to occupy similar regions in the chemical space. The analyses revealed that although CHC profiles of males and females show both a high disparity, the amount of this disparity was twice as large in the females, indicating that their CHC profiles not only are but probably have been much more divergent between closely related species than those of males. This was reflected as well in the phylochemospace of females, in which a larger overlap of the phylogenetic branches among subclades is distinguishable (Figure 6.9).

However, not only the application of (multivariate) methods (*e.g.* Mantel tests or Kmult) in which all the CHC profile is measured indicate a stronger phylogenetic signal in males. The calculation of phylogenetic signal (irrespective of the index used) on single compounds, as well as on a number of traits summarizing and characterizing CHC profiles, showed that males present stronger phylogenetic signal than females (both in the degree and in the number of variables). A high phylogenetic signal is an indication that traits have evolved in a Brownian motion manner, which could result either from genetic drift or from natural selection that is randomly fluctuating (Felsenstein, 1985b; Losos, 2008). In contrast, a weak or no phylogenetic signal indicates the existence of other evolutionary processes, among which diversifying and stabilizing selection are most commonly involved (Blomberg et al., 2003, but see Revell, 2008).

In other words, the results show that the evolution of CHC profiles of males is more phylogenetically constrained than those of females. This leads to ask what selective pressures may be responsible for these differences. CHC may evolve due to selective pressures acting on their protective function against desiccation or on their role in communication processes, or on both. Since the two sexes of cuckoo wasps occupy the same habitat and therefore share the same temperature and other climatic conditions, it is expected that the differences arise due to selection (or its constraint) acting on the role of CHC in communication. One likely hypothesis is that in females, coevolutionary arms race between female cuckoo wasps and their female hosts may drive rapid changes in their CHC profiles to chemically mimic the profiles of their hosts. This would in part explain, why the CHC profiles of females seem to evolve faster and show a much larger disparity, especially in recent history. Cuckoo wasps can exert a strong pressure on their hosts (*e.g.*, Simon Thomas & Simon Thomas 1972). To avoid parasitism, hosts of cuckoo wasps adopt behavioral (*e.g.*, Strohm *et al.*, 2001) as well as chemical adaptations (Wurdack *et al.*, 2015; Chapter 7), and thus hosts and parasites engage in an evolutionary arms race. This antagonist conflict, however, affects to a larger extent only female individuals (Chapter 7). Thus, natural selection

on the female CHC profiles (*e.g.* coevolution with their hosts) may be stronger than that acting on males' CHC profiles.

Despite these clear differences between sexes, phylogenetic signal was weak in many compounds (including compounds evolving in males), and values of disparity were still high for both sexes, suggesting that there must be other selective pressures driving the evolution of CHC profiles of males as well. In agreement with the findings of a large disparity in CHC profiles (positive MDI in both sexes), other studies have shown that traits used in chemical communication can exhibit large values of disparity (Zimmerman *et al.*, 2009; Pokorny *et al.*, 2015; Weber *et al.*, 2016) compared to other less labile traits (Blomberg, 2003). For example, DTT analyses comparing perfume signals and other non-signaling (*e.g.* morphological) traits in male orchid bees have revealed elevated disparity of traits involved in communication (Weber *et al.*, 2016). In this particular case, sexual selection has been suggested to be driving the evolution of CHC profiles of male orchid bees (Weber *et al.*, 2016).

CHC profiles are complex traits with a dual function that can be subject to both natural and sexual selection (Chung & Carroll, 2015). So far, experimental evolutionary studies to understand whether and how natural and sexual selection influence the evolution of dimorphic CHC profiles have been conducted only on *Drosophila* species. Breeding experiments on *Drosophila* have shown that both selective forces can influence the evolution of male and female CHC, albeit both sexes respond differently to each selective force. For example, it has nevertheless been demonstrated that male and female CHCs can evolve independently from each other (Rundle *et al.*, 2005), despite the presence of genetic constraints (Chenoweth *et al.*, 2007). Thus, there can be sex-specific responses to selection. Moreover, an experimental evolutionary study on the antagonistic effect of natural and sexual selection on CHC evolution of *Drosophila simulans* suggested that CHC profiles of males evolved due to sexual selection and female CHC profiles, in turn, responded more in the direction of natural selection (Sharma *et al.*, 2012). Despite the differences in the methods, the scope and the detail of analyses between this comparative study of Chrysididae and these controlled laboratory experiments on CHC evolution of *Drosophila*, these findings point also to different selective forces acting on the two sexes: female CHC profiles being affected by natural selection (see above) and sexual selection likely having a stronger effect on males' CHCs (see below).

The effect of sexual selection on the evolution of chemical signals has been empirically studied in relatively few insect groups (mainly on *Drosophila* and crickets) through selection experiments (as mentioned above) or by measuring selection gradients in a quantitative genetics framework (Lande & Arnold, 1985, see examples cited in Steiger & Stökl, 2014). Although I did not quantify selection, I argue, based on the life-history of cuckoo wasps, that sexual selection should have a stronger effect on males than on the opposite sex. Chrysididae wasps have a mating system similar to that of many aculeate solitary wasps, in which females are receptive upon emergence and mate only once in their lifetime (O' Neill, 2001). Few observations hint at males having a much shorter life-span than females do (Kimsey & Bohart, 1991; ON, *pers. comm.*), as in other Hymenoptera. Thus, males gain reproductive fitness by getting access to and fertilizing as many females as they can during their shorter life-span. Females, however, still need to invest resources in offspring production and in searching for a suitable host. Moreover, being haplodiploid, females can still produce male offspring being unmated, while males only gain reproductive fitness through being

able to fertilize females. Thus, the biology of the species suggests sexual selection should be stronger in male than in female cuckoo wasps.

6.5.2. Differences among compound classes

Whereas I can not disregard that sexual selection may influence the evolution of the CHC profiles of males (and possibly also on females) of cuckoo wasps, a separate line of evidence suggests that CHC dimorphism in cuckoo can possibly result of a stronger selective force acting on the CHC profiles of females.

When comparing the patterns of evolution of the main compound classes separately, striking differences between males and females were observed. Linear alkanes lack features which confer three-dimensional configurations (*i.e.*, double bonds, methyl-groups) and that are useful in the context of chemical communication and recognition (Dani *et al.*, 2001; Monnin, 2006). Instead, since they melt at higher temperatures than unsaturated compounds (Monnin, 2006) and methyl-branched alkanes (Gibbs, 2002), they are thought to regulate and help providing structure and stability to the CHC profile (Menzel *et al.*, 2017a). Hence, a more gradual mode of evolution is expected in n-alkanes. These expectations were confirmed only in the disparity through time (DTT) plots inferred from males but not from females. While the DTT plot of n-alkanes of males falls within the upper limit of the confidence interval of a BM model, the pattern of evolution in females is incompatible with BM model in the most recent clades, suggesting that there is strong selection for CHC profiles of females to differ in linear alkanes. This is an interesting result, since it emphasizes that strong selection should be acting on the female CHC profiles to differ in the expression of otherwise relatively constrained compounds. Linear alkanes are probably not playing an important role in intraspecific communication in cuckoo wasps, since they are less often selected as main compounds contributing to sexual dimorphism of CHC profiles (Chapter 4). Nonetheless, it is possible they correlate with other compound classes. Some host species may be able to shift the expression of CHC profiles to different compound classes or to longer chain lengths as an escape strategy from their parasites (Chapter 7). A shift in chain length, could be associated with the reduction in the expression or a shift in the production of alkanes accordingly to the chain length shift, and could be explained as an adaptation to chemically deceive its host. Congruent with this, phylogenetic signal of mean chain length was much larger in males than in females. Additionally, relative disparity was larger in the recent history of cuckoo wasps because these changes are likely a result of an ongoing coevolutionary arms race.

The comparison of the two other main compound classes showed that DTT plots of females exhibited larger disparity than those of males, especially in the comparison of alkenes. Sexual selection acting on the CHC profile of males (irrespective of the mechanism, *e.g.*, female mate-choice or male-male competition), has an effect on methyl-branched alkanes or alkenes. In fact, alkenes with an internal double bond position showed a strong phylogenetic signal, and seem to be among the main compounds contributing to the differences between females and males of cuckoo wasps (Chapter 4). It is therefore possible that alkenes of males are mainly used for intraspecific communication (to be recognized, accepted by a female). While sexual selection may contribute to differences among closely related species of males, the result is a pattern in which closely related species still show phylogenetic signal (Figure

6.9).

To my knowledge, no other study has compared CHC profiles (or other chemical signals) between females and males of a large number of species (> 20 spp). Pokorny and colleagues (Pokorny *et al.*, 2015) addressed the evolutionary patterns of the CHC profiles of orchid bees, unfortunately females of orchid bees are difficult to collect and to identify, which impeded them to compare males and females of a reasonable number of species. Therefore, it remains difficult to relate these results to another similar study. Nevertheless, research based on differences in signals of other modalities shows that it is also possible and maybe not that infrequent that sexual dimorphism arises due to changes in the female sex. For instance, in birds (in which a large number of studies have been conducted to test hypotheses on the origin of sexual dimorphism), females have changed more dramatically coloration plumages (Maluridae, Johnson *et al.*, 2013 and Icteridae, Price & Eaton, 2014), contributing to sexual dichromatism more than males have. In new world orioles, sexual dichromatism has originated due to losses of coloration in females and not due to gains of ornamentation in the males (Hofmann *et al.*, 2008). Even in cases in which sexual dichromatism of birds (*i.e.*, tanagers) seems to have evolved due to a greater number of changes in males, selection on female plumage has also played a role in shaping sexual dichromatism (Shultz & Burns, 2017). Recent studies are also showing that sexual dimorphism in singing behavior of birds may be the result of historical changes in females rather than in males and that changes in females can be faster (Price, 2015 and citations therein). In fact, Price (2015) argues that sexual dimorphism is the product of different selective forces acting on each sex and not only the result of strong sexual selection on males as previously accepted.

An alternative explanation would be that chemical sexual dimorphism has arisen due to changes on the males CHC profiles (possibly due to sexual selection). This explanation seems improbable, since there is stronger phylogenetic signal in males. Moreover, a recent study of a chemical coevolutionary arms race between cuckoo wasps of the genus *Hedychrum* and its Philanthinae hosts would contradict this explanation. *Hedychrum* males show stronger phylogenetic signal reflecting the evolutionary history of the species, while females CHC profiles have diversified into two groups according to the CHC profiles of their respective female hosts (Chapter 7). On the other hand, it seems also difficult to imagine a situation in which only males would be subject to the effects of genetic drift on their CHC profiles, and females not.

Despite certain limitations of the present study and the scant available information on life-history traits of these wasps, sexual dimorphism of CHC profiles in more than 50 species of a parasitoid Hymenopteran family were compared. This is the first of such studies to compare female and male CHC profiles within a phylogenetic framework that tries to elucidate the role of natural vs. sexual selection in the evolution of CHC profiles. Few studies have compared previously female and male CHCs but either using a small number of species (Alves *et al.*, 2010), or using just very few CHC compounds (Schwander *et al.*, 2013), and none of them with the aim to understand how sexual dimorphism in these "dual traits" (Chung & Carroll, 2015) may have arisen and is maintained.

One drawback of this study is the very little information available on life-history traits of cuckoo wasps and their hosts. Most of the host-parasite relationships of cuckoo wasps are not yet well known and life history traits are practically inexistent for many of the species, hindering the application of modelling approaches to test for

correlations between characteristics of the nest/other traits and the degree of sexual dimorphism using PGLS (Mundry, 2014). Nevertheless, these gaps in biological information may become filled in the near future as there is some revived interest in better understanding host-parasite relationships from trap nests studies (*e.g.* Pärn *et al.*, 2015; Martynova & Fateryga, 2015; Torretta, 2015, etc.) which provide a much reliable source of host-parasite relationships (from species nesting above-ground). Species nesting above ground are also among the most endangered species because they are less likely to find suitable nesting resources after habitats are fragmented in comparison to species nesting on the ground (Paukunnen *et al.*, 2017), in this sense the collection and publication of this valuable information should be promoted.

Four different indexes to calculate phylogenetic signal were employed. Results differed among the indexes. Different compounds were chosen as showing phylogenetic signal depending on the index used. Nevertheless, irrespective of the index used, more compounds and variables were selected and a stronger phylogenetic signal was revealed in males than in females. The reason for these differences may arise because the different indexes emphasize different aspects when calculating phylogenetic signal and may depend on different assumptions. Sample size has been indicated to cause variations in the results, especially for Blomberg's K (Munkemüller *et al.*, 2012). The present study however included more than 50 species, and sample size should therefore not have had major effects in the analyses. Another reason for this variation is that these indexes were all developed for continuous traits. Relative proportions of a compound constitute a semi-quantitative trait, which may have statistical influences on the application of the phylogenetic signal. For this reason, I also included the measurement especially suitable for binary traits (Fritz & Purvis, 2010). Nevertheless, phylogenetic signal has been applied many times to semi-quantitative traits in the literature providing consistent results (*e.g.*, Prieto-Benitez *et al.*, 2016, Menzel *et al.*, 2017b, García-Roa *et al.*, 2017). However, variation in the phylogenetic signals also has arisen in those studies in which more than one index was employed. My interpretation of phylogenetic signal is nevertheless, not specific to the compounds and the variables but to the general comparison between sexes, which seems to be strong enough (see Appendix).

In order to disentangle and prove the hypothesis, I suggest to explore whether there are correlations between a number of biological traits related to the host of cuckoo wasps (*e.g.*, type of nesting, whether the nest is left open or not, host prey, etc.) and the characteristics of the CHC profile. More interesting even would be to compare and relate the CHC profile of cuckoo wasps to those of their hosts and evaluate if the host profile can explain convergent evolution and the high disparity observed in CHC profiles. In the end, such an analysis would test the hypothesis that sexual dimorphism has arisen due to coevolution with the host profiles.

Sexual dimorphism is not uncommon in Hymenoptera (Stubblefield & Seger, 1994). Within this diverse order, many life strategies and life history types have evolved triggering the evolution of extreme cases of sexual dimorphism, which sometimes confused taxonomists to the point that different sexes of a species were allocated as being different species (Stubblefield & Seger, 1994). For instance, sexual size dimorphism is sometimes extreme as in some ant species, or one of the sexes is wingless (Stubblefield & Seger, 1994; Boulton *et al.*, 2015). Nevertheless, our knowledge of the effect of natural and sexual selection on shaping sexual dimorphism in traits other than size in Hymenoptera is still scant (Boulton *et al.*, 2015). While I have studied chemical

dimorphism in cuckoo wasps in light of the different effects of natural and sexual selection, it would be interesting to discover whether similar patterns of evolution are observed in other traits such as size and coloration. Both sexes of most cuckoo wasps are beautifully and strikingly colored, and whether color dimorphism may be rare (Kroiss *et al.*, 2009b) it does exist at least in some species (Kunz, 1994). Whereas the mechanisms of the iridiscent coloration in cuckoo wasps have been described, the ultimate causes of the evolution of coloration patterns in cuckoo wasps are not yet well understood (Kroiss *et al.*, 2009b). The same applies to size dimorphism, in which females are larger than males in some species but in others the differences in size between the sexes is less clear (Kunz, 1994). In Hymenoptera, it has been suggested that investment in parental care and nest construction has triggered the evolution of sexual size dimorphism (Shreeves & Field, 2008). However, it may be possible in the future to study the evolution of signaling traits of different modalities to better understand how natural and sexual selection may affect sexual dimorphism in these traits in cuckoo wasps.

6.6. Conclusion

In conclusion, this study has demonstrated that CHC profiles of female and male cuckoo wasps evolve differently, with females showing less phylogenetic signal, a larger disparity in their CHC profiles, and a higher rate of CHC evolution than males. The pattern of evolution of CHC compounds belonging to different compound classes (*e.g.*, alkenes, methyl-branched alkanes and linear alkanes) also varied strikingly between the sexes. Linear alkanes, which are assumed to be less affected by selection for communication and recognition processes, followed expectations only in males, whereas in females there was strong selection to evolve differences in these CHC compounds between closely related species. Overall, females have contributed the most to the divergence of CHC profiles among closely related species. Based on this evidence, and the parasitic lifestyle of the family, I argue that in the case of cuckoo wasps, chemical mimicry of the CHC profile of their female host by the female cuckoo wasp (in this case, natural selection on females) plays an important role, even more dominant, than that of sexual selection in generating sexual dimorphism in these important for recognition chemical signals.

Whereas I can not disregard that sexual selection has acted on males (and possibly on females) in species recognition, attraction and selection processes, I am inclined to state that the role of natural selection is comparatively stronger. However, the relative importance of both selection processes in driving CHC dimorphism in cuckoo wasps, still need to be studied. Nevertheless, this study sets the basis for further analyses on CHC profiles and has demonstrated that CHC profiles of females and males can not only be very dimorphic but that they indeed evolve differently and their evolution may be triggered by different strengths of sexual and natural selection.

7. Evidence for chemical coevolution between *Hedychrum* and their hosts

7.1. Abstract

By exploiting their hosts' parental care, brood parasites can exert strong selection on their hosts' populations. This can lead to co-evolutionary processes, in which adaptations by the brood parasites for example, for avoidance of detection face counter-adaptations by the hosts to improve brood parasite recognition. Here we present evidence for the occurrence of a historical and likely continuing coevolution between digger wasps of the genera *Cerceris* and *Philanthus* (Apoidea: "Crabronidae": Philanthinae) and their brood parasites, cuckoo wasps of the genus *Hedychrum* (Chrysidoidea: Chrysididae) that chemically mimic the cuticular hydrocarbon (CHC) profiles of their hosts. Digger wasps of the genera *Cerceris* and *Philanthus* hunt two types of prey: Coleoptera and Hymenoptera. Depending on the type of prey, an specific CHC profile must be maintained or not to avoid fungal infestation of the prey. In this study, we show that 1) there is less overlap in the CHC profiles of brood parasites of Coleoptera-hunters and their hosts, compared to that between Hymenoptera-hunters and their brood parasites, 2) CHC profiles of hosts preying on naturally preserved food are more diversified in females than in males, thus in the sex that is chemically mimicked by brood parasites, and 3) only female (not male) cuckoo wasps of Coleoptera-hunters are chemically similar to their female hosts. Altogether, these results show that female hosts that prey a naturally protected food item (Coleoptera-hunters) may have diversified their CHC profile as an adaptation to escape chemical mimicry from their brood parasites, a strategy that can not be adopted by wasps preying on Hymenoptera, more susceptible to fungal infestation.

Keywords: cuticular hydrocarbons, chemical mimicry, Chrysididae, evolutionary arms race, Philanthinae

7.2. Introduction

Coevolution or reciprocal genetic changes between interacting species is considered one of the major forces generating biological diversity (Laine, 2009). It plays an important role in the organization of communities (*e.g.*, shaping both symbiotic and parasitic interactions and co-evolutionary specialization among free living taxa; Thompson, 1994; 2009), and in promoting the evolution of key innovations (Thompson, 2012; Meyer et. al., 2012). Yet, demonstrating that a given trait has evolved in response to another trait from a different species and caused adaptations in return, is typically

not straightforward, and it is usually difficult to isolate the effects of external selection pressures on a particular trait of interest (Rothstein, 1990).

Among antagonistic interactions, interspecific brood parasitism is a widespread strategy of parasitoids and cleptoparasites (in the following simply brood parasites) where either the host immatures (the former) or the host's parental care (*e.g.*, provisions, the latter) are exploited to raise own offspring (Davies *et al.*, 1989; Rothstein, 1990; Winfree, 1999; Spottiswoode *et al.*, 2012). Various remarkable examples of coevolution occur in such systems. In vertebrates, these examples include avian brood parasites (*e.g.*, cuckoos, Rothstein, 1990) that lay eggs visually mimicking their host's eggs, and whose hosts respond with different strategies (*e.g.*, by increasing egg colour polymorphism, Spottiswoode & Stevens, 2011).

Other examples of co-evolutionary histories between brood parasites and their hosts are found in eusocial insects and their social parasites (Davies *et al.*, 1989; Kilner & Langmore, 2011). A common adaptation of brood parasitic insect species is their ability to chemically deceive their hosts when sneaking into their nests for oviposition. In insects, recognition systems are primarily mediated by cuticular hydrocarbons (= CHC; van Zweden & d'Etorre, 2010). This diverse group of hydrophobic molecules play a central role in the life of insects by restricting water loss to the environment and by conveying a plethora of information (*e.g.*, age, sex, reproductive status, caste and colony membership; Blomquist & Bagneres, 2010a).

Insect brood parasites are known to adopt one of at least three different strategies to successfully deceive their host's recognition system (Lenoir *et al.*, 2001): (1) chemical insignificance: the brood parasitic species has a very low amount of CHC on its cuticle and thus provides the host few recognition cues (Lambardi *et al.*, 2007; Kroiss *et al.*, 2009a); (2) chemical camouflage: the brood parasite physically adopts the host's CHC profile by grooming; this strategy is commonly applied in social parasites (D'Etorre *et al.*, 2002; von Beeren *et al.*, 2011); (3) chemical mimicry: the brood parasite synthethizes *de novo* a CHC profile that is very similar to that of its host (Howard *et al.*, 1990). The three above mentioned strategies are not mutually exclusive and in some species, a combination of them is applied by a given species of brood parasite. For example, the butterfly *Phengaris (Maculinea) rebeli,* whose larvae develop in ants' nests, uses both chemical mimicry and chemical camouflage to deceive its host (Akino et. al., 1999). Hosts can counteract the above strategies by brood parasites with an improved ability to recognize and discriminate chemical cues and by expressing chemical phenotypes that differ from those of their brood parasites (*e.g.*, via negative frequency-dependent selection, Spottiswoode & Stevens, 2011; Jongepier & Foitzik, 2016).

Despite the importance of chemical mimicry for brood parasites, very few studies provide evidence for this strategy to result in a co-evolutionary arms race between brood parasites and hosts. Most of these studies have focused on species of the large and diverse order Hymenoptera. For example, adjustments of the CHC profiles of brood parasites as a result of coevolution have been shown in slave-making ants (Brandt et al, 2005; Errard *et al.*, 2006; Guillem *et al.*, 2014), cuckoo bumblebees (Martin *et al.*, 2010) vespid wasp social parasites (*Polistes* species, Lorenzi, 2006) and chrysidid cuckoo wasps (Strohm *et al.*, 2008; Wurdack *et al.*, 2015). In most of the studied cases, parasitic species use a chemical camouflage strategy (*e.g.*, ants, *Polistes*, cuckoo bumblebees), often preceded by an insignificance strategy. Furthermore, most of these cases involve host and parasites that are phylogenetically closely related, a

phenomenon known as Emery's rule (Emery, 1909). Chemical mimicry, on the other side, seems to be known only where hosts and parasites are distantly related, with parasites being forced to evolve an *ex novo* chemical profile to mimic the host (Akino et. al., 1999; Wurdack *et al.*, 2015). While some examples of CHC adjustments to host profiles are known in a co-evolutionary context, it is much more difficult to show counter-adaptations of the hosts to escape the evolution of such chemical strategies by the brood parasites. Besides behavioral or morphological counter-adaptations aimed to reduce parasitism (Achenbach *et al.*, 2010; Pamminger *et al.*, 2011), hosts may escape parasitism by evolving changes in their own CHC profile (Brandt *et al.*, 2005; Jongepier & Foitzik, 2016; Lorenzi *et al.*, 2014; Wurdack *et al.*, 2015). Such changes in CHC profiles were shown to include increasing CHC diversity (Jongepier and Foitzik 2016) or even the divergence of CHC profiles into strikingly distinct phenotypes (Wurdack *et al.*, 2015). These studies, however, investigated co-evolutionary patterns across populations of a single host species. To the best of our knowledge, there have been no comparative studies to unveil co-evolutionary patterns of CHCs in insect host-brood parasite systems across closely related species of hosts as well as their parasites.

In the present study, we investigated co-evolutionary patterns of CHC across species, using as models cuckoo wasps of the genus *Hedychrum* (Hymenoptera: Chrysididae: Elampini) and their distantly related hosts, digger wasps of the genera *Cerceris* and *Philanthus* (Hymenoptera: "Crabronidae": Philanthinae). Females of these solitary hosts dig brood cells in the ground and provision their larvae with paralyzed prey, which are either Coleoptera or Hymenoptera, depending on the host species (in the following text, we refer to Coleoptera hunting wasps and Hymenoptera hunting wasps as COLw and HYMw, respectively). These host-parasite systems were chosen both because of the peculiar host foraging biology of Philanthinae, which had important consequences on the evolution of their CHC, and because of the known patterns of host specialization in *Hedychrum* cuckoo wasps.

Concerning hosts, females of HYMw are known to embalm their prey with a secretion from their postpharyngeal gland to delay/prevent fungal infestation of the prey (Strohm & Linsenmair, 2001; Herzner *et al.*, 2013; Weiss *et al.* 2015b; Wurdack *et al.*, 2017). These secretions consist primarily of unsaturated long-chain hydrocarbons (*e.g.*, alkenes), which form a hydrophobic oily layer that prevents water condensation and impairs mold development on the prey (Herzner & Strohm, 2007; Herzner & Strohm, 2008). Moreover, the hydrocarbon composition of the postpharyngeal gland of HYMw strongly matches the CHC composition of the cuticle (Strohm *et al.*, 2010), similar to what was previously observed in ants (Bagneres & Morgan, 1991), and it is highly similar among species, suggesting that their profile is adaptive for prey preservation (Wurdack *et al.*, 2017). This embalming behavior on the other hand, is absent in COLw, as coleopteran prey is more resistant to fungal development (Wurdack *et al.*, 2017). Being released from this chemical brood-care strategy, COLw may have considerably diversified their CHC profiles, which now include a large number of methyl-branched alkanes (Wurdack *et al.*, 2017).

Concerning cuckoo wasps, each *Hedychrum* species is known to be specialized in attacking one or two host species (HYMw or COLw exclusively, see Appendix). Based on the only two studied species to date, chemical mimicry (Strohm *et al.*, 2008) and chemical insignificance (Kroiss *et al.*, 2009a) seem to be the strategies adopted by *Hedychrum* cuckoo wasps to sneak into hosts nests.

In this study, we thus investigated whether the modification of the CHC profiles of COLw by inclusion and diversification of methyl-branched compound represents a strategy to escape chemical mimicry by their brood parasites. HYMw cannot evolve such a strategy, given that these hosts have to maintain an alkene-rich CHC profile for preserving their prey. We hypothesized that 1) chemical mimicry of COLw hosts by their cuckoo wasps is less precise compared to that between cuckoo wasp species parasitizing HYMw and (any of) their HYMw hosts, 2) chemical strategies in the hosts are expected to be more pronounced in females than in males, because only female hosts are under selective pressure to evolve counter-adaptations to chemical mimicry by cuckoo wasps, and 3) female cuckoo wasps are under stronger selection pressure to mimic their hosts' CHC profile and should therefore show improved mimicry than conspecific males.

7.3. Methods

7.3.1. Collection and origin of the insect samples

We used between five and 14 individual wasps per group (each sex of each species) for the chemical analyses (Appendix). Following the collection of wasps by netting, each live specimen was placed in a glass vial (1.5 mL) and transported to the lab where the specimen was killed by freezing. All specimens were stored at -20°C until CHC extraction was conducted (see below). Tissue material from one or two individuals of each species was used for extracting DNA (see below) after CHC extraction. Specimens are stored at the Zoological Research Museum Alexander Koenig in Bonn.

7.3.2. Molecular procedures and phylogenetic analyses

7.3.2.1. DNA Extraction

DNA was extracted from the muscle tissue using QiagenKit "DNeasy Blood & Tissue Kit" (Qiagen GmbH Hilden, Germany).

7.3.2.2. Target DNA Amplification with Polymerase Chain Reaction

We used a set of twelve degenerated oligonucleotide primers to amplify twelve single-copy protein-coding nuclear genes in cuckoo wasps (Hartig *et al.* 2012; Appendix).

Polymerase chain reactions (PCR) were run in 20 µL volumes using the Multiplex PCR Kit by Qiagen (Venlo, The Netherlands). We applied a touch-down temperature profile that started with an initial denaturation and QIAGEN HotStarTaq DNA polymerase activation step at 95°C for 15 min followed by 15 cycles of 95°C (30 s), 60°C (30 s), and 72°C (1.5 min), during which the annealing temperature was decreased by 1°C (for each). This was followed by 25 cycles of 95°C (30 s), 50°C (30 s), and 72°C (1.5 min), followed by a final elongation step at 72°C for 10 min. All PCRs were run on a 2720 Thermal Cycler (Applied Biosystems, California, US). PCR products were separated by a 1.5% agarose gel using GelRed (Biotium). All PCR products were purified with the Illustra ExoStar PCR and Sequence Reaction Clean-Up Kit (GE Healthcare Life Sciences, Amersham, UK). Purified PCR products were sent

to Macrogen (Amsterdam, The Netherlands) for bidirectional direct Sanger sequencing with the sequencing primers HOG-Seq-A-F, HOG-Seq-A-R, HOG-Seq-B-F and HOG-Seq-B-R (Appendix).

7.3.2.3. DNA Sequence Processing

Forward and reverse DNA strands obtained from sequencing the amplicons mentioned above were assembled to contigs, trimmed (to exclude binding sites of PCR primers) in Genious version 6.1 (http://geneious.com, Kearse *et al.*, 2012). Subsequently all sequences were aligned with the l-insi algorithm of MAFFT (v7.123; Katoh and Standley 2013). Intron and exon regions were annotated manually by aligning a reference sequence for each gene from the 1KITE transcriptome of the cuckoo wasp *Chrysis terminata* and identifying canonical splice sites in the genomic sequencing (*i.e.* the dinucleotide pair GC-AG). We manually removed uninformative and ambigously aligned sites from the intron sequences. Finally, we concatenated all exons and introns into a supermatrix and defined three partitions: (1) 1st and 2nd codon position of exons; (2) 3rd codon position of exons; (3) introns.

7.3.2.4. Phylogenetic analyses

For phylogenetic analysis under the Maximum Likelihood optimality criterion, we used Modelfinder (Kalyaanamoorthy *et al.* 2017) implemented in IQ-TREE (v 1.5.5, Nguyen *et al.* 2015) in order to select the best fitting substitution model for each partition with the corrected Akaike information criterion (AICc). The substitution models selected for each partition is given in the Appendix. We used the results thereof in order to infer a phylogenetic tree with IQ-TREE. Statistical branch support was estimated from 1,000 non-parametric bootstrap replicates. For Bayesian phylogenetic analysis we based the selection of substitution models again on ModelFinder, but only tested models included in MrBayes (v 3.1.2; Huelsenbeck and Ronquist 2001 , Ronquist and Huelsenbeck 2003), which we subsequently used for tree inference. We started two parallel runs, both with a random starting tree, over 107 generations. We sampled trees every 105 generations and treated the first 106 of generations of both runs as burn-in and excluded these. We used the remaining trees from all Bayesian analyses in order to build a 50 % majority rule consesus tree. Convergence was visually assessed using Tracer (v 1.6, Rambaut *et al.* 2014).

We used the host phylogeny inferred by Wurdack and colleagues (Wurdack *et al.*, 2017), trimming it to the species analyzed in this study.

7.3.3. Chemical Analyses

7.3.3.1. Gas Chromatography/Mass Spectrometry

Frozen insects were allowed to thaw for about five minutes and subsequently submerged in n-hexane in order to extract the CHCs. After 10 minutes, the extract was transferred into another vial, concentrated with a gentle stream of CO_2 until approximately 80–100 µL of hexane remained and the CHC extract was stored at -20°C. The insect was stored separately in 100% ethanol to preserve the sample's DNA.

A HP 6890 gas chromatograph (GC) coupled with a HP 5973 Mass Selective Detector (MS) (Hewlett Packard, Waldbronn, Germany) or an Agilent 7890/5975 GC/MS

System were used for analyzing the extracts. The GC (split/splitless injector in splitless mode for 1 min, injected volume: 1 µl at 300°C injector temperature) was equipped with a DB-5 Fused Silica capillary column (30 m x 0.25 mm ID, df = 0.25 µm³, J&W Scientific, Folsom, USA). Helium was used as carrier gas with a constant flow of 1 ml/min. We applied the same temperature program irrespective of what specific GC-MS we used: start temperature at 60°C, with an increase of 5°C/min until 300°C, and isotherm at 300°C for 10 min. An ionization voltage of 70 eV (source temperature: 230°C) was set for the acquisition of the mass spectra by electron ionization (EI-MS).

In order to identify the double-bond position of alkenes, one to five extracts of each sex and species (depending on the amounf of CHC extract) were pooled and used for a dimethyl-disulfide (DMDS) derivatization following the protocol provided by Carlson and colleagues (Carlson *et al.*, 1989). The double bond positions of alkadienes remained unidentified due to low amounts of this substance class. Alkadienes were separated according to their retention indices.

7.3.3.2. Characterization of CHC profiles

We analyzed the cuticular hydrocarbon composition of cuckoo wasps and their hosts using a semiautomatic procedure which consisted of running batch jobs in AMDIS (Automated Mass Spectral Deconvolution and Identification System), and processing them using built-in codes in R version 3.02 (R Core Team, 2013). Prior to the use of AMDIS, we created a mass spectral library (which contains more than 600 identified mass spectra of common hydrocarbons and their retention indices). We confirmed the identified methyl-branched alkanes in this library by comparison of their retention indices with those given by Carlson and colleagues (Carlson *et al.*, 1998b). AMDIS uses mass spectra similarities and retention indices to select target compounds. AMDIS' result files were curated in R before further analyses. The parameters used in AMDIS were as follows: component width = 22, adjacent peak subtraction = 2, resolution = medium, sensitivity = low and shape requirements = medium.

We excluded non-hydrocarbon compounds (*e.g.*, esters, acetates, alcohols) since they were not the focus of our study and they did not occur in all individuals. After all compounds were identified, we calculated their relative abundance by dividing the total ion count of each peak relative to the total ions count of all peaks within a CHC profile in the range of C21–C33. To ensure that a compound occurred in the majority of samples of any species and did not represent an artifact of concentration differences or of the sensitivity of the GC/MS, we set a threshold for the consideration of any compound within a group. This minimum threshold requirement is met if the compound occurs in at least 50% of all specimens (per sex and species) and the mean relative quantitative abundance is at least 0.1%. If not stated otherwise, we analyzed the CHC extracts of males and females separately.

We estimated the total number of compounds and the number of compounds by compound class (*e.g.*, linear alkanes, unsaturated compounds and methyl-branched alkanes). Additionally, we calculated the mean chain length of each individual by summing up the relative amount of each peak (within the range C21–C33) weighted by its retention index. This value indicates the retention index at which half of the relative amount of the CHC profile occurs.

7.3.4. Statistical analysis

CHC profiles were compared and differences visualized using multivariate methods. We conducted a Nonmetric Multidimensional Scaling (NMDS) using Bray-Curtis dissimilarity to visualize CHC profile similarity in two-dimensional graphs (Kruskal, 1964a; 1964b). All inferred stress values fell below 0.15. We then assessed the degree of similarity and overlap between CHC profiles of the different groups using an analysis of similarity (ANOSIM, Clarke, 1993), a non-parametrical test that operates on a ranked dissimilarity matrix (*i.e.* Bray-Curtis). The test statistic R ($-1<R<1$) indicates the degree of similarity between and within groups. An R value close to 1 indicates complete separation between the tested groups and values close to 0 indicates more similarity between the groups (greater overlap, less separation). Negative values of R are less common and do not have a biological interpretation.

We used Welch corrected t-tests (Welch, 1938; Ruxton, 2006) to compare various traits between HYMw and COLw and their brood parasites. Specifically, we compared Bray-Curtis dissimilarities between hosts and their brood parasites, the proportion, number, and diversity of CHC compounds and chain length.

For most of the analyses, we used the package vegan (Oksanen *et al.*, 2015) in R version 3.02 (R Core Team, 2013). Additional R packages used for plotting procedures were xcms (Smith *et al.*, 2006), flagme (Robinson, 2010), ade4 (Dray & Dufour, 2007), phytools (Revell, 2012) and ape (Paradis *et al.*, 2004).

7.4. Results

7.4.1. Molecular phylogeny and evolutionary relationships

On one hand, our inferred cuckoo wasp phylogeny shows that species parasitizing COLw (*Hedychrum chalybaeum*, *H. nobile* and *H. niemelai*) are more closely related to each other than to those parasitizing HYMw (*H. longicolle*, *H. gerstaeckeri* and *H. rutilans*) and viceversa, thus forming two distinct groups according to host specialization. On the other hand, the digger wasp phylogeny (Wurdack *et al.*, 2017) shows that host species preying on Hymenoptera belong to two separate clades (Figure 7.1).

7.4.2. Cuticular hydrocarbon composition

We identified 112 different hydrocarbons across the 277 analyzed samples (Appendix). Among these, there were twelve linear alkanes, 30 alkenes, nine alkadienes, 39 monomethyl- and 22 dimethyl-branched alkanes. Linear alkanes constitute about 30–45% of the total CHCs in all species (except in females of *Hedychrum niemelai* whose alkanes made up 60% of the total CHC profile). Unsaturated compounds (mainly alkenes) make up 60–65% of the CHC profiles of HYMw species whereas the proportion of methyl-branched alkanes in HYMw accounts for less than 3% of the total CHCs and these species do not possess any dimethyl-branched alkanes. In contrast, COLw show between 20% (males of *Cerceris interrupta*) and more than 60% of methyl-branched alkanes (females of *C. quinquefasciata*, figure 7.2).

The CHC profiles of females HYMw are very similar to each other across species and are predominantly composed of alkenes (60-65%) and linear alkanes (35%). Males of HYMw species are similar to their female conspecifics in CHC class composition

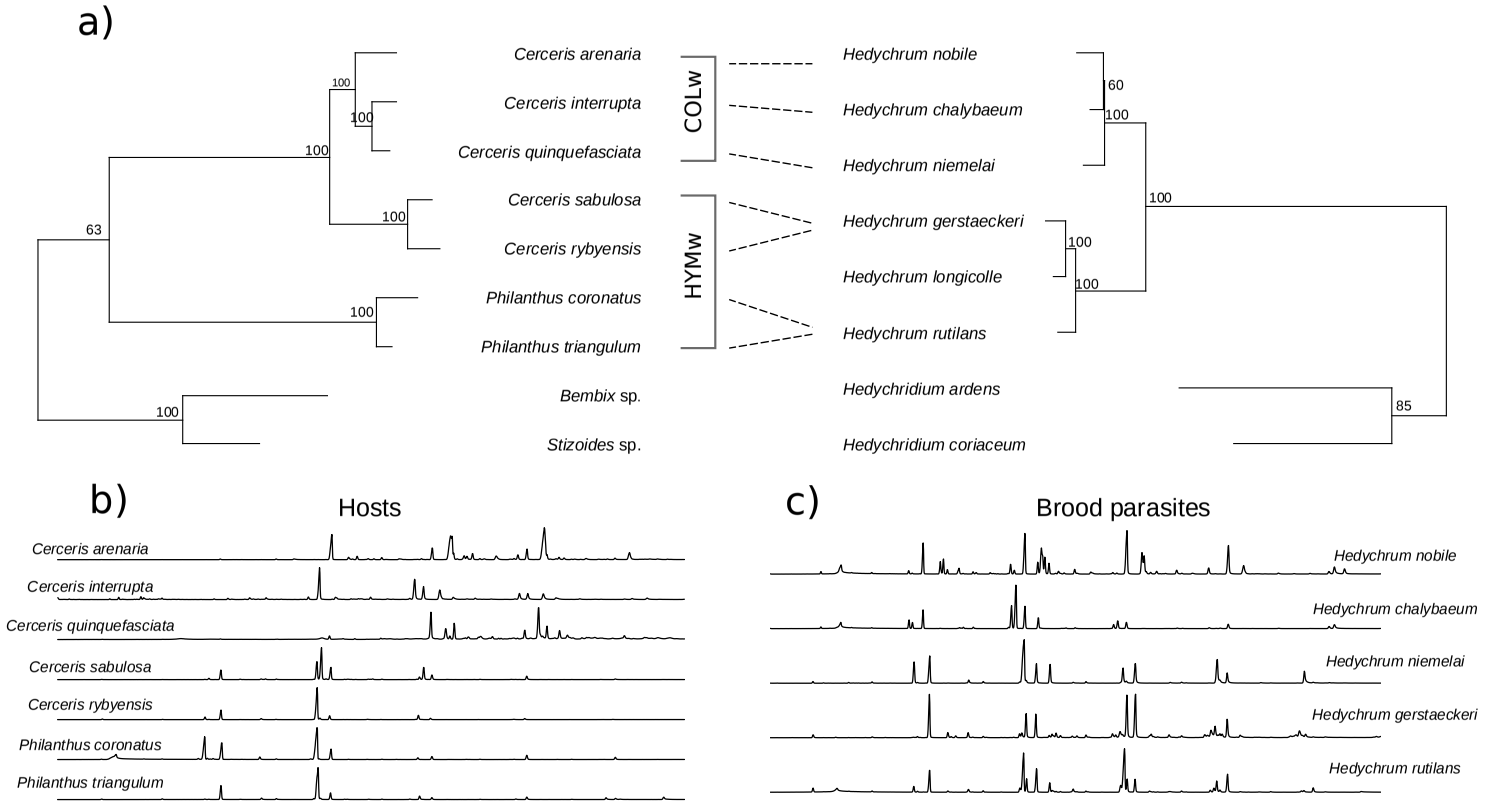

Figure 7.1.: a) Phylogenetic relationships of the analyzed hosts (left, adapted from Wurdack *et al.*, 2017) and their brood parasites (right) shown with representative chromatograms of female individuals of the hosts (b) and brood parasites (c). The trees were inferred using the Maximum Likelihood optimality criterion and bootstrap support values are given above each node. Host species are mentioned in front of their brood parasites, except for outgroup species (*Stizoides* sp. and *Bembix* sp., and *Hedychridium* spp.).

(Figure 7.2), but differ in the relative amount of alkenes with different double bond position. In general, both female and male HYMw hosts exhibit less diversified profiles with an average of 24.7 ± 3.49 and 21.9 ± 4.46 different CHCs in their profile, respectively, in contrast to 30.0 ± 6.17 and 34.5 ± 3.65 in females and males of COLw (difference between female HYMw and female COLw t(42): 4.32, $p < 0.001$; difference between male HYMw and male COLw t(65): 13.69, $p < 0.001$). The low number of compounds in HYMw is due to the very low number of methyl-branched compounds they express (on average less than six compounds). Among COLw, only in *Cerceris arenaria* females possesed more diversified profiles than males (35.9 ± 1.12 and 31 ± 1.26) . In the two other *Cerceris* COLw species, males have a larger number of CHC compounds in their profiles than their conspecific females (Figure 7.3a and 7.3b). Females of COLw possess a larger proportion of methyl-branched alkanes in their CHC profiles than their conspecific males (50% vs. 38%, t(54): 3.03, $p = 0.004$; Figure 7.3c, Appendix).

The profiles of cuckoo wasps show all substance classes of CHCs. Species that parasitize HYMw tend to show larger proportions of alkenes (in comparison to those parasitizing COLw, t(108): 4.99, p-value < 0.001), whereas species that parasitize COLw feature larger proportions of methyl-branched alkanes (in comparison to HYMw: t(88):-2.88, $p = 0.005$), with the exception of *H. chalybaeum* that exhibits larger proportions of alkenes (> 60%). Female brood parasites of COLw have a large proportion of methyl-branched alkanes (except *H. chalybaeum*), but conspecific males produce relatively large quantities of alkenes (> 60%), similar to the proportion of alkenes in the profiles of female and male brood parasites of HYMw (see figure 7.2). Female and male brood parasites of HYMw differ in the type of alkenes. Females have primarily alkenes with double bonds at position 7 and 9 while their conspecific males exhibit mainly alkenes with double bond at positions 9 and 11.

7.4.3. Changes in the chain length of COLw

On average the mean chain length of COLw was approximately two carbon atoms longer than that of HYMw and all species of the genus *Hedychrum* (Figure 7.4). Both females and males of COLw species have significantly less relative amount of hydrocarbons occurring before the RI 2400 (tetracosane) than HYMw (t(664): -4.22, $p < 0.0001$). In general, females exhibit a longer mean chain length than males (t(259): 3.58, $p < 0.001$). When looking at the different species groups, this pattern was found when comparing males and females of COLw hosts (t(54): 3.23, $p = 0.002$) and of all brood parasites (t(96): 7.99, $p < 0.001$), but not of HYMw (t(50): 1.47, $p = 0.15$).

7.4.4. Characterization of the chemical space

The NMDS shows a clear separation between female HYMw and COLw with the host types forming distinct groups with little overlap of their chemical spaces (ANOSIM R: 0.943, mean rank distance within groups: 1279, mean rank distance between groups: 1890, $p = 0.001$, 999 permutations, figure 7.5). This separation results from the differences in the relative amounts of methyl-branched and unsaturated compounds in both types of hosts. *Hedychrum nobile* and to a lesser extent *H. niemelai*, produce large amounts of methyl-branched compounds and therefore are separated from the other cuckoo wasps occupying a chemical space that falls between their hosts and other

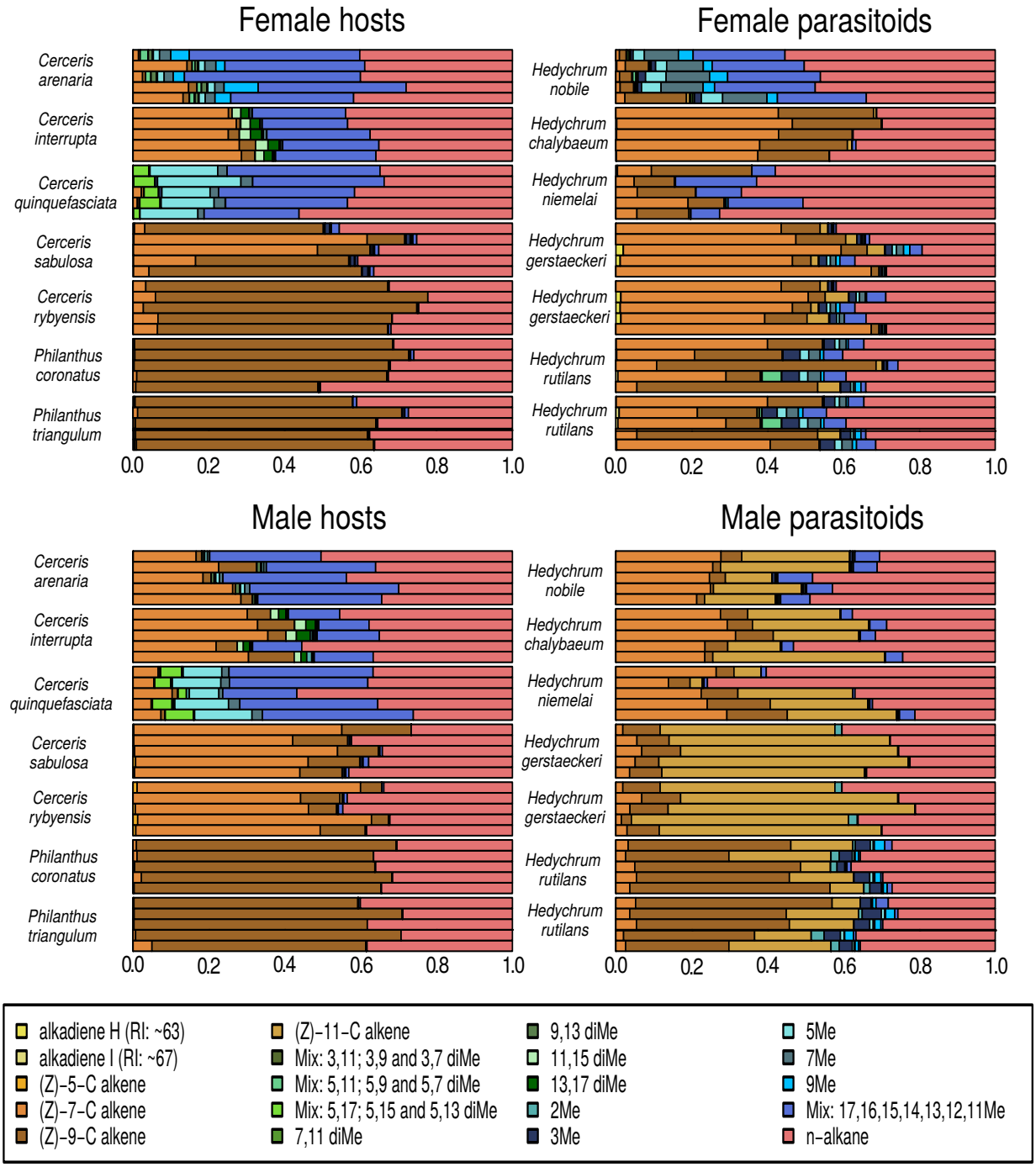

Figure 7.2.: Composition of CHC profiles sorted according to the most common functional groups in the analyzed species. Left columns refer to hosts and right columns refer to brood parasites; top graphs refer to females, bottom graphs to males. Five randomly selected samples of each species and sex illustrate within group CHC variability. Color hues indicate different CHC classes, also sorted from right to left: pink indicates pure n-alkane (C21–C33), blues are used to indicate monomethyl-branched alkanes, greens indicate dimethyl-branched alkanes, orange-brown colors are used to indicate alkenes and yellow hues indicate alkadienes.

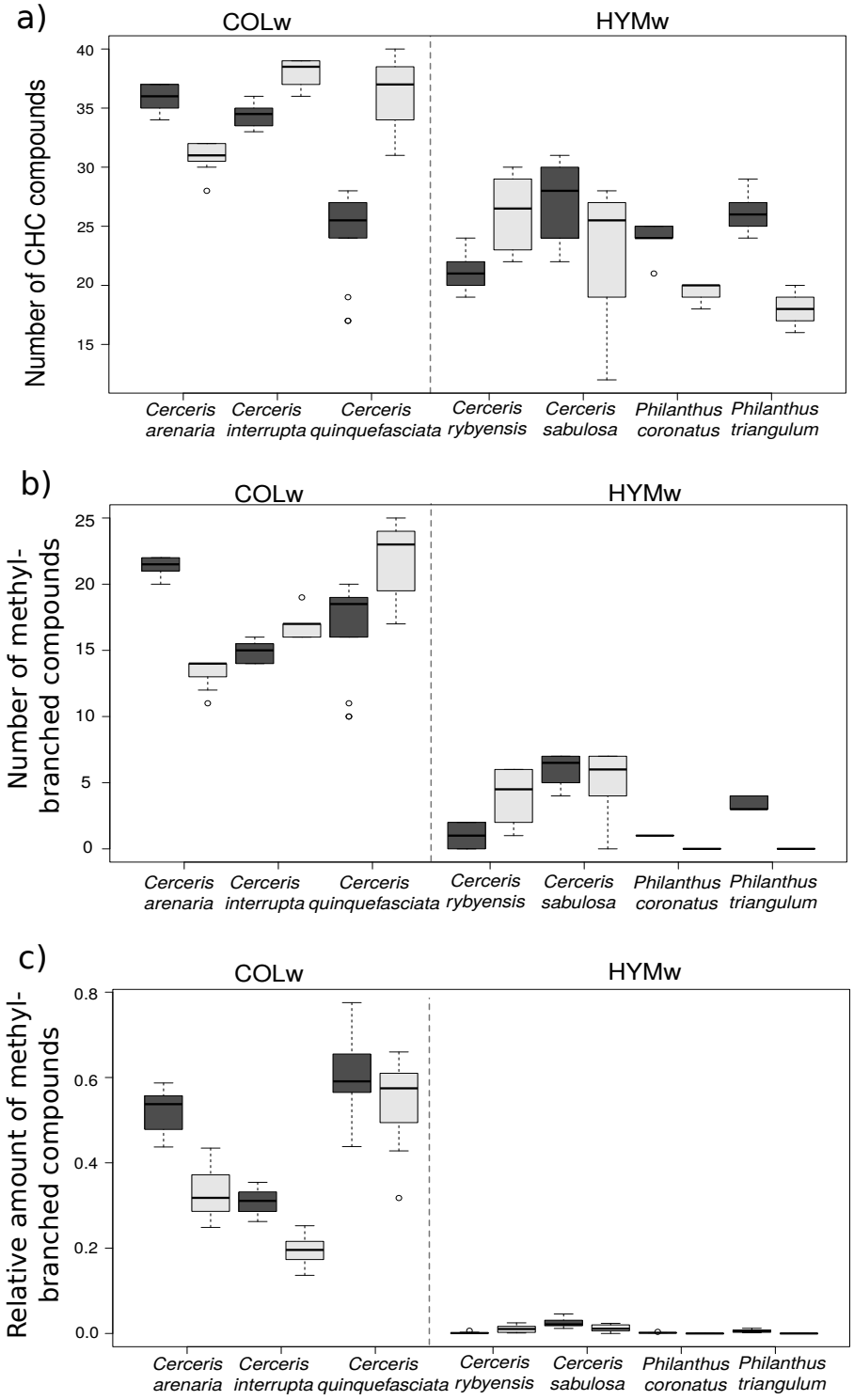

Figure 7.3.: Number of a) total CHC compounds, b) methyl-branched compounds and c) relative proportion of methyl-branched compounds in CHC profiles of females (dark gray) and males (light gray) of COLw and HYMw host species.

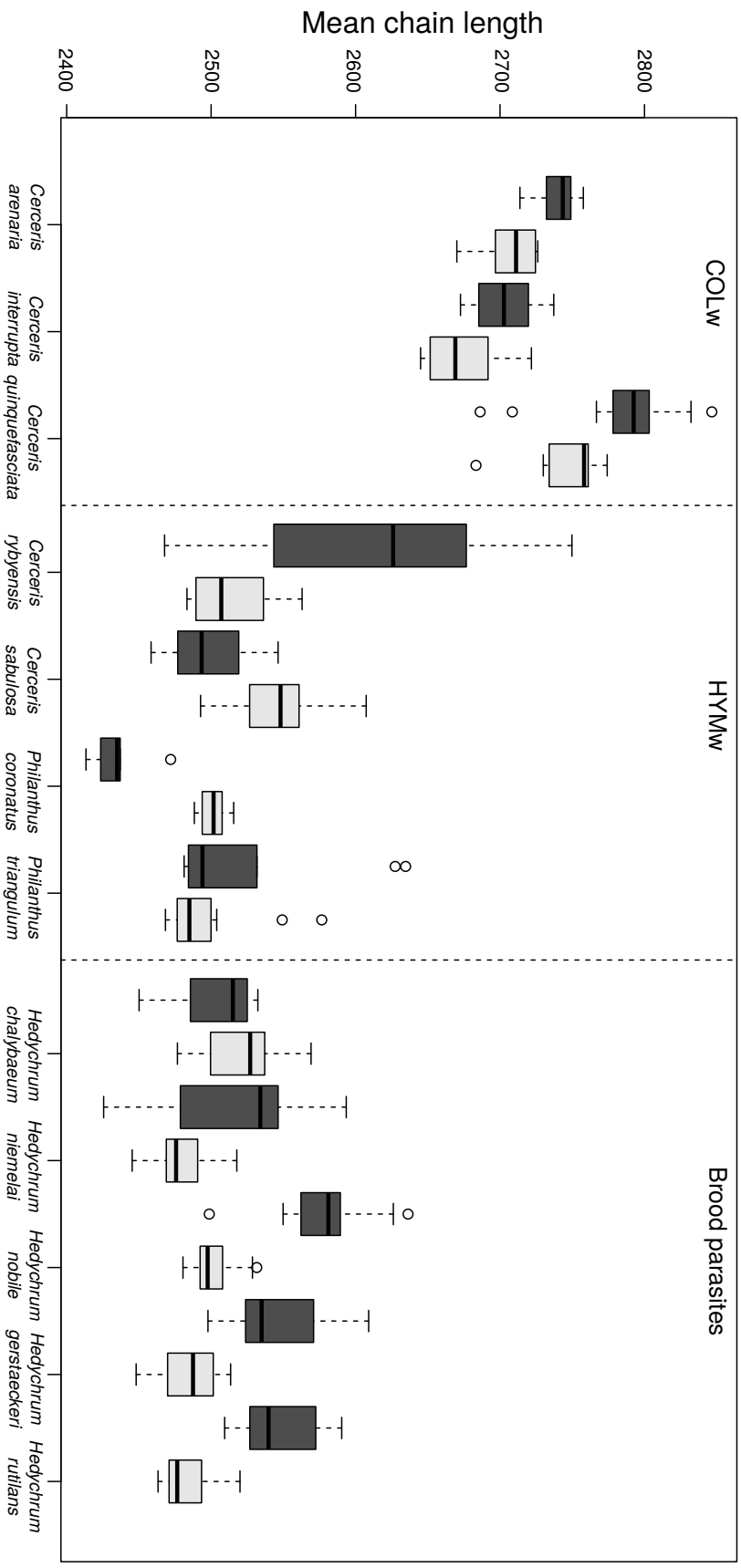

Figure 7.4.: Mean chain length of the different species in this study separated by sex: females (dark gray) and males (light gray).

HYMw. In contrast, all HYMw and their brood parasites occupy a comparatively smaller range of chemical space and have overlapping chemical signatures (*i.e.*, are relatively similar to each other, Fig. 7.5a). Indeed, the R statistic of ANOSIM was larger than 0.99 for all COLw-brood parasites comparisons, whereas it ranged between 0.66 and 0.93 in HYMw-brood parasite comparisons. A different pattern is observed when the CHC profiles of male brood parasites are plotted with those of their female hosts. Males of cuckoo wasps that parasitize COLw species are chemically similar among each other and separated from their COLw hosts (Fig. 7.5b, ANOSIM among male brood parasites of COLw hosts, R: 0.4873, p = 0.001; ANOSIM between male brood parasites of COLw and their female COLw hosts, R: 1, p=0.001), whereas female brood parasites of COLw are very distinct from each other (ANOSIM R: 0.9686, p: 0.001). With the exception of *H. rutilans*, male cuckoo wasps overlap more in their chemical profiles than females (ANOSIM between female brood parasites of COLw and HYMw, R: 0.4524, p = 0.001; ANOSIM between male brood parasites of COLw and HYMw, R: 0.7184, p = 0.001). The overlap between female COLw and their female brood parasites was larger than that between female COLw and male parasites (ANOSIM between female COLw and their female brood parasites, R: 0.5873, p: 0.001; ANOSIM between female COLw hosts and their male brood parasites, R: 0.7316, p: 0.001). However, in HYMw the oppposite pattern was observed: the overlap between female brood parasites of HYMw and their female hosts was smaller than between male brood parasites of HYMw and female hosts (ANOSIM between female HYMw and their female brood parasites, R: 0.4297, p: 0.001; ANOSIM between female HYMw hosts and their male brood parasites, R: 0.2957, p: 0.001).

7.4.5. Host-parasite chemical distances

Female host/female brood parasite distances are smaller than female host/male brood parasite distances (Bray-Curtis dissimilarity index calculated with all compounds present) in all comparisons except when considering two HYMw species that are parasitized by *Hedychrum rutilans* (Figure 7.6). We did not find differences between distances of female HYMw hosts to their female brood parasites, and distances of female COLw hosts to their female parasites (t(820): 1.36, p < 0.173). Distances of COLw female hosts to their female brood parasites were smaller than the distances to their male brood parasites (0.58 vs. 0.74, t (659): -19.02, p < 0.001). However, distances of HYMw female hosts to their female brood parasites were similar to that to their male brood parasites (0.57 vs. 0.58; t(1162): -0.97, p = 0.33). Relatively small chemical distances were found between females of *Cerceris arenaria* and their brood parasite *Hedychrum nobile* and between males of *Hedychrum rutilans* and the two *Philanthus* species (figure 7.6).

7.4.6. Intra and interspecific variability of HYMw and COLw hosts

When looking at the NMDS, we observed a pattern indicating differences in inter- and intraspecific variability of CHC profile. Therefore, we calculated Bray-Curtis dissimilarities between and within species. Interspecific differences (among species preying on the same type of host) were significantly larger in female COLw species than in female HYMw species (average 0.57 vs. 0.47 Bray-Curtis distance; t(897):

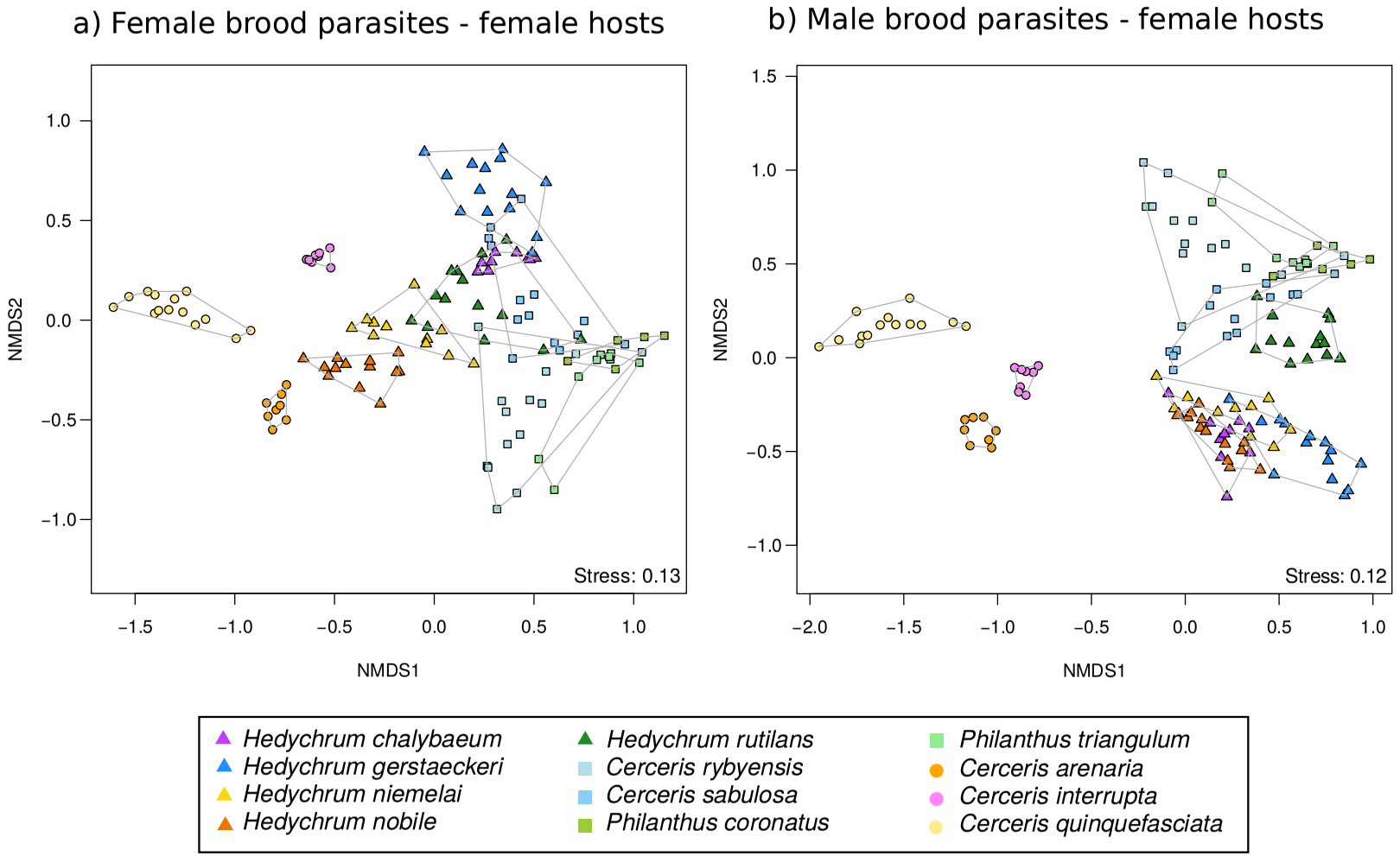

Figure 7.5.: Separation of CHC profiles of the studied species by Nonmetric Multidimensional Scaling. Coleoptera hunters are indicated by circles, Hymenoptera hunters by squares and brood parasites by triangles. Similar hue colors are used to indicate host-brood parasites species pairs: orange (*Hedychrum nobile-Cerceris arenaria*), purple (*H. chalybaeum-C. interrupta*), yellow (*H. niemelai - C. quinquefasciata*), green (*H. rutilans - Philanthus triangulum* plus *P. coronatus*), blue (*H. gerstaeckeri – C. rybyensis* and *C. sabulosa*). a) Female brood parasites and their female hosts and b) male brood parasites and their female hosts.

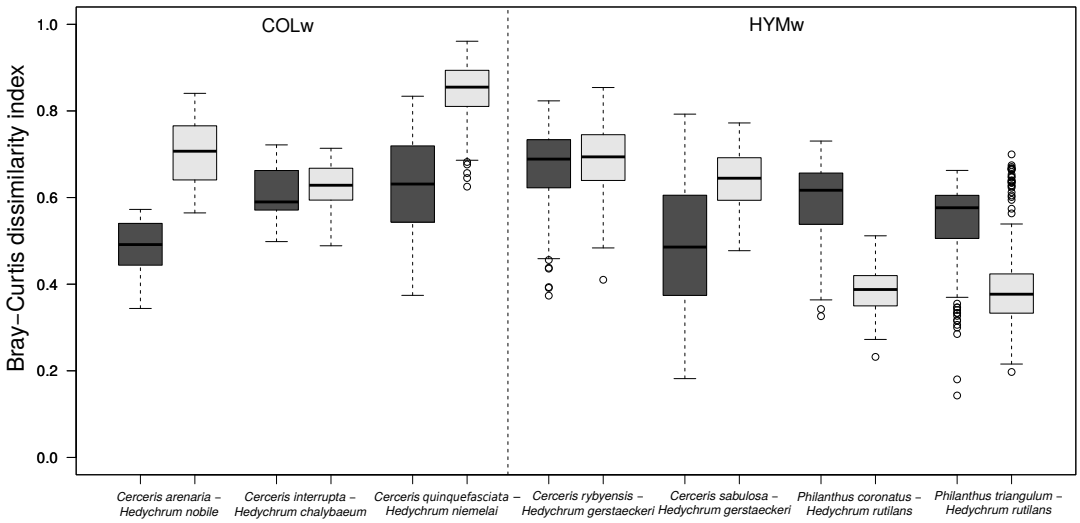

Figure 7.6.: Host–brood parasite chemical distances. Dark gray boxes indicate female host–female brood parasite distances, light gray boxes female host–male brood parasite distances.

11.39, $p < 0.001$). In contrast, intraspecific variability was smaller in female COLw hosts than in female HYMw hosts (on average, Bray Curtis distances between samples within COLw species were 0.17 ± 0.08 whereas within HYMw species was 0.32 ± 0.19 ; t(334): -10.83, $p < 0.001$). Hence, the difference between intra- and interspecific variability was larger in COLw female hosts than in HYMw female hosts, indicating that the latter showed a larger intraspecific profile variance and thus a much larger dissimilarity between individuals belonging to the same species. This trend was not observed in their male counterparts (Figure 7.7). In general, all COLw and HYMw males showed a smaller intraspecific variability and both types were just marginally different (COLw distances were 0.17 ± 0.07 vs. HYMw distances 0.15 ± 0.09; t(302): 2.01, $p = 0.046$). To test whether these results were not an artifact of the difference in sample size among the species used, we repeated the analyses using only five samples per species (the minimum number of available specimens within a group) and obtained basically the same results (Appendix). In addition, we randomly picked two CHC profiles of HYMw females and of COLw females separately, with each pair of samples coming from the same population. We found that intraspecific differences in HYMw females are significantly larger than in COLw females (t(1324): 22.2, $p < 0.001$). However, when repeating the analysis using the CHC profiles of HYMw and COLw males, no such differences were observed (Appendix).

7.5. Discussion

In this study, we confirmed the existence of clear differences between HYMw and COLw as found by Wurdack and colleagues (Wurdack *et al.*, 2017). HYMw species exhibit profiles more similar among each other consisting primarily of unsaturated compounds, whereas COLw species possess more diverse CHC profiles with a larger proportion of mono- and dimethyl-branched alkanes. If by chemically mimicking the CHC profiles of their hosts, cuckoo wasps gain an advantage (*e.g.*, *H. rutilans* by

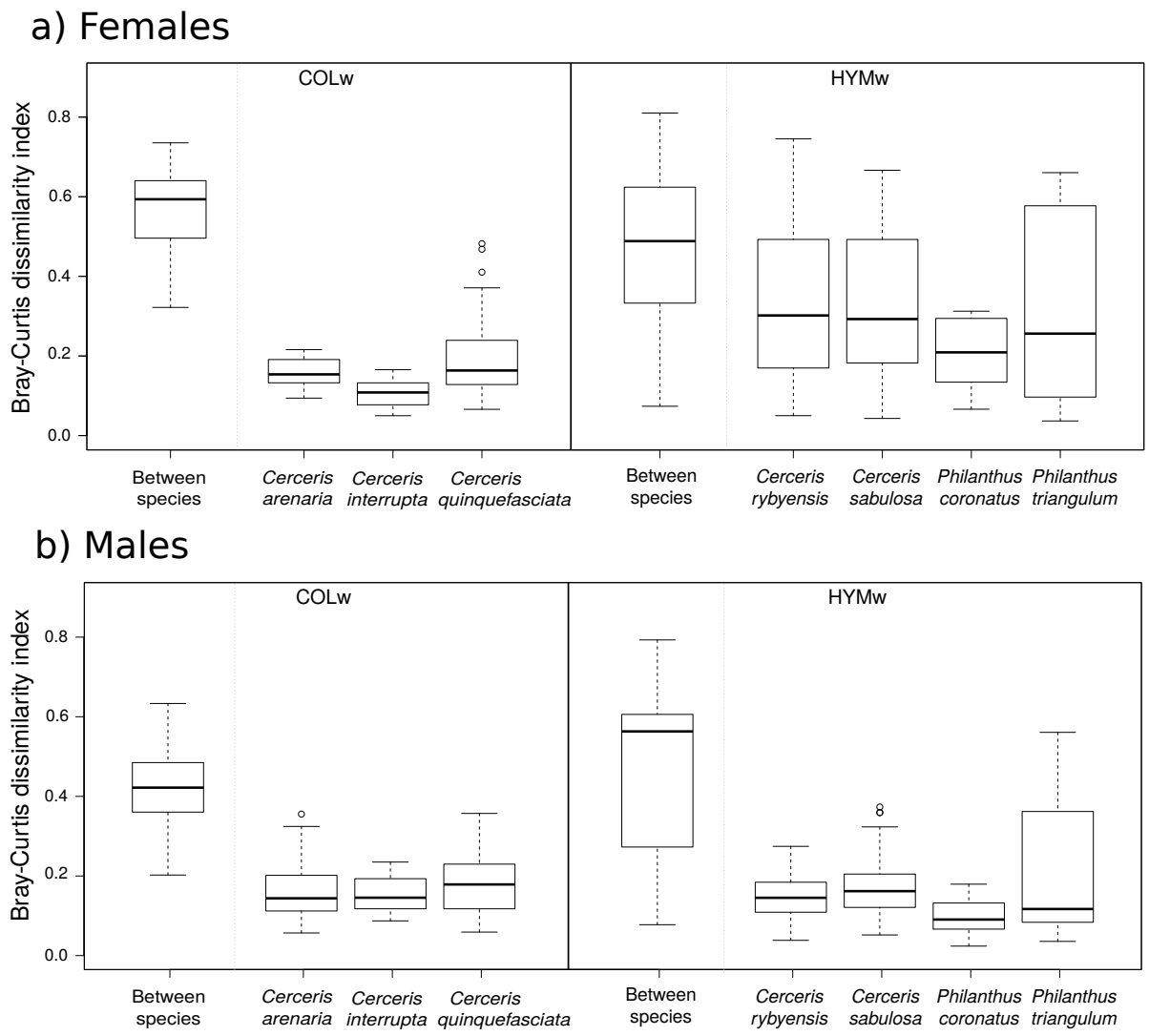

Figure 7.7.: Intra- and interspecific variability of cuticular hydrocarbon profiles in a) female and b) male individuals of all host species. Bray-Curtis dissimilarities were calculated between all individuals of species hunting the same type of prey ("between species") and between individuals of the same species (species name indicated).

mimicking *P. triangulum*, Strohm et. al., 2008), the diversification of the hosts' profile may allow the hosts to escape that chemical mimicry and may be part of an ongoing evolutionary arms race. This change of a CHC profile dominated by unsaturated compounds towards a new one dominated by methyl-branched compounds could have evolved as the result of neutral evolution (*e.g.* genetic drift) in which methyl-branched compounds would have appeared in the common ancestor of COLw species and were maintained in the population because they were not detrimental (*e.g.*, Kimura, 1991). Alternatively, it may have evolved independently as a result of selection acting on the female hosts to escape parasitism. Whether the first scenario is possible, we advocate here that selection on the hosts has been the dominant force.

We follow here the conclusions of Wurdack and colleagues (Wurdack *et al.*, 2017) and assume that preying on Hymenoptera may have been ancestral to preying on Coleoptera, despite the phylogenetic tree does not conclusively suggest which prey was ancestral in *Cerceris* species (see Wurdack *et al.*, 2017).

7.5.1. CHC diversification in COLw reduces the chemical overlap between COLw and their brood parasites potentially facilitating parasite escape

Our first hypothesis was that brood parasite species attacking COLw species should have difficulties mimicking their host whereas brood parasites species attacking HYMw hosts should be able to almost perfectly mimic the CHC profile of their HYMw hosts and thus show a strong overlap in CHC profile. We expected this because HYMw are constrained to produce a relatively fixed and alkene-enriched CHC profile to cope with prey embalming. Chemical dissimilarity between HYMw and COLw and their respective brood parasites can either be attained by changes (or the lack of it) in the host or in the parasite.

Our results provided partial support to our first hypothesis because although there was a larger overlap between HYMw and their female brood parasites than COLw and their parasites, we found relatively large chemical distances between HYMw and their parasites, and we did not find larger distances between COLw-parasites than in HYMw-parasites as we expected. In the following, we discuss our findings and we propose two different strategies that hosts do to escape their brood parasites.

Although the overlap between the CHC profiles of some individuals of HYMw female hosts and their brood parasites was larger than the overlap between female COLw and their brood parasites, Bray-Curtis dissimilarities between HYMw and their brood parasites were not much smaller than those between COLw female hosts and their brood parasites. In all our HYMw-parasite comparisons, the female brood parasites produce similar amounts of the same compound classes as their hosts. However, there are major differences in the double bond positions of alkenes, which contribute to larger chemical distances between the CHC profiles of female HYMw and their female brood parasites than we expected. In addition, CHC profiles of female brood parasites of HYMw (*i.e., H. gerstaeckeri* and *H. rutilans*) include low amounts of other compounds (*e.g.*, methyl-branched compounds) that the CHC profile of their hosts lack and which also contribute to the relatively large chemical distances we observed between HYMw and their brood parasites. Whether the diverse CHC compounds that occur in low abundance on the cuticle of the brood parasites of HYMw can be

recognized by their hosts is unclear, especially when considering that these cues may dilute by and be confused with scents of the environment (*e.g.*, nest ground, nest material, provided prey, etc.). On the contrary, alkenes in HYMw species may be used as cues by their parasites to detect their nests as it occurs in *Hedychrum rutilans* while searching the nest of its host *Philanthus triangulum* (Kroiss *et al.*, 2008), so that a reduction of alkenes could limit host detection by the parasites.

On the other hand, whereas overlap of CHC profile between COLw and their associated brood parasites is minimal, the parasites produce a similar chemical composition as their hosts (*e.g.*, *H. nobile* and *H. niemelai*). Female brood parasites of COLw hosts (with the exception of *H. chalybaeum*) produce less (amount of) unsaturated compounds (but not as little as their respective hosts) and also the same type of methyl-branched alkanes (same branching positions regardless of chain length, so-called homolog series), and often the same compounds as their hosts. In fact, some studies suggest that CHC profiles with more methyl-branched alkanes prevail in highly parasitized populations of other Hymenoptera. For example, parasitized ant colonies of *Formica fusca* show a higher diversity of dimethyl-branched alkanes than non-parasitized ones and this increase in compound diversity correlates with increased recognition abilities in the host populations (Martin *et al.*, 2011). Lorenzi and colleagues (2014) compared the CHC profiles of three populations of the paper wasp *Polistes biglumis* that differ in the degree of parasitism by the social parasite *Polistes atrimandibularis*. They showed that the proportion of methyl-branched hydrocarbons in CHC profiles was larger in highly parasitized populations.

Females of one cuckoo wasp species parasitizing COLw, *H. chalybaeum* still produce an alkene-enriched CHC profile, similar to what is observed in species attacking HYMw. Its host, *Cerceris interrupta*, was the species showing the least proportion of methyl-branched hydrocarbons among all COLw. This maybe indicates that this species has started the latest to evolve a CHC profile specializing on methyl-branched compounds, and/or that it has taken it longer time to evolve them. As a result, its parasite may also lag in its response to evolve these substances. One possible reason explaining the hypothesized less rapid evolution of the diversification of the CHC profile in *C. interrupta*, might be that this species together with its parasite are species with restricted distributions and small populations, being both listed as threatened (Red List of Hymenoptera, Schmid-Egger, 2010), in comparison to other more widespread and common *Hedychrum* and their respective hosts that we have studied. Theory predicts that the rate of evolution is positively correlated with the effective population size (Ne) in cases where natural selection fixing advantageous mutations and removing deleterious ones, is dominating evolution (Lanfear *et al.*, 2014). In contrast, neutral and effectively neutral mutations which have fitness effects of 0 are driven by genetic drift and have stronger effects in smaller Ne (Lanfear *et al.*, 2014). Simulations on empirical adaptive landscapes also show that larger population sizes are adaptively more advantageous than smaller ones (Vahdati & Wagner, 2017).

In addition to the diversification of the CHC profile in COLw, we found that the mean chain length of COLw species was significantly larger than that of HYMw species. Brood parasites of both host types have chain lengths similar to those of HYMw species, that is COLw and their brood parasites show a difference in mean chain length while HYMw and their parasites do not. Since both types of hosts inhabit similar habitats, an increase in mean chain length may not be associated with adaptations to warmer/drier conditions (*e.g.*, Gibbs & Pomonis, 1995, Gibbs *et al.*,

1997, Roualt *et al.*, 2004). It appears likely though that HYMw species require alkenes of a specific chain length, because the chain length is linked to the ability to maintain a semifluid texture (Herzner & Strohm, 2008) for the secretions that are spread on their prey for preservation. In this sense, it is possible that increasing chain length may represent an additional escape strategy from chemical mimicry for COLw as this increases differences between host and parasite CHC profiles. A shift of the mean chain length could then be beneficial if it allows the hosts to better recognize and detect the differences between the CHC profiles of a potential brood parasite in their nests and those of its own species. Moreover, an increase of the mean chain length of CHC would likely not require the evolution of new enzymatic pathways (Blomquist, 2010a).

As we mentioned, HYMw were not as easily mimicked by their brood parasites as we had expected. Whereas, some individuals of HYMw were chemically very similar to their female parasites, many were not and within intra-specific variability was large. In fact, we suggest that HYMw hosts may have evolved another strategy to cope with chemical mimicry by their brood parasites. Chemically, they are restrained to maintain the same proportion of alkenes in their CHCs because of the advantages they confer to prey preservation. We found large differences in intraspecific variation between females of COLw and HYMw species analyzed here, with females of HYMw having a greater within species variability. In the case of HYMw, negative frequency-dependent selection may be favoring the existence of rare host chemotypes. Polymorphic or highly variable CHC profiles may thus result in some individuals in a population exhibiting chemotypes that are likely not being perfectly mimicked by a brood parasite. A similar phenomenon (*i.e.*, increasing among clutch variation) has been observed in hosts of avian cuckoos (Spottiswoode & Martin, 2011). Females of some cuckoo host species have evolved the ability to lay eggs with different color hues (*i.e.*, Spottiswoode & Martin, 2011). It has been suggested that cuckoo hosts that are in an evolutionary arms race with their brood parasites should increase inter-clutch variation (*i.e.*, differences among eggs within a population, eggs laid by different females) but at the same time should keep intra-clutch color variation low (Oien *et al.*, 1995) in order to make mimicry more difficult for the brood parasites. Similar adaptations for increasing within species variation in chemical signals have also been observed in insects. The within population variation in the proportion of hydrocarbons is higher in the highly parasitized populations of *Polistes biglumis*, probably because of negative frequency-dependent selection of rare phenotypes (Lorenzi *et al.*, 2014). Similarly, when the CHCs of *Temnothorax longispinosus* ants were compared within and between colonies in populations with and without the slavemaking ant *Protomognathus americanus*, the CHC profiles of the host ant species were more variable in parasitized populations (Jongepier & Foitzik, 2016). We have shown here that two randomly chosen CHCs of a HYMw female host coming from the same population are in general more dissimilar than two randomly chosen CHCs of a COLw host (Appendix). CHC variation in HYMw females is largely quantitative. For example, in two out of nine female individuals of *Philanthus triangulum* in our analysis the main compound being produced was (Z)-9-C27:1 whereas in the remaining individuals (Z)-9-C25:1 was more abundant (Figure 7.5a). This polymorphic variation in the production of one or the other alkene in females of *Philanthus triangulum* within the same population has already been observed (Strohm *et al.*, 2008, Kroiss *et al.*, 2008) and it would be interesting to test the hypothesis that this chemical polymorphism is

an adaptation to partly escape chemical mimicry by *H. rutilans*.

7.5.2. CHC profile diversification is stronger in female COLw hosts than in their conspecific males

We hypothesized that if COLw species are able to evolve CHC profiles that differ from that of their brood parasites, we should see this pattern more pronounced in female than in male individuals . In general, the CHC profiles of the analyzed hosts do not show striking sex-specific qualitative differences, irrespective of whether or not we analyzed COLw or HYMw. However, we found quantitative differences between female and male profiles. Specifically, females of COLw are characterized by synthesizing larger proportions of methyl-branched alkanes than their conspecific males. In *C. arenaria,* females furthermore synthesize a larger number of methyl-branched compounds than their males. However, methyl-branched compounds that occurred exclusively in males contributing to the larger diversity of CHC in male COLw (*C. interrupta* and *C. quinquefasciata*, Figures 7.3a, 7.3b) compared to their conspecific females made up less than 3% of the total CHC profile. These findings support our hypothesis that hosts changed their profile as a response to parasitism.

7.5.3. CHC profiles of female cuckoo wasps, but not those of male cuckoo wasps, are similar to those of their hosts (only in COLw)

Only cuckoo wasp females track the nest of their hosts. Thus, if brood parasites differ in their CHC composition between the two sexes, chemical mimicry should be more pronounced in the female than in the male sex, since only the brood parasites' females interact with the host. As predicted, we found that cuckoo wasp females parasitizing COLw species (with the notable exception of *H. chalybaeum*) largely synthesize the same type of compounds as their female COLw hosts (especially the same homolog series of methyl-branched alkanes). Male cuckoo wasps, irrespective of what type of host they develop from (*i.e.*, COLw, HYMw), show a CHC profile that is compositionally more similar to the CHC profiles of HYMw (probably ancestral to COLw, see Wurdack *et al.*, 2017). Males of brood parasites of COLw hosts are chemically very similar to each other (Figure 7.5, see Results). Females of the different species of brood parasites of COLw however are chemically very distinct from each other, with two of these analyzed species having evolved CHC compounds that are similar as those of their hosts (mostly monomethyl-branched compounds), but whose conspecific males do not produce those substances yet. Nevertheless, whereas this held in brood parasites of COLw hosts, the opposite pattern was true in HYMw, because males of *Hedychrum rutilans* are more similar to their female hosts than their conspecific females (see Figure 7.6). This is a rather unexpected result and has not been observed before. On the other hand, this could also result because there is a larger intraspecific variability of *Philanthus triangulum* females (see above), which increases the overall chemical distances between female hosts and female cuckoo wasps.

7.6. Conclusions

Brood parasitism can exert strong natural selection on the host to improve its ability to detect brood parasites. The brood parasites, in return, are counter-selected for traits that enable them to remain undetected by their host. The switch from Hymenoptera to Coleoptera prey that took place in Philanthinae wasps freed the latter group (those preying on Coleoptera) from producing CHC profiles enriched with unsaturated hydrocarbons, a requirement for embalming Hymenoptera prey. We demonstrated that this relaxation allowed COLw species to evolve distinct species-specific CHC profiles dominated by a diversity of methyl-branched alkanes as a strategy to escape chemical mimicry by their *Hedychrum* brood parasites. Several lines of evidence support this hypothesis: 1) COLw CHC profiles are conspicuously distinct from the unsaturated (olefin)-enriched CHC profiles necessary for prey preservation and very distinct between each species with species-specific types of methyl-branched alkanes; 2) CHC profile overlap between COLw and their brood parasites is smaller than between HYMw and their brood parasites; 3) there is a larger proportion of methyl branched alkanes in female COLw CHC profiles compared to conspecific males; 4) female cuckoo wasps show a CHC profile that resembles that of their COLw host species (except for *H. chalybaeum*), whereas the CHC profile of their male conspecifics resembles (the probably ancestral) CHC profile of HYMw. In addition, we found COLw species showed CHC profiles with longer chain lengths, which may constitute an additional strategy to escape chemical mimicry by their brood parasites. Altogether, these conclusions support our hypothesis that a diversification of CHC profiles with methyl-branched alkanes has started to evolve in COLw species and their cuckoo wasps are "following" their hosts in chemical space. Furthermore, our data also suggests that HYMw could possibly counteract brood parasitism by cuckoo wasp attack by exhibiting a larger intraspecific CHC profile variability.

8. A proposed pipeline for the analysis of cuticular hydrocarbons

8.1. Abstract

Cuticular hydrocarbons (CHC) play central roles in insects as waterproofing barriers and as semiochemicals in a number of intra- and inter-specific processes. The analysis of CHC has become a routinaire procedure in the field of chemical ecology. CHC are extracted from the insect's cuticle, by submersing the animal in a nonpolar solvent. CHC compounds are then analyzed using gas chromatography - mass spectrometry (GC/MS), which can generate a large amount of data. The data obtained (chromatograms) need to be available in a form that allows its further analysis using statistical methods. The statistical analysis of CHC data, regarded as compositional, presents a number of challenges which have been previously addressed, and for which some solutions have been proposed. However, the data-mining and preprocessing of chromatogram's information has been less often discussed, despite being often not devoid of associated problems. In fact, one of the most time-consuming and error-prone steps of the processing of GC/MS raw data is the alignment of the corresponding peaks from several chromatograms. Here, I propose a workflow to process GC/MS data files using the freely available program AMDIS, which has the advantages of deconvoluting mass spectra and identifying compounds that are provided in a mass spectral library. The preliminary identification of compounds allows the alignment of chromatograms. The processing and extraction of the chromatogram's information by AMDIS can be faster and as accurate as that offered by other methods (manual integration using commercial programs). Nevertheless, the data need to be curated before statistical analyses can be done. Here, I explain all steps conducted to optimize results provided by AMDIS and correct for potential errors derived from the analysis using AMDIS. The procedure described here was employed to analyze CHC profiles of the 59 species of cuckoo wasps and 7 of their hosts, which have been subject of study in this thesis.

8.2. Analysis of cuticular hydrocarbons

Hydrocarbons present on the cuticle of insects (CHC) serve two major roles in an insect's life: they act as a waterproofing layer hindering water loss from the body, and they are used extensively in communication (Blomquist & Bagneres, 2010). It is especially the pursuit to understand this last role of CHC in chemical communication which has generated an increasing number of studies in the last decades (Blomquist & Bagneres, 2010). Thus, there are currently many investigations using CHC to answer different interesting questions in the fields of chemical ecology and evolutionary biology. In this respect, the analysis of CHC has become a standard procedure, which

starts with the extraction of CHC from the insect's body (either by solvent extraction or by solid phase micro-extraction, SPME, see Figure 8.1). Capillary gas chromatography - mass spectrometry (GC/MS) using nonpolar columns is often employed for analyzing CHC (Blomquist, 2010). After the CHC extract has been analyzed with GC/MS, the resulting chromatogram provides information on: the number of peaks, the retention time at which each peak has eluted in the column (e.g., heavier molecules appear at a later retention time), their relative abundance (the area of each peak) and (when MS is present) mass spectra which allow the identification of the compounds in the chromatogram. Note that a peak may be composed by more than one coeluting nonpolar CHC compounds; therefore, the number of compounds in the chromatogram may be larger than the number of peaks conforming it. Additionally, although nonpolar hydrocarbons are the dominant surface lipids of the cuticle (Hadley, 1994), other polar molecules (e.g., wax esters, fatty acids, ketones, oxygenated derivatives of hydrocarbons and even contaminants) may also elute and be part of the chromatogram (Blomquist, 2010; Buckner, 2010).

Chromatograms can be considered raw data because their information needs to be extracted and available in some sort of worksheet format (especially if several samples are to be compared). Thus, the steps followed after the GC/MS analysis form part of the data processing and can be summarized as follows: 1) the export of the peaks' information of each chromatogram into a spreadsheet program. This information consists minimally of the retention time and the area of the peak. 2) the alignment of the previously mentioned peaks' information from the different chromatograms, and 3) the identification of peaks (or compounds) using mass spectra and a calculated retention index, RI. Many factors affect retention time making this measurement very variable and not useful for comparisons among different samples (D'Acampora Zellner *et al.*, 2008, Mallard, 2014). Instead, retention indices are frequently calculated for each peak by interpolating retention times with well established methods (e.g., Kovats RI, Kovats, 1958 for isothermal GC conditions; or the method by van den Dool and Kratz when variable temperature programs are used, van den Dool & Kratz, 1963). After the conversions, the same CHC compounds appear to elute at very similar RI across different samples, thus RI is often an additional helpful information for the identification of CHC compounds (Carlson *et al.*, 1998b, Mallard, 2014). Note also that step 3, the identification of compounds, is not necessary for analyzing data. In fact, it may be done *a posteriori* or not be achieved at all, if not required by the aims of the study (e.g., Sharma *et al.*, 2012, Ingleby *et al.*, 2013). However, it is just after having aligned the information contained in all different chromatograms (*i.e.*, sampling units) of a study, when data exploration and further statistical analyses can be conducted. While there are a number of problems associated with the posterior statistical processing of CHC/chemical data, especially because they involve the analysis of "blends" of compounds (e.g., compositional data, Aitchison, 1986) that restrict the application of standard parametric statistics (Aitchison, 1986; Brückner & Heethoff, 2016), these problems have been addressed few times (e.g., Martin *et al.*, 2009, Ranganathan & Borges, 2011, Brückner & Heethoff, 2016) and some methodological solutions for dealing with such complex data sets have been provided (e.g., Brückner & Heethoff, 2016). However, problems faced during the first part of the data processing have been much less discussed or acknowledged in the literature. In fact, most details regarding the processing of data, that are described in the methods section of publications, mainly deal with the identification of the compounds. This is in part

understandable considering that the identification of CHC compounds, using diagnostic ions and retention indices (Carlson *et al.*, 1998b) is not always straightforward and can be a time-demanding task (e.g., for polymethyl-branched compounds, Chapter 9), sometimes even requiring the execution of additional methods (e.g., derivatization of unsaturated compounds, Dunkelblum *et al.*, 1985). Nonetheless, how the alignment of the information of peaks from each chromatogram is achieved, is rarely, if at all, described in the methods section of most publications (see Ottensmann *et al.*, 2017). Due to variability introduced during the CHC extraction or GC/MS analysis of the sample (the amount of solvent used, the time employed for solvent extraction) or to natural variation present in insects (e.g., size of the specimen), not all chromatograms belonging to a group (e.g., members of the same species) will have the exact same number of peaks. Therefore, a rapid alignment using retention times information is not possible (Bartelt *et al.*, 1986). For this reason, samples to be included in an analysis are often run in the GC/MS sequentially, or an internal/external standard can also be used to adjust retention times (e.g., Peterson *et al.*, 2007, Greene & Drea, 2014). Frequently, the alignment of peaks is conducted manually (or visually aided) by comparing the mass spectra among the different samples and manually moving the peaks of each sample in an spreadsheet program, often aided by some standardization/correction of the retention time information. The procedure is probably so widespread and a tacit understanding among chemical ecologists that it is rarely or just briefly mentioned in the publications. However, depending on the number of chromatograms that need to be manually aligned, it is a time-consuming process and it can be prone to error (Cerdán-Calero *et al.*, 2012, Ottensmann *et al.*, 2017). One worth noting exception in mentioning the cumbersome process of chromatograms alignment prior to the analyses is that by Bartelt and colleagues, who explained in detail the procedure they used to match peaks of chromatograms (e.g., calculating the equivalent chain length from retention times, see Bartelt *et al.*, 1986). Moreover, these authors wrote a computer program to do this "matching of peaks", probably the first computational alignment tool in the analyses of CHC (Bartelt *et al.*, 1986). Sadly, their idea had to wait over 20 years to be implemented, and most researchers of the field do, if not mentioned otherwise, a manual alignment of GC/MS peaks until now (Ottensmann *et al.*, 2017).

The alignment tools (e.g., SpectConnect, Stycinski *et al.*, 2007; Metab, Aggio *et al.*, 2011) available at the start of my analyses of GC/MS data were not considered suitable because they were mainly designed for metabolomics data sets. For this reason, I developed a semi-automatic workflow which is the subject of this chapter. The procedure followed for the analysis of CHC in this thesis (described in the methods section below), takes advantage of some of the properties of a freely available program (AMDIS, Automated Mass Spectral Deconvolution and Identification System, http://chemdata.nist.gov/mass-spc/amdis/, Stein, 1999) and allows the use of the output files from AMDIS for posterior curation using the statistical language R (R Core Team, 2013). Thus, by using this procedure an automatic alignment of all identified CHC compounds from the chromatograms is possible. AMDIS permits the deconvolution of compounds (the acquisition of "putative pure spectra from overlapping peaks", Styczinski *et al.*, 2007) and automatically identifies all target compounds by comparing the deconvoluted peaks' mass spectra to those available in a library provided by the user (which may also contain retention data, Stein, 1999, Mallard, 2014). AMDIS was developed to detect and identify low levels of chemical agents

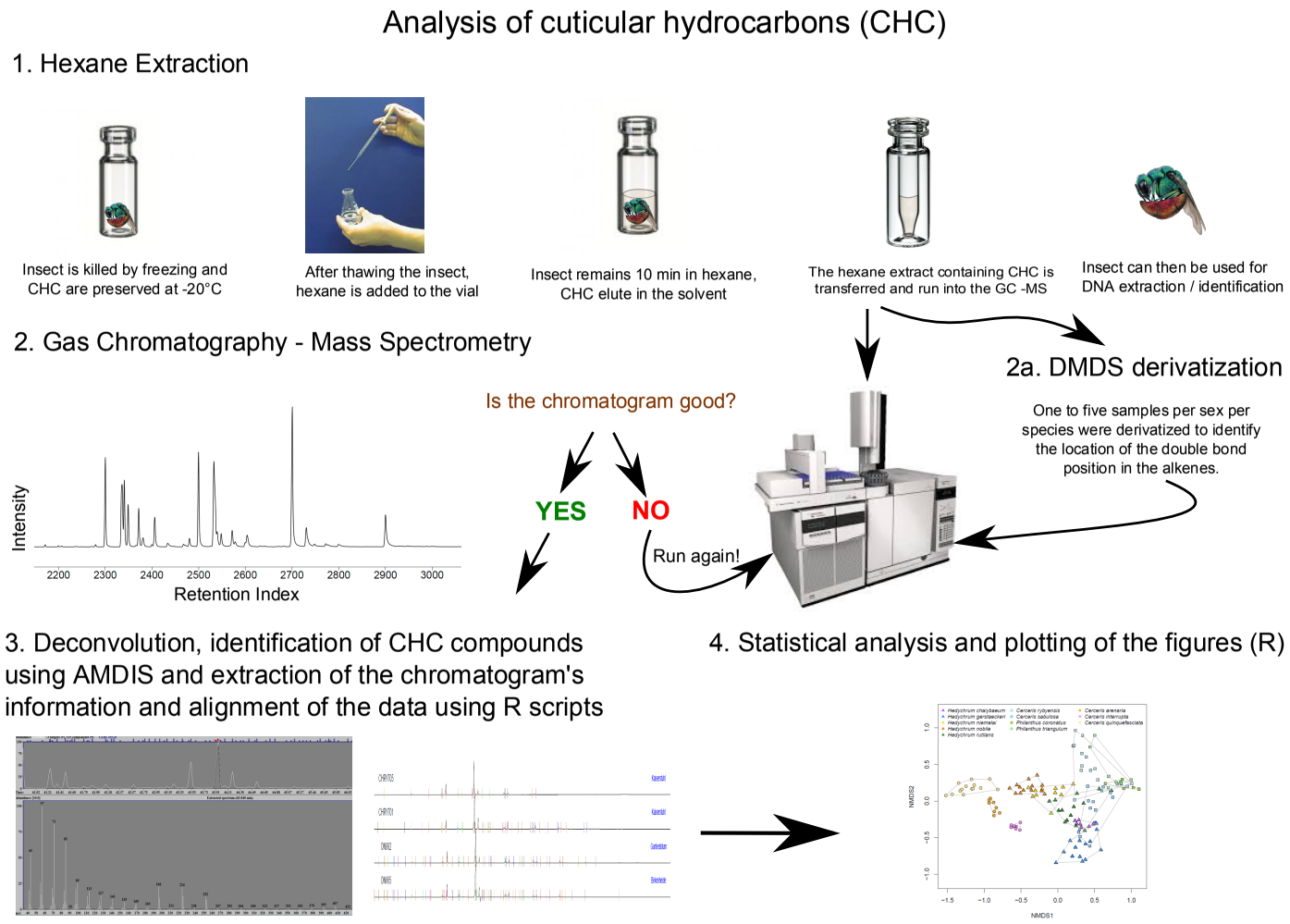

Figure 8.1.: Procedure for the analysis of cuticular hydrocarbons. CHC are extracted with a nonpolar solvent. The CHC extraction is analyzed with a GC-MS, and the resulting chromatogram contains all information about the CHC composition in a given insect. This information is further analyzed and extracted here using AMDIS. A series of R scripts curate AMDIS results and allow the alignment of CHC composition from different sampling units. Just then can the statistical analysis and data exploration start (see text).

that could be used as chemical weapons, as part of the inspection regime established under the Chemical Weapons Convention (Mallard, 2014). Some of the strengths and weaknesses of the program, as well as an explanation of its functioning have been discussed somewhere else (Stein, 1999; Meyer *et al.*, 2010; Mallard, 2014). Despite the specific objective for which it was created, AMDIS is a general-purpose tool with broad applications beyond its role in the detection of chemical weapons (Mallard, 2014). In metabolomics, the need for a rapid processing of the growing amount of raw data has enhanced the development of several software tools written in different programming languages (Katajamaa & Oresic, 2007). The many advantages offered by a free program as AMDIS (such as its relative flexibility with many adjustable parameters, its deconvolution properties, its speed compared to other methods, the possibility to import chromatograms in different formats, etc.) promoted the creation of tools that curate the data generated by it (e.g., GAVIN, Behrends *et al.*, 2011) even as an R package (the R packages flagme, Robinson., 2010, Metab, Aggio *et al.*, 2011 and a suggested pipeline in Smart *et al.*, 2010). While the combination of AMDIS with another tool (SpectConnect, Stycinski *et al.*, 2007) has enabled a straightforward characterization of hydrocarbons present in algae that could be used as biofuels (Barupal *et al.*, 2010) the use of AMDIS in the field of chemical ecology for the identification and analysis of cuticular hydrocarbons has been occasional and comparatively limited. Notable exceptions are the analyses of cuticular hydrocarbons of ants by Witte and colleagues (Morrison & Witte, 2011; von Beeren *et al.*, 2011; von Beeren *et al.*, 2012). These authors used AMDIS to identify and quantify CHC compounds in few species sharing a large number of compounds in their chromatograms. However, the application of AMDIS for analyses of multiple GC/MS data files from a large number of species has not been tried before. Here, I intend to demonstrate that the integration of AMDIS as a routine analysis procedure may be possible, though not exempt from errors. I provide a protocol for the analysis and explain how and when various adjustments and corrections were conducted on the resulting output files of AMDIS. The aim of this chapter is to offer a pipeline for the rapid analysis of several and diverse chromatogram's data that may be adopted by others and eventually greatly improved.

8.3. Description of the proposed pipeline

The identification and quantification of CHC compounds of all GC/MS chromatograms of insects utilized in the chapters of this thesis have been done using AMDIS. I created batch jobs to run and process several chromatograms together. The whole procedure required adjustments and the curation of the obtained data further in R version 3.02 (R Core Team, 2013) to correct for some errors in the analysis by AMDIS. In the following, I explain the steps of this procedure which are summarized in Figure 8.2.

8.3.1. Creating a mass spectral library

The most important but time-consuming step is creating a good mass spectral library. Hence, the benefits of using AMDIS should exceed the time a researcher may need to invest on producing a good mass spectral library. Creating a library in AMDIS is easier when relatively few target compounds need to be detected. For example,

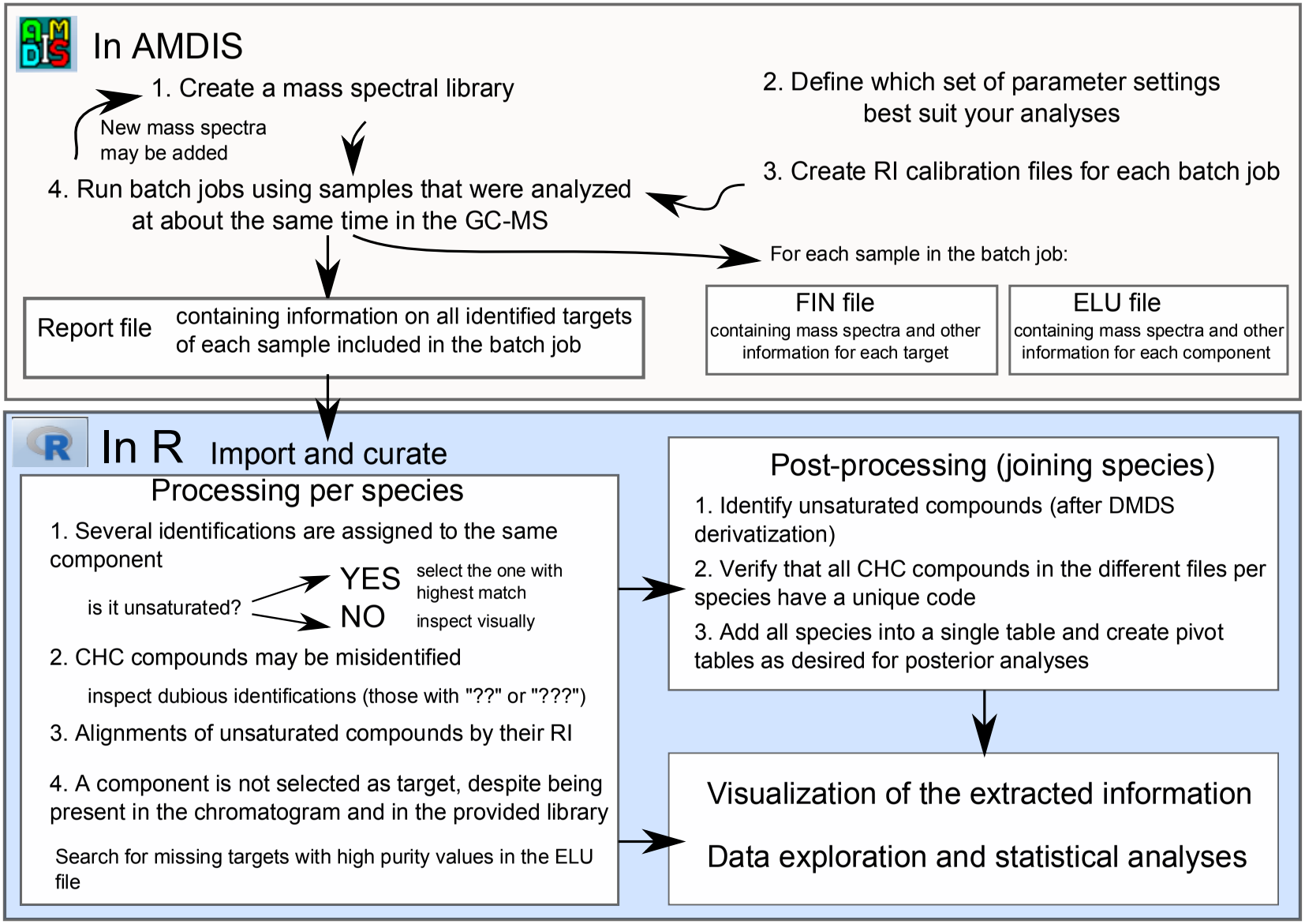

Figure 8.2.: Procedure used to analyze and extract information from chromatograms using AMDIS and R

von Beeren and colleagues created a mass spectra library containing 109 CHC compounds to identify and quantify GC/MS chromatograms of one host ant species and its cleptoparasitic silverfish (von Beeren *et al.*, 2011). The majority of the compounds contributing to the total ion count (99%) occurring in workers and in the cleptoparasitic silverfish did not exceed 32 compounds, which is a relatively small number of compounds to be detected. Moreover, this same library has been used repeatedly to analyze different castes and colonies of the same and closely related species (e.g., Morrison & Witte, 2011; von Beeren *et al.*, 2012).

For other uses, for example the characterization of the CHC profiles of new species (the aim in this thesis), the task of creating a mass spectral library might be overwhelming. Each new species may contain new compounds that need to be added to the library, so that AMDIS will be able to detect these compounds in the analyses. Mass spectra can be added from an existing library or from gas chromatograms of any insect. Except for n-alkanes that were added from the default NIST library in AMDIS, all other compounds were manually added from different chromatograms of Hymenoptera, mainly cuckoo wasps. Mass spectra of small peaks may not be of good quality, and replacing these spectra with better suited ones takes not only time but it may be in some cases impossible. Hence, in few cases, the spectra were manually corrected by adding missing ions. However, the advantage of creating a good mass spectral library may be appreciated in the long term, when the library already contains the most commonly occurring compounds. A closely related species may be characterized faster because the majority of its CHC compounds might already exist in the library. Currently, the library used to characterize the CHC profiles of 59 species of cuckoo wasps and seven of their hosts contains mass spectra of almost 950 hydrocarbon compounds, among which there are 214 monomethyl-branched compounds, 335 dimethyl-branched compounds, all n-alkanes from C18 to C37 and about 272 mass spectra of unsaturated compounds.

To identify target compounds, AMDIS uses up to two sources of information: the mass spectral information (diagnostic ions) and the retention index. Note that in the program, the retention index is an optional parameter since a wide range of compounds may be analyzed (and identified) using only mass spectral information. However, I used in all cases the analysis that included the retention index to increase the reliability of the identification for CHC compounds. All compounds present in the mass spectral library get a unique code (automatically created by AMDIS) so that target compounds do not need to be identified *a priori*. The identification of a compound may be conducted at any further step. However, a compound that is not present in the library may not be selected as a target compound by the program. Nevertheless, it is selected as a component of the chromatogram, and its extraction from the files produced by AMDIS may still be possible (see below).

Mass spectra of n-alkanes and methyl-branched compounds are unique because the presence of diagnostic ions (together with the retention index) allows (theoretically) the unambigous identification of a compound (see Chapter 9). On the other hand, the mass spectra of unsaturated compounds (e.g. alkenes, alkadienes, alkatrienes) obtained from a GC/MS analysis are all very similar, changing only in chain length. Thus, the different isomeric forms of unsaturated compounds can not be readily identified from the original chromatogram unless a derivatization procedure is performed (e.g. Dunkelblum *et al.*, 1985, Carlson *et al.*, 1989) or a different analytical equipment is used (e.g., Kroiss *et al.*, 2011). Moreover, mass spectra of unsaturated compounds

occurring at the same retention index may slightly differ across individuals of the same species. Because of these slight differences between the mass spectra in the chromatogram and that of the corresponding compound in the library, sometimes unsaturated compounds are not selected as target compounds by AMDIS. To solve this, repeated mass spectra of alkenes occurring at almost similar retention indices but showing slight differences can be included in the library with the intention that all (or most) of the peaks of unsaturated compounds will be selected by the program. For this reason, the number of spectra of unsaturated compounds in the mass spectral library used here exceeds the number of real different unsaturated compounds.

In addition to hydrocarbons, I also added common non-hydrocarbon compounds into the library (mostly esters, fatty acids, ketones, etc.). The majority of these compounds were, however, not identified since they have not been used in any of the analyses.

8.3.2. Running the batch files

Once the library has been created, AMDIS can be used to process many samples in a semi-automatic procedure. As mentioned before, AMDIS may, in addition to the mass spectral information, use the retention index to select target compounds. While this is an optional setting in the program (an analysis in AMDIS does not necessarily require retention data), I used this option in every analysis because it provides a higher accuracy in the identification of compounds. Thus, a comparison between a peak in the chromatogram and a target compound existent in the library is based on two sources of information. AMDIS compares this information and provides match values: the higher the match, the higher the certainty that the peak selected in a chromatogram is indeed a target peak (a peak present in the library used). To assure that AMDIS handles retention information appropriately, chromatogram files that were run at more or less the same time in the GC/MS, may be processed together within the same batch file in AMDIS. Hence, all these files are assigned a calibration file (created by the user) in which the n-alkanes and the retention times at which they occur are provided. Thus, AMDIS can calculate retention indices for all files within this batch using the information from retention times in the calibration file. In this thesis, most chromatograms used belonged to samples that were collected in the field, extracted and subsequently run between the years 2005 and 2014. Some chromatograms were also run after this time, but batch files in AMDIS were always created with samples that were run at adjacent dates, despite belonging to very different species.

After AMDIS has processed a chromatogram, it detects peaks. Each detected peak is called a component (an inverted triangle symbol). When a component is found in the library and it gets a match value larger than the minimum match value in the settings, then AMDIS additionally assigns a T symbol, and this component is selected also as a target (see Figure 8.3). After the batch processing, AMDIS creates two output text files for each sample that was run within a batch job: an ELU file and a FIN file. The FIN file contains information on the targets identified by AMDIS (e.g., integrated peak area, retention time, retention index, net score, the model ions, the name of the compound, if it was previously identified) whereas the ELU file contains information on the components (e.g., information about the peak quality and peak area and retention data).

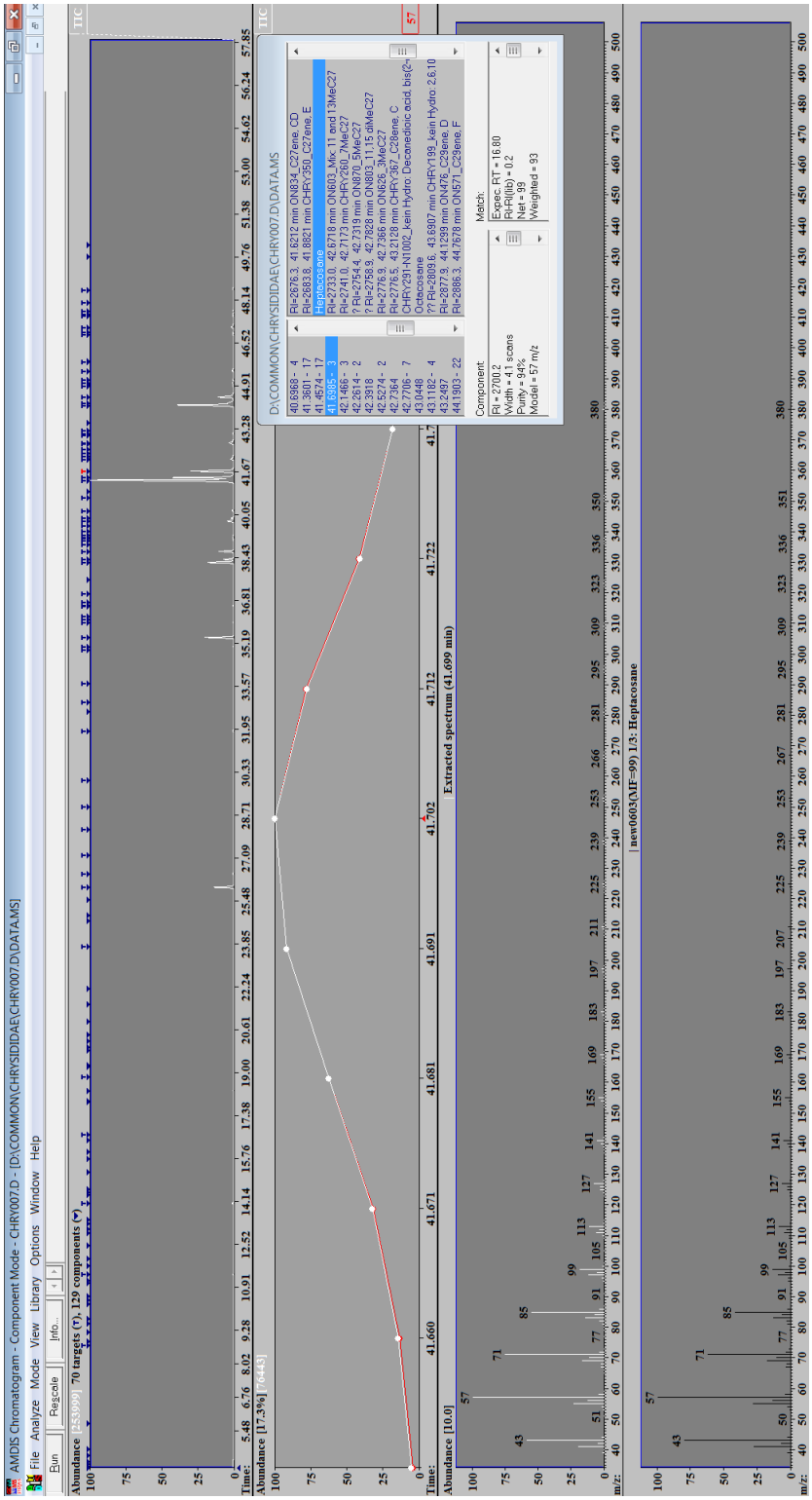

Figure 8.3.: Screenshot of AMDIS, showing the results of an analysis. The upper panel shows the chromatogram display, showing the relative percentage of the total ion count (TIC) of the chromatogram on the y-axis and the retention time on the x-axis. The number of targets (70) and total components extracted (129) is also indicated at the top left. The selected target is marked in red and is heptacosane (see lower panels). The second panel is the Profile display, which shows the major ions of the deconvoluted compound (in this case, heptacosane) over the selected retention time. The third and fourth panels display the mass spectra of the selected compound. When a component is selected only the extracted spectrum (third panel) is displayed. When however, a target is selected an additional mass spectrum (fourth panel) that shows the mass spectrum in the library is shown. The floating window at the right shows different types of information regarding the component/target selected. Note for example, the match value (Net = 99). All this information is exported into the respective ELU and FIN files after the analysis in AMDIS (and can be read into R).

8.3.3. Parameters setting

AMDIS provides the user with several settings options. This is an advantage, because it makes the program very flexible. However, finding the set of parameters that produces the best result requires some expertise. The most important parameters to deal with are: 1) the match factor (the default value is 60). This parameter sets the minimum match factor that will be used by the program to select any component (all peaks detected after a run in AMDIS) as a target compound (a compound present in the library). Details about the calculation of the match factor can be read elsewhere (Stein, 1999; Mallard, 2014). 2) The component width (default value is 12), which equals the number of scans across a well resolved peak at half-height (D'Arcy & MAllard, 2004). By testing a set of different chromatograms of different species, I found that a component width of 22 worked best for most chromatograms utilized in this thesis, the match factor was kept at is default value of 60. Additionally, three parameters determine how many components and targets will be selected. If the "sensitivity" is too high, there will be too many peaks selected, with some very small peaks being selected even if they are noise. If sensitivity is too low, on the contrary, few components will be selected. In order to select new compounds for creating a library, high sensitivity values were used, however when the library was created a sensitivity of medium or low was more appropriate. On the other hand, setting the parameter "Shape requirements" to high reduces the number of false positives, since it requires a stricter match between the mass spectra of any component and that of the mass spectra in the library. However, this may also hinder the selection of peaks that occur at low abundances, whose spectra are not so good. In general, when a good mass spectral library is available, the default value parameters of AMDIS perform well. Different parameter settings were used for creating the library of mass spectra, and for processing the files. The default parameters used in the analyses were: resolution = medium, sensitivity = low and shape requirements = medium. A number of other parameters and setting options are available in AMDIS, which I do not mention here. I refer the reader to the manual of the program for further details (D'Arcy & MAllard, 2004). Additionally, a number of parameters apply penalties for reducing the match factor when the retention index of the compound differs from that one in the library. The most recent version of AMDIS also allows the application of a filtering which can restrict the number of components that the program selects as well (see Mallard, 2014).

8.3.4. Correcting the result files of AMDIS

All the steps described above involved working directly with the program AMDIS. As mentioned before, AMDIS has many advantages. However, some mistakes may appear when doing the processing of the data and these need to be detected and corrected. ELU and FIN files can be read into R for further correction using functions implemented in the package flagme (Robinson, 2010). Modifying functions available in the latter package, I created a number of R scripts to curate and process the output files of AMDIS. There are five main problems that may appear during an analysis with AMDIS and I explain how I dealt with each of these stepwise:

8.3.4.1. A component may be assigned more than one identification

In some cases two or more identifications occurred for a detected component. This happened for unsaturated compounds (whose spectra are very variable, and for which several mass spectra were added to the library, see above) and for mixtures of methyl-branched alkanes (*e.g.*, 11Me and 13Me, or 12Me and 14Me). The R scripts detected these cases (*i.e.*, by selecting those scans of the chromatogram that were repeated). If all identifications of a component were unsaturated compounds, only one of them (the one with the highest match factor) was selected by the scripts and the other targets were discarded. If there was a methyl-branched compound among the suggested identifications, information regarding these targets was exported into a table for visual inspection to select the correct identification among the existing options.

8.3.4.2. The (mis)identification of the peak

Whether a given peak is correctly identified depends on the quality of the mass spectrum and the accuracy of the retention index provided in the library. If a given compound has a high-quality mass spectrum in the library (its signal to noise ratio was very high and the spectrum includes most ion peaks), AMDIS can detect this substance even if it appears in very low amounts (Mallard, 2014; Meyer *et al.*, 2010). However, in a few cases, especially if the mass spectrum of the library does not include enough ion peaks, AMDIS may misidentify a compound. The program I created provides graphic output which allows the visualization of the identified targets by AMDIS. Target compounds that are the same across different samples are assigned identical colors and can be plotted together. Thus, if a given color does not match the others located in the same peak, it is easy to spot disagreements in the identification (Appendix). Moreover, the program is also able to export a table with dubious identifications (those which have "??" or "???" behind the target name) and those whose difference of RI to the RI in the library is larger than a threshold selected by the user (in my case, 10). Each of these misidentified targets may be then visually inspected and corrected if necessary.

8.3.4.3. Several mass spectra of alkenes and alkadienes occurring at similar RI are included in the library

Since there were several mass spectra in the library that could be selected for the same unsaturated compound (see Creating a mass spectral library), different target names could be assigned to the same unsaturated compound. The identification of the different isomeric forms of alkenes was conducted at a later phase. In this sense, alkenes (and alkadienes) could only be separated based on RI. Samples belonging to the same species were often processed in different batches. Nevertheless, the alignment of unsaturated compounds by their RI was often correct. Nonetheless, the script allowed the detection and manual correction of potential errors in the alignment of unsaturated compounds.

8.3.4.4. Sometimes a given component is not selected as a target

For some (unexplained) reasons, occasionally a given component in the analyzed chromatogram is not selected as a target, despite having a corresponding high-quality

mass spectrum in the library. This happens seldom, generally with peaks representing alkenes or alkadienes. I created a code that compares the selected targets and unselected components in the ELU files, and inspects if there is a medium/high quality (purity > 50) component that was not selected by AMDIS and should have been selected. I then investigated all these components in the respective chromatogram and assigned the correct identification.

8.3.4.5. Quantification of peaks

Some authors have expressed their concern about the way how AMDIS integrates peak areas (*e.g.* Smart *et al.*, 2010, Zushi *et al.*, 2013, Smits *et al.*, 2016). Whereas the integration procedure by AMDIS may not be optimal, I expect this not to have influenced the results. Non-metric multidimensional analysis was a common tool for the analysis of CHC compounds in many studies of this thesis. This method uses ranks which were calculated from the relative amounts of each compound. Thus, a precise quantification was not necessary as long as AMDIS correctly distinguished the relative amount of ions in each peak within a sample. In the field of metabolomics, however, accurate quantification for comparisons across samples may be required. Moreover, I have used several replicates (sampling units) whenever possible for each group compared (*e.g.*, each sex of each species). Hence, results represent often average amounts, possibly reducing the potential error of an eventual inaccurate quantification of peaks. Furthermore, errors in quantification of peaks may also occur in other commonly used programs if the integration method is not correctly applied. Besides, many of these programs do not correct for deconvolution, possibly increasing the potential for quantification errors since mass spectra may not be clean (see Introduction in Stein, 1999).

8.3.4.6. Checking for other errors

The final step was to evaluate via graphic output (see below) if there was another error. Individual chromatograms belonging to the same group (*e.g.*, one sex of a given species) could be plotted in alignment because AMDIS correctly interpolated RI (despite the samples could have been run in different years and the retention times were very discordant). This allowed the rapid detection of possible misidentifications, which otherwise would require opening or printing chromatograms individually using other programs. In all these instances, the unwanted chromatograms were removed if necessary.

8.3.5. Post-processing the data

All correction steps described in the previous section applied to the result files of AMDIS were performed for each species separately. After the curation of the data was done, a corrected file was created for each species. The next step involved aligning all or several species together for further comparisons. Two issues demanded however post-correction before different species could be grouped into a single file. First, alkenes (and alkadienes) needed to be identified. Second, a unique unifying code for each CHC compound was necessary in all corrected files.

Alkenes can not be readily identified from the mass spectra of the original chromatogram. To correctly identify the position of the double bond in alkenes, a deriva-

tization with dimethyl-disulfide (DMDS) was conducted for a pool of one or more samples from each sex and each species (Dunkelblum *et al.*, 1985, Carlson *et al.*, 1989). Derivatization of alkenes was done in a later phase of the project. Therefore, the correcting R scripts of the previous sections align alkenes based on the retention index but do not assign an identification. The identification of alkenes was necessary to assemble data from different species. Once the derivatization of the hexane extracts with DMDS was conducted and the alkenes were characterized in all species, an extra R-script renamed all alkenes of a given species with the corresponding alkene found in the derivatization. Since alkadienes have not been identified, alkadienes were assigned a name according to their retention index. This name included the chain length at which this alkadiene occurred and a letter indicating a range of retention index at which it occurred. For example, alkadienes with retention index between 2849 and 2851, would receive the arbitrary name of "C29 alkadiene E". Nevertheless, since the number of compounds increase with each disrupting feature that is added to the chain (*e.g.*, there are 300 monomethyl-branched compounds between C21 and C40, but around 3000 dimethyl-branched compounds potentially exist within the same range of carbon atoms, see Chapter 9), it is possible that different alkadienes with very similar retention indexes have been grouped together. The identification of alkadienes is a very time-demanding process, and since they are relative rare compound classes, their isomeric information is often not determined (Kather & Martin, 2015). In cuckoo wasps, alkadienes were relatively abundant in only six species (Chapter 4), and they may need to be identified in the future, if hypotheses concerning the importance and use of alkadienes in chemical communication in these species need to be understood.

The second problem to deal with was that the identification codes of all CHC compounds in the corrected files per species needed to be the same. Since the created mass spectral library used in the analyses has been built during the whole processing of data, new mass spectra (new CHC compounds not yet in the library, or a better spectrum of an already available CHC compound in the library) were added as they were encountered. For this reason, the identified unique code of each assigned compound were not necessarily the same among corrected files belonging to different species. An extra R script permitted the renaming of the CHC compounds and associated information, in the corrected files using the last updated mass spectral library.

After these two problems were succesfully dealt with, a table containing all available information for species (*e.g.*, species name, sex, sample name, locality of collection) and CHC compounds (*e.g.*, relative amount, retention index, and a number of AMDIS parameters) was assembled from which pivot tables could be created as desired to further conduct any required analyses.

8.3.6. Graphical visualization of the corrected data

The R scripts used in the analysis and processing of the result files of AMDIS allowed also the graphical visualization of all the steps described above. In fact, the files imported in R could be visually inspected to spot for example the wrong identification of a species or of a sex. This could be more easily detected in the graphs created in R than in other programs for chromatograms processing (AMDIS, ChemStation, Openchrom), because many samples need to be open and visualized together. Interestingly, this simple visualization allowed me to discover a mistaken identification of

Hedychridium roseum and *Hedychridium valesiense*, two closely related species which are difficult to tell apart morphologically (see Chapter 5).

Summarizing patterns and creating boxplots or barplots indicating which compounds are more often occurring in each sex of a given species could also be done in R. A final NMDS was created to visualize how females and males of a given species cluster together after the correction of the data. This allowed again to evaluate if there was a possible outlier in the dataset.

Several R packages were used to build the different R scripts used in the analyses. Among them, the most important ones were: flagme (Robinson, 2010), XCMS (Smith *et al.*, 2006), reshape2 (Wickham, 2007), stringr (Wickham, 2012) for the processing of the AMDIS output files and vegan, ade4 and gclus for preliminary exploration of the resulting information. Additionally, in order to plot chromatograms in R with functions available in the package XCMS, netCDF files were required. Thus, these netCDF files were converted from the original chromatograms using the free software OpenChrom (Wenig & Odermatt, 2010).

8.4. Discussion and concluding remarks

One of the advantages of this procedure is that analyzing a large number of samples belonging to one species is possible and does not demand much more time than analyzing few samples. In comparison, the required time for the analysis of chromatograms, extracting information with the commercial program ChemStation and then manually aligning the chromatograms in a spreadsheet program like Microsoft Excel (see Otterman, 2017), increases linearly with the number of samples used. Another advantage is the possibility to save either graphs or tables at every step allowing for a better tracking of what exactly was done. Moreover, AMDIS saves all the information into the ELU and FIN files, which can always be accessed for verifications if necessary (*e.g.*, mass spectra, matches between the library spectra and the spectra, ion count of each peak, etc.). This is impossible to do with a program such as ChemStation because all processed information is lost once the program is closed. Finally, an important convenient by-product of using AMDIS is that samples are automatically identified.

One remaining task is to assess the applicability of this procedure by comparing the results obtained when using AMDIS and the here proposed protocol with those obtained by using the most often employed procedure (*e.g.*, using ChemStation, and manually aligning samples) on the very same set of samples. For example, Cerdán-Calero and colleagues have compared the performance of AMDIS to that of manual analysis in raw GC/MS data files for identification and quantification of volatile and non-volatile compounds present in orange juice (Cerdán-Calero *et al.*, 2012), finding that if parameters settings are optimized, AMDIS could provide accurate and fast results. Doing such a comparison for CHC data, would not only permit to compare the speed at which these analyses can be done by the different methods, but also assess the degree of correlation of the results, and evaluate the capability and accuracy of the suggested workflow.

The advent of the 'omics' era at the end of the last century has been enhanced by the emerging advance of new analytical technologies which can generate large amount of data (Ward & White, 2002). Mass spectrometry is one of the key analytical technologies used by many of these "omic" approaches (*e.g.*, metabolomics, genomics,

lipidomics, proteomics, Di Girolamo *et al.*, 2013). However, in order to encompass these advances, the development of new computational tools that allow the processing and mining of these data is required (Ward & White, 2002; Di Girolamo *et al.*, 2013). While in metabolomics, many commercial and non-commercial software tools have been developed for different steps of the data processing (see Review of Katajamaa & Oresic, 2007), the generation of software tools that could be applied in the analyses of cuticular hydrocarbon has been comparatively scarce (*e.g.*, GCaligner, Dellicour & Lecocq, 2013). Nonetheless, recent software tools created for the field of GC/MS-based metabolomics could be applied in chemical ecology (*e.g.*, eRah, Domingo Almenara *et al.*, 2016, GCalignR, Ottensmann *et al.*, 2017). As in other fields, the time is ripe for bioinformatics aiding in the processing of data for cuticular hydrocarbon analyses. The workflow and the simple program created here may eventually be extended with contributions from other experts in the field.

9. A useful tool for the quick identification of methyl-branched hydrocarbons

9.1. Abstract

Hydrocarbons (CHC) on the external layer of an insect cuticle provide important functions to insects. Their non-polar properties have anti-desiccation and protective functions. In addition, CHC convey a plethora of information that facilitates their use in intra- and inter-specific communication. The interest in the role of CHC in chemical communication has originated an important, exciting and growing field of research, which has translated into an exponential increase in the number of publications on CHC. Although CHC are relatively simple molecules composed of only carbon and hydrogen, they occur in a great diversity of compounds. Hydrocarbons can be classified into three main substance classes, depending on whether the molecule presents certain features such as double bonds and methyl groups inserted along their chain (*e.g.*, linear alkanes, unsaturated hydrocarbons (olefins), and methyl-branched compounds). Linear alkanes occur in a limited number and are easily identified due to the characteristic pattern of their mass spectra. However, identifying the large diversity of unsaturated and methyl-branched compounds requires certain expertise. A subsequent derivatization is necessary for being able to identify unsaturated compounds while methyl-branched compounds can be readily identified using retention indices and diagnostic ions in their mass spectra. Whereas the bases for interpreting mass spectra of methyl-branched hydrocarbons were set several decades ago, the interpretation of their mass spectra can still be a difficult and time-consuming task, especially if the compound has not been described before and has more than two methyl groups. Here, I propose a tool, in the form of an R script, that may help characterizing mass spectra of one of the most commonly occurring classes of CHC (methyl-branched hydrocarbons). This simple tool will be especially useful for researchers for whom access to specialized literature and mass spectral libraries may be limited.

9.2. Introduction

Hydrocarbons of chain lengths between C21 and C50 are present in the cuticle of almost all insects (Blomquist & Bagnères, 2010; Ginzel & Blomquist, 2016). Due to their hydrophobic nature, their primary function is to confer desiccation resistance (Gibbs, 1998; Gibbs & Rajpurohit, 2010; Stinziano *et al.*, 2015) as well as contributing to protect the insect against infections (Golebiowski *et al.*, 2008; Golebiowski *et al.*, 2011; Ortiz-Urquiza & Keyhani, 2013; Herzner & Strohm, 2007; Wurdack *et al.*, in press) and/or external pollutants (Anyanwu *et al.*, 2000; Balabanidou *et al.*, 2016).

In addition, because of their extreme diversity and their potential to be used as cues or signals, cuticular hydrocarbons (CHC) play a central role in intra- and interspecific communication conveying different types of information (*e.g.*, species and nestmate recognition (Singer, 1998; van Zweden & d'Ettorre, 2010), chemical mimicry (Bagneres & Lorenzi, 2010), sexual communication (Ingleby, 2015; Lane *et al.*, 2016), colony integration (Greene, 2010)). The function of CHC as signals and cues has originated a fecund new area of research and has conferred them a central role in the field of insect chemical ecology.

In spite of their relative simplicity (their molecules consist of just carbon and hydrogen), CHC can be very diverse. There are three main substance classes of hydrocarbons: linear alkanes (a straight chain); unsaturated compounds, which have one (alkene) or more (*i.e.*, alkadienes, alkatrienes) double bonds somewhere along the chain; and methyl-branched alkanes, which possess one or more methyl groups somewhere along the chain (Blomquist & Bagneres, 2010b). Other more complex types exist but are rare (*e.g.*, methyl-alkenes and methyl-alkadienes, Menzel *et al.*, 2008; Martin & Drijfhout, 2009a; Kather & Martin, 2015; or allenic hydrocarbons, Fletcher *et al.*, 2001). With the exception of alkanes in which there is only one possible compound per carbon atom, the diversity of possible compounds increases with chain length especially for methyl-branched alkanes because with every new carbon atom in the chain there is a substantial increase in the number of possible internally branched methyl-hydrocarbons. In this sense, hydrocarbons represent in the chemical world of insects an analagous example to the various coloration patterns bird plumages exhibit (Blomquist & Bagneres, 2010b, Ginzel & Blomquist, 2016).

The first insect hydrocarbons were identified in the 1960s shortly after the gas (liquid) chromatography (GC) was invented in 1952 (James and Martin, 1952; in Bartle & Myers, 2002). The first complete hydrocarbon profile identified was that of the American cockroach (*Periplaneta americana*, Baker *et al.*, 1963 in Blomquist & Bagnères, 2010). The choice of the cockroach was a fortunate decision because of its simple profile with only 3 major compounds accounting for more than 90% of the total profile, which facilitated their identification (Baker *et al.*, 1963; Blomquist & Bagnères, 2010b). However, it was only with the development and application of mass spectrometric detection in combination with GC (GC-MS, Gohlke, 1959), that a rapid and efficient analysis of insect hydrocarbons was possible (Blomquist & Bagnères, 2010b). Indeed, the number of studies using hydrocarbons has expanded greatly in recent years (Figure 9.1) corroborating their specific role in chemical ecology.

One crucial step in CHC characterization is the identification of the different compounds with mass spectrometry. By ionizing any compound, this microanalytical technique can detect and separate ions based on their mass-to-charge-ratio (m/z, the mass of the ion divided by the number of charges it carries, Watson & Sparkman, 2007). The resulting histogram that depicts the intensity vs. the m/z of any analyte – the mass spectrum – can be interpreted to determine the structure and elemental composition of the molecule allowing its identification (Watson & Sparkman, 2007). Linear alkanes are easily identified because of their distinctive composition of ions, which determines a particular mass spectral pattern, and the characteristic diagnostic ions indicating their molecular mass. In contrast, to identify unsaturated compounds a derivatization with dimethyl disulfide (DMDS, Buser *et al.*, 1983; Dunkelblum *et al.*, 1985; Carlson *et al.*, 1989) or other methods (Francis & Tande, 1978) is usually conducted with the aim of identifying the position where the double bond occurs.

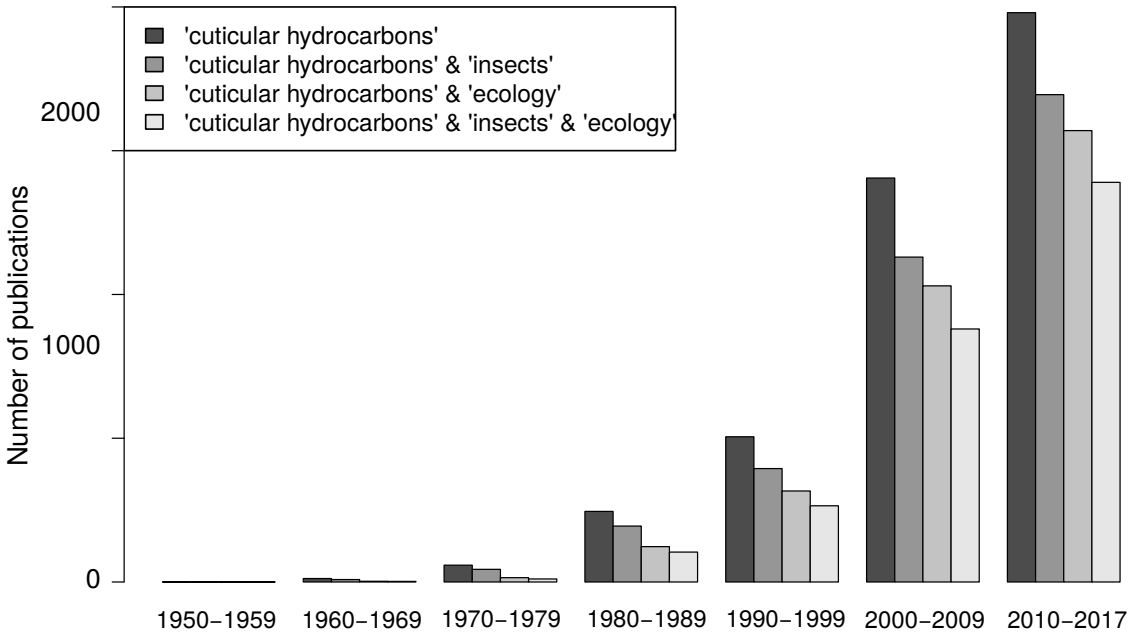

Figure 9.1.: Increase in the number of publications citing "cuticular hydrocarbons" and/or "insects" and "ecology" since the invention of the gas chromatography. The search was done in GoogleScholar on the 15th of May 2017, restricting the search only to publications in English.

Identification of alkenes after derivatization is relatively straightforward (Howard, 1993) and some methods have been developed to permit their rapid identification, even without requiring a derivatization method (Kroiss *et al.*, 2011). Nevertheless, this last method entails the use of a gas chromatography–ion-trap mass spectrometry (Kroiss *et al.*, 2011). The identification of alkadienes, alkatrienes and higher levels of unsaturated compounds demands more expertise (Blomquist *et al.*, 1987). However, alkadienes and alkatrienes often constitute minor components of the profiles (Blomquist *et al.*, 1987). Moreover, a derivatization conducted on scarce compounds hinders the acquisition of mass spectra of good quality to correctly determine the positions of the double bonds. Thus, their identification is often not possible (Blomquist *et al.*, 1987).

In the case of methyl-branched hydrocarbons, two publications set the bases for their identification in the late 1960s. The effect that the position of the methyl branch in the carbon chain of monomethyl-branched alkanes has on retention times was studied by Mold and colleagues (Mold *et al.*, 1966). These researchers discovered that 3Me compounds elute later in time than all other monomethyl-branched alkanes and that the more internal the branch position is, the earlier the elution time of the compound. Cleavage of the carbon-carbon bond at the branch point may sometimes result in a doublet of peaks in the mass spectrum: the odd-mass ion and an even-mass ion consisting of one unit mass less due to the loss of a hydrogen atom. Even-mass ions are sometimes more dominant than odd-mass ions, and the explanation of these patterns in the cleavage of the bonds and their significance in aiding in the inter-

pretation of mass spectra of methyl-branched compounds was provided by McCarthy and collaborators in 1968 (McCarthy *et al.*, 1968). They showed that the intensity of these even mass-ions depended on the size of the molecule and the position and number of methyl groups (reviewed in Nelson *et al.*, 1993). Currently, the identification/interpretation of methyl-branched hydrocarbons is achieved by evaluating the diagnostic ions, characteristic of the carbon-carbon bond cleavage at the branch point, and by confirming the mass spectrum with the retention data (Kovats retention index) at which the methyl-branch compound elutes (Carlson *et al.*, 1998b). The retention time is the time that a compound analyte needs to pass through the column before it gets detected. In general, different factors can affect retention time: linear velocity, temperature, length of the column and phase ratio. As a result, retention time may vary a lot across samples and not be useful for peak identification (D'Acampora Zellner *et al.*, 2008). To overcome these problems, Kovats proposed to use what he named the retention index, which constitutes an additional useful value to confirm the identification of methyl-branched compounds. Using a homologous series of alkanes as reference peaks allows the adjustment of the retention times when isothermal GC conditions are applied (Kovats, 1958). However, when the temperature program varies during the run, the equation of van den Dool and Kratz (van den Dool & Kratz, 1963), should be instead used for the calculation of the retention index (D'Acampora Zellner *et al.*, 2008).

The interpretation of the fragmentation patterns of methyl-branched alkanes is relatively simple when only one methyl-group is present. However, the difficulty of identification increases with each additional methyl-group because several isomers with similar or slightly different retention indices can co-occur (Schulz, 2001, see figure 9.2). Moreover, in comparison to unsaturated compounds and alkanes, methyl-branched compounds represent the most numerous and diverse compounds in insects' cuticle, at least from what is known from previous meta-analyses of CHCs (Martin & Drijfhout, 2009a; Kather & Martin, 2015). In fact, methyl-branched hydrocarbons represent up to 85% of the total number of compounds identified in 78 species of ants (848/993 in Martin & Drijfhout, 2009a).

Despite the predominance of methyl-branched hydrocarbons and their role as important, sometimes unique components of many contact pheromones in insects (*e.g.*, Lacey *et al.*, 2008; Silk *et al.*, 2009), their identification is still based on comparing their fragmentation patterns to already published and described methyl-branched alkanes. Although the interpretation of mass spectra is not a complex task, it can be time consuming, especially if the researcher is new into the field, or if he/she is dealing with tri- or tetramethyl-branched alkanes, which are more difficult to identify. Moreover, more than one methyl-branched compound may elute at the same time and be represented by one mass spectrum that is a mixture (*i.e.*, it contains diagnostic ions for all those compounds), thus complicating their identification. In fact, the overlap of elution times in the GC may occur in methyl-branched compounds that differ by one or two carbons in the backbone (Carlson *et al.*, 1998b), confusing their identification even more. Given the inherent difficulties in interpreting their mass spectra, others have proposed ways to model and predict retention indexes based on their chemical structure (Katritzky *et al.*, 2000). However, these equations are not often used since their implementation was conducted in outdated programs and the models used require the incorporation of too many parameters.

Most of the current interest in the research on methyl-branched alkanes of insects

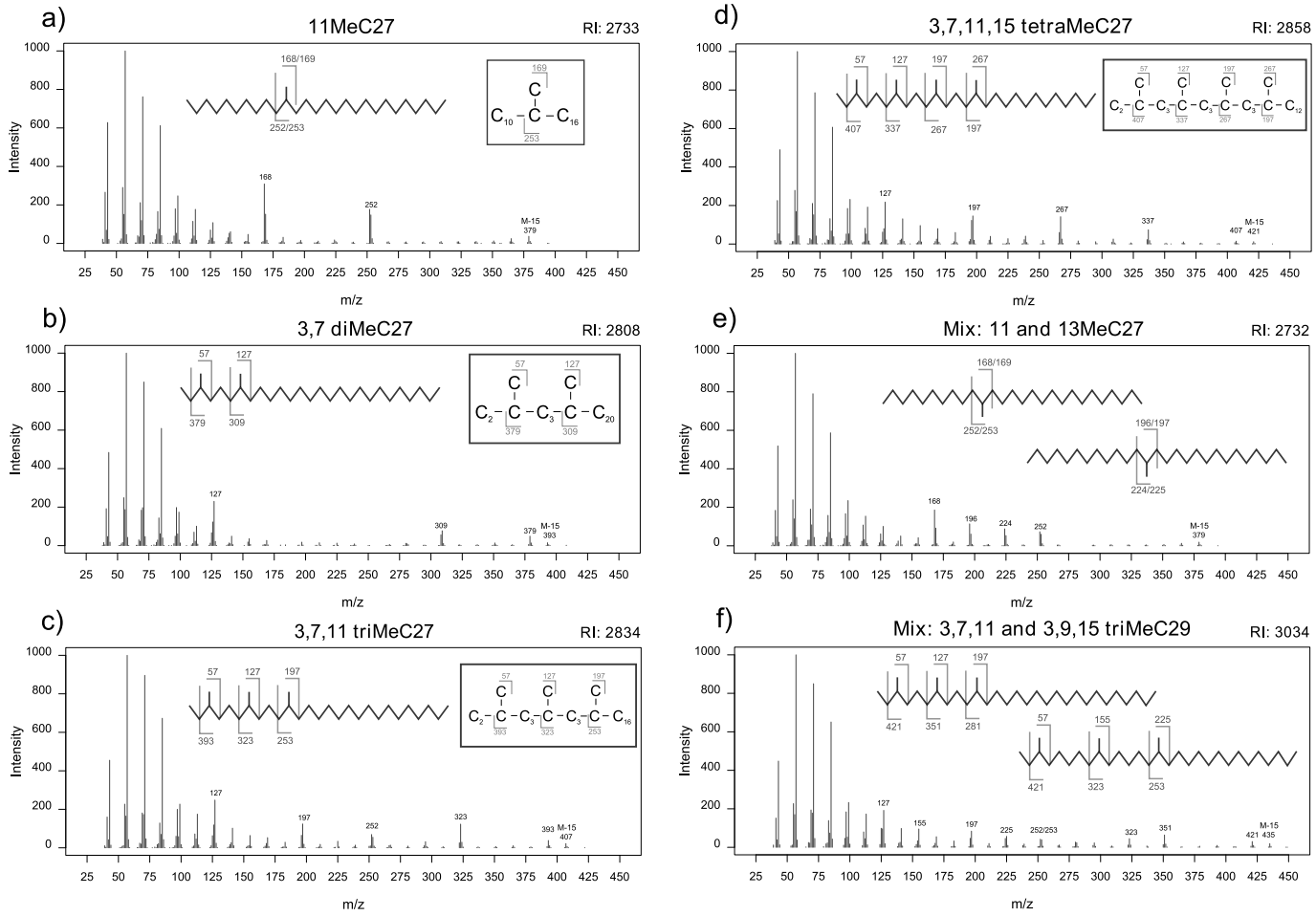

Figure 9.2.: Mass spectra of a a) monomethyl-branched b) dimethyl-branched, c) trimethyl-branched, d) tetramethyl-branched hydrocarbon showing the cleavage of the molecules after ionization and their diagnostic ions. Graphs above the mass spectra show how the diagnostic ions are calculated. e) and f) show mass spectra for mixtures of compounds. e) depicts a commonly encountered mixture of two monomethyl-branched compound (an example of a relatively easily identifiable mix of compounds), f) shows a much more complex mixture, that of two co-occurring trimethyl-branched compounds. Retention indices at which those compounds often elute are shown at the top right of each graph. Identification of all compounds was achieved with the program described here. Diagnostic ions of b), c) and d) can be confirmed with Nelson *et al.*, 2003b. Molecules are named using the short-nomenclature employed in all chapters of this thesis (see chapter 4). For example: 11MeC27 is 11-monomethyl-heptacosane.

aims to understand their synthesis and elucidate their absolute tridimensional configurations in the cuticle, which may have implications in their bioactivity and their function as signals (Ablard *et al.*, 2012; Kühbandner *et al.*, 2013; Bello *et al.*, 2015). While these constitute important research areas, the interpretation and discovery of new methyl-branched alkanes receives less attention. Nevertheless, some researchers still publish newly identified methyl-branched alkanes of insects and provide a mean of identifying them (*e.g.*, the ions used in the interpretation of the mass spectra). This suggests the need for creating a way to elucidate mass spectra of methyl-branched alkanes more readily. As previously mentioned, confirming the identification of any methyl-branched alkane usually requires the comparison to a specialized mass spectral library or specialized literature which provides some, but not all, of the possible methyl-branched hydrocarbons. A downside of this is that neither specialized software nor literature are easily available for the common researcher and access to specialized mass spectral libraries may be expensive for researchers of developing countries. Thus, here, I aim to provide a useful tool which may help in the identification of spectra of any methyl-branched hydrocarbon occurring in the cuticle of insects.

9.3. Methods

I wrote a script in R (R Core Team, 2013) that allows the identification of methyl-branched compounds (with up to 4 methyl groups) for any chain length between C20 and C40 (range is user-defined). R is a freely available programming language used primarily for statistical analyses and with the potential to create any graph with publication quality, whose use has been widely adopted in biological sciences. With its more than 5000 applied packages (Pathak, 2014), it is the tool of choice for analyzing data in ecology, having largely replaced other commercial statistical programs in the last years (Touchon & McCoy, 2016). The script is composed of two functions. One creates through simple arithmetic rules a table listing all theoretically possible combinations of methyl-branched compounds (up to 4 methyl groups), separated by an odd number of carbon atoms, starting with a minimum of one (*e.g.*, the code produces 3,5 diMe and 3,7 diMe, but not 3,4 diMe or 3,6 diMe), since dimethyl-branched alkanes with adjacent methyl branches or with methyl branches separated by an even number of carbons are considered unusual in insects (Blomquist *et al.*, 1987). The resulting table provides as information the molecular mass of the compound, its chain length, its name, and the diagnostic ions indicative of the position of the branching point. Optionally, the script can also calculate an experimental Kovats Retention Index (Junkes *et al.*, 2002, see below). The second function allows the filtering of compounds according to criteria provided by the user: observed diagnostic ions, molecular weight, chain length and/or the observed retention index. To simplify the interpretation and the script functioning, only odd-ion combinations are provided by the script (see below). I have not used even-mass ions (sensu McCarthy *et al.*, 1968), despite the possibility that they may in some cases be more dominant than the odd-mass ions (see Introduction, Figure 9.2). The list of the ions that need to be provided by the user are listed in the Appendix.

As already mentioned above, in order to identify methyl-branched compounds, two pieces of information are necessary: 1) the mass spectra (*e.g.*, all diagnostic ions or a subset of them) and 2) the retention index. The details of their implementation are

explained in the following paragraphs.

9.3.1. Implementation of diagnostic ions of methyl-branched hydrocarbons

The diagnostic ions resulting from the cleavage of the carbon atoms next to the inserted methyl groups can be calculated knowing two pieces of information: the total molecular weight (MW) and the positions at which the methyl groups are inserted along the chain. Keep in mind that the incorporation of an additional carbon to the hydrocarbon chain (not bearing a methyl group) increments the MW of a compound by 14 units (molecular weight of CH_2). An example illustrates this procedure better (Figure 9.3). Starting with a methyl group in the second position and ending with the most internal branched compound possible for a given chain length, the program is able to calculate the MW and the diagnostic ions for all possible combinations of methyl-branched hydrocarbons (containing up to 4 methyl groups) in a user-defined range of carbon atoms. Refer to figure 9.3 for an example of this calculation and to the Appendix for details on the implementation.

9.3.2. Implementation of retention index

I used a semi-empirical topological index proposed by Junkes and colleagues (Junkes *et al.*, 2002) to predict and calculate approximate retention indexes for each methyl-branched alkane created with the script. The topological index is calculated based on the chain length, the position(s) of the methyl group(s) and the number of methyl groups that are attached to the backbone of the molecule. Some researchers (Katritzky *et al.*, 2000; Junkes *et al.*, 2002) have shown that the retention indexes of methyl-branched compounds can be reliably predicted based on quantitative structure-property relationships (QSPR). I used the equation of Junkes and colleagues (2002) to calculate an experimental retention index for each compound.

$$I_{ET(opt)} = I_{ET} - \tfrac{1}{3}\{(log_{n1+1}) + (log_{n2+8}) + (log_{n3+24}) + (log_{n4+14})\}$$

where n_1, n_2, n_3 and n_4 are the positions of connections of first, second, third and fourth methyl groups. Log is logarithm of base 10. The formula presented is the extended version used in the case in which a maximum of four methyl groups are present in the chain, each of them represented by the corresponding term in the subtracted fraction. If less methyl groups are present, the formula reduces the number of terms accordingly. In short, a topological index is calculated by providing a value for all different types of carbon atoms that there can exist within any methyl-branched compound (a maximum of 6 types, see Junkes *et al.*, 2002) and summing that up to obtain I_{ET}. A carbon atom within the molecule does not receive the same weight if it is located at the middle of the molecule or at an extreme or if there is a methyl group attached to it. I_{ET} is calculated as follows: $I_{ET} = \sum C_i + \delta_i$, where $\delta_i = logC_1 + logC_2 + logC_3 + logC_4$. Ci is the value attributed for each carbon atom and δ_i is the sum of the logarithms of the values for each adjacent carbon atom (C_1, C_2, C_3 and C_4, Junkes *et al.*, 2002). For further details on the implementation, refer to the original publication (Junkes *et al.*, 2002) and to an example of the calculation (Figure 9.3).

According to the authors of this topological index, this semi-empirical topological retention index has a good predictive value with a standard deviation of 4.3, which

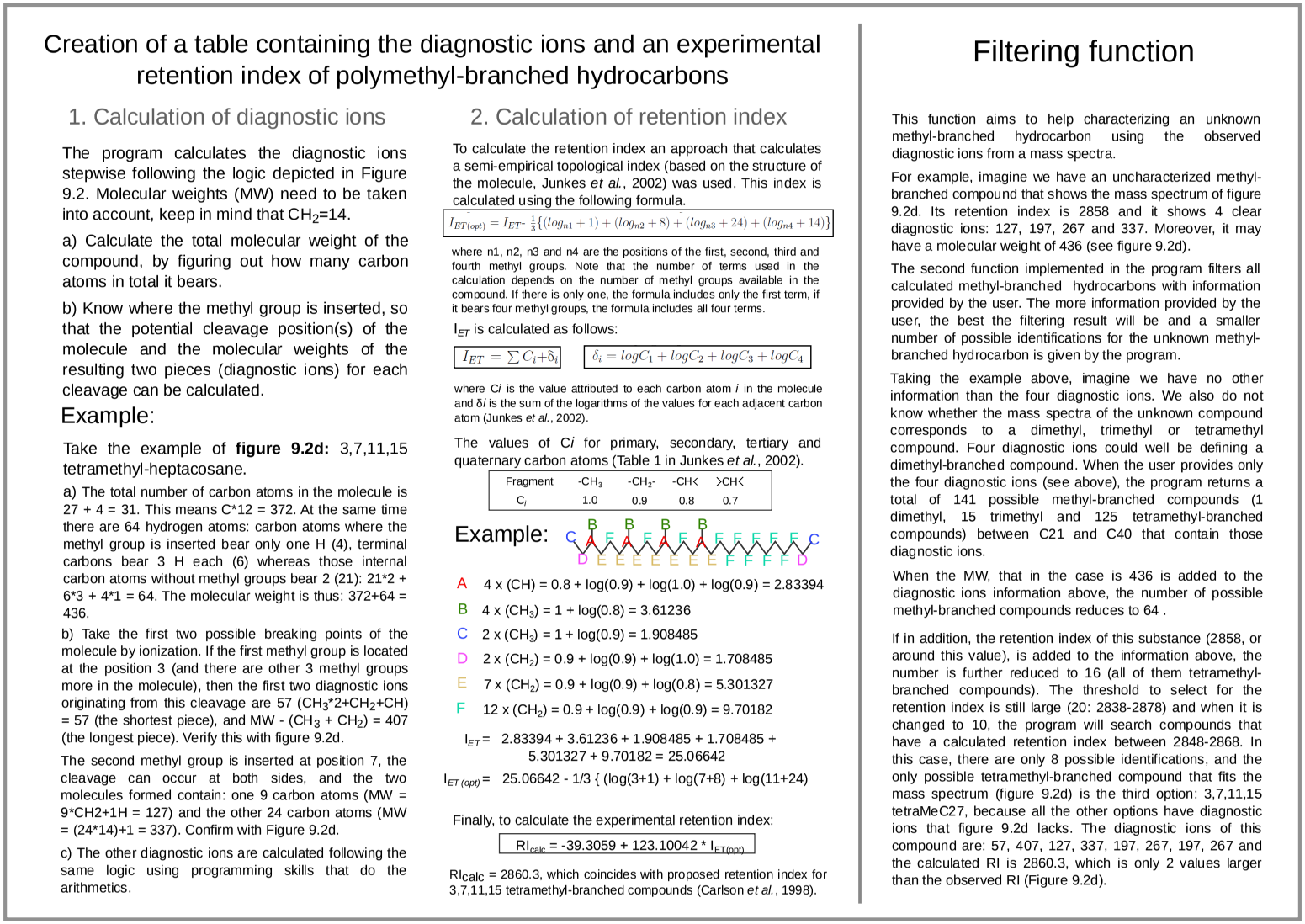

Figure 9.3.: Explanation of the functioning of the identification tool of this chapter. The R script consists of two functions. The first one creates a table containing the diagnostic ions and the retention indices of all methyl-branched hydrocarbons within a range of carbons (user-defined). The second one allows filtering this table based on diagnostic ions, retention index and/or molecular weight (provided by the user). Through this filtering function, mass spectra of unknown methyl-branched compounds can be much rapidly identified.

is smaller than the standard deviation from a previous model by Katritzky and colleagues (Katritzky *et al.*, 2000), who did compare their values to retention indexes observed in insects.

To evaluate the fidelity of the diagnostic ions provided by the script, diagnostic ions provided by the program were compared with previously described mass spectra of various polymethyl-branched hydrocarbons (*e.g.*, Nelson, 2001; Schulz, 2001; Nelson *et al.*, 2003a; Nelson *et al.*, 2003b). The code is entirely available in the supplementary material and is open to improvements, provided it is properly cited.

9.4. Results

The code available in the supplementary material provides a function that can be run as a source file in R and helps in routine identification analysis. Producing the main table (that contains up to 4 methyl-branched alkanes of C20–C40) with the calculation of the retention indexes may take 80 minutes in a regular computer, but it may be only run once and saved as a csv.file. This file can then be read from R and used with the filtering function when trying to identify new complex methyl-branched hydrocarbons. Its use is simple and straightforward. Moreover, given that sometimes hydrocarbons occur in mixtures, it can allow the discovery of these compounds very easily, thanks to the filtering function. Thus, the researcher would not need to spend much time trying to discover which new methyl-branched hydrocarbon he/she is dealing with. In total, the code calculated 42,359 methyl-branched compounds within the range of carbon atoms mentioned above. Figure 9.4a shows how the number of compounds increases exponentially with each methyl group in the molecule. Whereas there are 299 different monomethyl-branched compounds for carbon chains ranging between C20 and C40, there are as many as 30,630 tetramethyl-branched compounds that could possibly occur in that same range of carbons. The number of possible combinations of methyl-branched compounds reduces as the methyl group is inserted more internally (Figure 9.4b).

9.5. Discussion

The R code presented here (Appendix) reduces the time requirement for interpreting mass spectra of new methyl-branched hydrocarbons. This tool may be especially useful for identifying complex hydrocarbons (consisting of more than two methyl groups) or mixes of methyl-branched hydrocarbons. Although in nature, some methyl-branched hydrocarbons are much more common than others (*e.g.*, odd-numbered carbon chains are more common and the insertion of the methyl group occurs more often at odd positions, Blomquist *et al.*, 1987; Katritzky *et al.*, 2000), it is theoretically possible to discover not previously described complex mass spectra in species whose CHC have not been yet characterized. Complexity increases tremendously with the addition of each methyl group to the backbone, to the point that in the range of hydrocarbons studied here (C20–C40), the number of theoretically possible methyl-branched hydrocarbons exceeds 42,000 (Figure 9.4).

Whereas the great majority of compounds calculated with this script have not been observed in insects, this may as well be the result of the low number of species that have been so far analyzed and for which CHC compounds have been reported. In

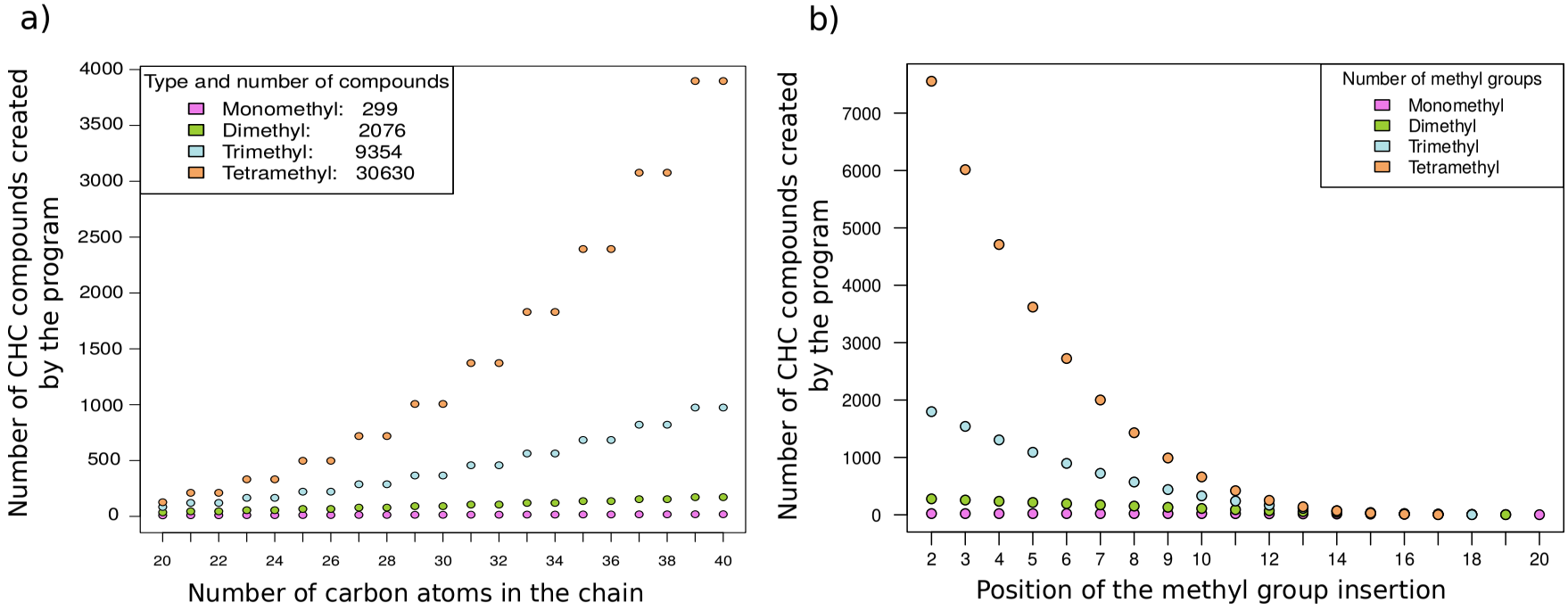

Figure 9.4.: a) Number of methyl-branched hydrocarbons created by the program colored by compound class plotted against carbon chain. Note the exponential increase in the number of methyl-branched compounds with the addition of each methyl group. b) Number of methyl-branched hydrocarbons plotted against the carbon position at which the methyl group is inserted. There are many more possible methyl-branched compounds when the insertion of the methyl group(s) is at more external carbons.

many cases, branched hydrocarbons are not identified due to the complexity of the interpretation of their mass spectra. Thus, this tool may help to readily identify and report methyl-branched compounds of insects not characterized before, even if these compounds occur in small abundances. In comparison to olefins, branched hydrocarbons can be identified from the original gas chromatogram with no need to conduct additional GC-MS analyses. Hence, this tool may help to avoid reporting unidentified methyl-branched compounds in the future (*e.g.*, Sharma *et al.*, 2012, provided retention times, formulas and molecular weight but did not identify many CHC observed in their study on the effect of natural and sexual selection on the cuticular hydrocarbons of *Drosophila simulans*).

Retention indexes are usually a further criterion for correctly identifying multiple methyl-branched compounds. The Open Source code provided here, calculates an experimental retention index which is inferred from structural information. This retention index is not accurate and may deviate from a retention index calculated from a chromatogram by as much as 10-15 units. For this reason, the retention indexes provided by the code should not be reported. Nevertheless, they provide valuable additional information that helps restricting the number of possible methyl-branched compounds that may match some combinations of ions and are therefore useful.

By changing one single parameter in the first function, the code provided can calculate hydrocarbons within a broader range (C15-C50 for example, dimethyl-branched alkanes have been found to occur within this range of carbons in certain insects, Blomquist *et al.*, 1987). Additionally, following the same logic used in the programming, the code may also be extended, if necessary, to estimate ion combinations for methyl-branched hydrocarbons of more than 4 methyl-groups. Whereas hydrocarbons with more than four methyl groups occur less often in Hymenoptera (the insect group I have worked with, see Kather & Martin, 2015), methyl-branched alkanes with more than five methyl groups may not only be occurring but may represent major compounds in CHC profiles of other species (*e.g.*, *Antitrogus parvulus*, Chow *et al.*, 2005). Thus, the provided script may be extended to increase the complexity of the list of hydrocarbons, and hence help in the identification of more complex methyl-branched hydrocarbons. Nonetheless, this script does not permit to say anything about the stereochemistry or the chirality of the compound. To identify these, other procedures are necessary and this escapes the aim of this tool.

9.6. Conclusion

I have provided an open source script written in R that helps to reduce the time invested in the interpretation of mass spectra of methyl-branched compound in insects. The script calculates all possible combinations of methyl-branched compounds between the range of C20–C40 containing up to four methyl groups in the molecule, providing an astonishing number of over 42,000 different possible compounds. The script provides information regarding the diagnostic ions for each compound as well as it optionally calculates an experimental retention index, based on structural information of the molecule (Junkes *et al.*, 2002). It is expected that with the use of this tool, all methyl-branched compounds encountered may be identified and reported by researchers, including investigators of developing countries who may not have the resources to access specialized mass spectral libraries or specialized literature against

which they could eventually compare mass spectra of methyl-branched hydrocarbons. Thus, this tool may be useful for all researchers in the field of insect chemical ecology working with CHC.

10. Discussion

The process by which reciprocal selection in two or more interacting species leads to evolutionary changes is called coevolution (Janzen, 1980, Clayton *et al.*, 2016), and is often credited for generating phenotypic and biological diversity (Janz *et al.*, 2006; Smith & Benkman, 2007; Laine, 2009; Medina & Langmore, 2015a; Speed et al 2015; Medina *et al.*, 2016). Among coevolutionary interactions (*e.g.*, mutualism, predation, competition, parasitism), brood parasitism appears suitable to test how this interaction may lead to diversification (Rothstein, 1990). In particular, abundant evidence on coevolved traits caused by this type of antagonistic interaction comes from avian cuckoos and their hosts, which are frequently cited as a textbook example of an evolutionary arms race (Davies & Brooke, 1989a; Rothstein, 1990; Feeney *et al.*, 2014). To succesfully exploit the parental care of their hosts, cuckoos evolved adaptations that enabled them to avoid recognition of their eggs by their hosts (*e.g.*, mimicking coloration, size and marking patterns of their host´s eggs, Brooke & Davies, 1988, Oien *et al.*, 1995). Hosts, on the other hand, overcome these adaptations by evolving better defense strategies against cuckoos (*e.g.*, improving egg discrimination abilities, Spottiswoode & Stevens, 2011; mobbing against adult parasites, Welbergen & Davies, 2009; cooperative breeding, Feeney *et al.*, 2013).

In this thesis, I have introduced another model for an evolutionary arms race, potentially driving the diversification and evolution of cuticular hydrocarbons (CHC), which are important traits in insects (see Introduction). As parasitoids and kleptoparasites (in the following also referred as brood parasites) of mainly other hymenopteran hosts, cuckoo wasps (Hymenoptera: Chrysididae) are particularly interesting because of the many adaptations they have evolved to cope with their parasitic lifestyle (reviewed in Chapter 2). Among these, chemical adaptations (*e.g.*, chemical mimicry of the CHC profile of their hosts, Strohm *et al.*, 2008) are relevant since CHC are known to be involved in intra- and interspecific recognition. CHC are however, not only used as signals in communication but also act as a barrier against water loss, being subject to both natural and sexual selective forces in a likely complex manner. Thus, studying their evolution is an interesting but also challenging task.

10.1. Coevolution of brood parasites and their hosts as a driver of CHC evolution

The evolutionary outcome (adaptations and counteradaptations) of the antagonistic interaction between host and parasite, depends on the fitness costs that the interaction inflicts on one or the other actor (Rothstein, 1990, Medina & Langmore, 2015b). Host adaptations should evolve more rapidly when selection is strong, for example, when the parasite is very virulent (Medina & Langmore, 2015c). If, in turn, hosts evolve a defence strategy that can have a negative impact on their parasites (Medina & Langmore, 2015c), an evolutionary arms race may take place (Dawkins & Krebs,

1979; Rothstein, 1990).

Using closely related species in the genus *Hedychrum*, I have studied the effects of an arms race in the CHC profiles of parasites and their hosts. Cuckoo wasps are insufficiently studied, partly because of their comparatively low abundances in spite of their relative ubiquity, their susceptibility to habitat degradation and their difficult taxonomy (Paukkunen *et al.*, 2015; Paukkunnen *et al.*, 2017; see Chapter 2). Nevertheless, some species of the genus *Hedychrum* are comparatively well known with respect to other cuckoo wasps, and have been the subject of some previous investigations, especially the particular common *Hedychrum rutilans* and its host, the European beewolf, *Philanthus triangulum* (*e.g.*, Strohm *et al.*, 2001; Strohm *et al.*, 2008; see also review in Chapter 2). Selection imposed on the host by the parasite can be high, for example, it has been reported that *H. rutilans* can cause local extinction of its host (Simon-Thomas & Simon-Thomas, 1972). In general, hosts of *Hedychrum* are digger wasps of the tribes Philanthini and Cercerini (Hymenoptera: Crabronidae), whose females hunt either Hymenoptera (HYMw) or Coleoptera (COLw) prey as provision for their nests (Bohart & Menke, 1976). This particular specialization on the type of prey of these digger wasps has been shown to drive chemical adaptations of their CHC profiles (Wurdack *et al.*, 2017). Females of HYMw embalm their Hymenopteran prey with alkene-enriched secretions from the post-pharyngeal gland (Strohm & Linsenmaier, 2001; Herzner *et al.*, 2007). This embalmation confers better protection against fungus infestation by reducing the condensation of water on the surface of the immobilized prey (Herzner & Strohm, 2007; Wurdack *et al.*, 2017). Since the hydrocarbon compositions of the cuticle and the post-pharyngeal gland are highly correlated (Bagneres & Morgan, 1991; Strohm *et al.*, 2010), CHC profiles of HYMw are commonly dominated by unsaturated compounds (mainly alkenes, Wurdack *et al.*, 2017). The switch of prey to Coleoptera, apparently relaxed the constraint to produce a relatively alkene-dominated CHC profile (Wurdack *et al.*, 2017) and the diversification of CHC profiles with methyl-branched alkanes may have evolved as a strategy to escape brood parasitism from cuckoo wasps (Chapter 7, Wurdack *et al.*, 2017, see below).

10.1.1. Adaptations in the parasites

Brood parasitism often results in complete loss of the host's progeny. Therefore, it is in the host's interest to be able to detect the presence of their enemies. Cuckoo wasps of the genus *Hedychrum* enter the nest of their hosts to oviposit. When inside the nest, chemical cues may be left unintentionally, and recognition of the presence of the female cuckoo wasp in the nest can have detrimental effects: if the host discovers the cuckoo wasp inside the nest, it might display aggressive defense behavior against the parasite (Kimsey & Bohart, 1991). Alternatively, if the female host recognizes the nest has been parasitized by finding chemical cues from the cuckoo wasp, the host may remove the parasitized prey evicting the cuckoo egg with it (Strohm *et al.*, 2008). Thus, cuckoo wasps have evolved adaptations to counteract chemical detection of their CHC profiles. The first cuckoo wasp for which chemical mimicry (or the *de novo* production of a CHC profile resembling that of the female host, *e.g.*, Howard *et al.*, 1980) and chemical insignificance (a reduction in the total amount of CHC produced, Lenoir *et al.*, 2001) was confirmed was *Hedychrum rutilans* (Strohm *et al.*, 2008; Kroiss *et al.*, 2009a). Although, chemical mimicry has been demonstrated so far only in

three cuckoo wasp species (*H. rutilans, Pseudospinolia neglecta* and *Chrysis mediata*, Strohm *et al.*, 2008; Wurdack *et al.*, 2015), unpublished evidence suggests that it might be more widespread in cuckoo wasps (Bandorf, 2017). Chemical camouflage (the physical acquisition of the chemical cues by parasites, commonly found in social parasites, Lenoir *et al.*, 2001), is however not to be expected in cuckoo wasps, since cuckoo wasps are solitary, and contact between them and their hosts might at all times be avoided (Strohm *et al.*, 2008).

There are (at least) four different ways how an odor blend (*e.g.*, the CHC compounds in a profile) may vary. I follow a graphical model employed by Raguso (2008) to explain them: 1) the same blend is produced but in a different abundance (*e.g.*, producing less of the same blend may result in insignificance), 2) the ratio of the different (or some of the) compounds varies, 3) new compounds are produced (or in defect, some compounds are not produced), 4) the environmental context in which the odor occurs varies (Raguso, 2008). In this thesis, only changes in the relative amounts of distinct CHC compounds were studied. Therefore, these changes can only reflect situations exemplified by the previous second and third points, in which relative variations of the different CHC compounds can be detected. Overall, parasites have adapted their profiles to those of their hosts, but using different strategies. On one hand, *Hedychrum* wasps parasitizing HYMw hosts (those constrained to produce alkene-enriched CHC profiles because of the prey embalming, Wurdack *et al.*, 2017) exhibited cuticular profiles with the same proportion of compound classes as those of their hosts, sharing the same chemical space with them. However, the double bond position of the most abundant alkenes differed between hosts and parasites yielding higher chemical distances between parasite-host pairs than we expected (Chapter 7). Furthermore, whereas parasites of COLw wasps do not overlap in the chemical space with their hosts, they have started to produce methyl-branched dominated CHC profiles, possibly following the diversification of CHC profiles of their hosts (Wurdack *et al.*, 2017, Chapter 7). In fact, in some cases, cuckoo wasps produced exactly the same CHC compound as its hosts, which made their profile much more similar in the chemical space (Chapter 7).

With only one exception, cuckoo wasps resembled the cuticular profiles of their respective hosts. However, the degree to which chemical mimicry was attained varied among species, and might reflect both constraints imposed to evolve adaptations (or a time lag in evolving them), and the strength of selection imposed by the evolutionary arms race between host and parasite (Thompson, 1986). A long history of coevolutionary interactions between host and parasite may lead to better chemical mimicry, as that found between *H. rutilans* and its host *P. triangulum* (Strohm *et al.*, 2008), which has also evolved chemical insignificance (Kroiss *et al.*, 2009a). Surprisingly, one of the best cases of chemical mimicry was exemplified by the association *Hedychrum nobile* - *Cerceris arenaria*, in which the kleptoparasite has evolved to produce several of the same CHC compounds of its female host. Strohm and colleagues had previously suggested no mimicry of *H. nobile* on its host (Strohm *et al.*, 2008). However, although in their analyses the profiles of *H. nobile* and *C. arenaria* did not overlap in the chemical space, many CHC compounds were shared between the two species, and they were both the most similar among the species in their study. Unpublished evidence suggests that *H. nobile* are more closely resembling the profiles of their hosts than those of any other closely related species of *Cerceris* (Chapter 7). Moreover, in a large comparison of CHC profiles, the two above mentioned cuckoo wasps clustered

with their respective hosts in a large cladogram that included almost 250 species of Hymenoptera (Kather & Martin, 2015), confirming the high degree of chemical similarity between cuckoo wasps and their respective hosts despite being phylogenetically distant (Kather & Martin, 2015).

Hedychrum chalybaeum, was the only cuckoo wasp species whose cuticular profile was very different to that of its host. Differing from the other closely related species that parasitize COLw hosts, *H. chalybaeum* showed an alkene-dominated CHC profile. A number of non-exclusive reasons have been proposed for this lack of mimicry (Chapter 7). One possibility is that the association between host and parasite may be young, and we have not been able to observe its response yet (evolutionary lag hypothesis, Dawkins & Krebs, 1979). A more complex explanation may be that the adaptive rate of evolution is slower in smaller populations. Both *H. chalybaeum* and its host *C. interrupta* are relatively uncommon, and their population sizes may be comparatively smaller and patchier than those of other species in our study. In fact, both species are considered threatened and rarely encountered wasps (Reder & Burger, 2009; Schmid-Egger *et al.*, 2010). Theoretical and empirical studies are showing now that, contrary to past predictions, higher rates of positive selection are more apparent in larger populations than in smaller ones (Jensen & Bachtrog, 2011, Lanfear *et al.*,2014, Vahdati & Wagner, 2017). In any case, *C. interrupta* appears to be the species having started a possible diversification of their CHC profile later because it still shows 30% of their profiles composed of unsaturated compounds, and its parasite is maybe running behind in the arms race.

The hypotheses mentioned above predict that constraints may impede the evolution of chemical mimicry. However, there might be other cases in which chemical mimicry will not evolve because it does not represent a benefit for the parasite. For example, the cuticular profile of *Chrysis viridula* is very distinct to any of the chemotypes of its host, the eumeninae wasp *Odynerus spinipes* (Wurdack *et al.*, 2015). *C. viridula*, is however, an orthoparasite (sensu Malyshev, 1968), laying its eggs at the host stage at which their larvae will develop (*e.g.*, when the host larvae is about to form the cocoon, Martynova & Fateryga, 2015). Orthoparasites oviposit after the nest has been provisioned and closed, and their flight periods and those of their hosts rarely coincide (Martynova & Fateryga, 2015). Thus, these parasites would not be selected to evolve chemical mimicry. Furthermore, other wasps may not benefit from resembling their host (but maybe their prey), since they are usually ovipositing into the host's preys before the latter ones are caught and brought into the nests by their hosts (*e.g.*, many members of Elampini, see Chapter 4, *e.g.*, Veenendaal, 2012, Winterhagen, 2015, Paukkunen *et al.*, 2015). This idea needs to be studied, but unpublished evidence suggests that cuckoo wasps that are "Trojan horse" parasites (Strohm & Liebig, 2008) are also not chemically similar to their hosts (Bandorf *et al.*, 2017).

In general, *Hedychrum* cuckoo wasps may benefit from resembling the profiles of their hosts, and although the degree of mimicry varied, it was in no case perfect, not even in the well studied model system *H. rutilans* - *P. triangulum* (Strohm *et al.*, 2008). Mimicry, however, needs not to be perfect as long as it still provides an advantage to the mimic. In fact, theory and empirical examples, have often shown that imperfect mimicry commonly occurs (Kikuchi & Pfennig, 2013, Dalziell & Welbergen, 2016). Cases of perfect mimicry are rare, and are mainly occurring in parasites that need to be integrated into an eusocial host's colony, often ants (see reviews by Thomas *et al.*, 2005, Akino, 2008 and Nash & Boosma, 2008; these parasites include *e.g.*,

other species of ants, Guillem *et al.*, 2014, *Microdon* hoverflies, Howard *et al.*, 1990, *Phengaris (Maculinea)* butterflies, Akino *et al.*, 1999, rove beetles, von Beeren *et al.*, 2018), but also other eusocial hosts (*e.g.*, termites, Howard *et al.*, 1980, wasps, Cervo *et al.*, 2008). In these cases, parasites often acquire the cues from their hosts, but a combination of strategies, including chemical mimicry, may be operating, and their profiles are almost perfectly identical (*e.g.*, Akino *et al.*, 1999, Guillem *et al.*, 2014). That is because in social integration, the parasite is subject to recognition by all nestmates, during an extended period of time, which depending on the parasite's type, may include its whole life cycle. Moreover, selection in opposite directions may select against perfect mimicry (*e.g.*, Pekar *et al.*, 2011). For instance, female cuckoo wasps should be as close to their hosts' profile, so that their presence in the nest is not detected. But, they still might need to signal their specificity to their conspecific mates.

In addition, mimicry needs to be evaluated from the receiver's point of view. Mimicry does not need to be perfect as long as it deceives the expected receiver. So far, behavioural assays conducted with *Philanthus triangulum* showed less aggressive behavior towards chemical cues from its specialized parasite than towards chemical cues of another cuckoo wasp (Strohm *et al.*, 2008), despite the existence of important differences between the profiles of both species. For example, *H. rutilans* females possess several low-amount species-specific methyl-branched compounds that their hosts lack, and larger amounts of (Z)-7 alkenes than their hosts. It is possible that some of these low-amount CHC compounds get confounded with other odors in the nest environment. In the future, it would be interesting to test how CHC profiles of the host interfere with those of their prey and if cuckoo wasps benefit from this. This may also explain why host switches are usually associated with the type of prey of the host species (*e.g.*, *Trichrysis cyanea* parasitizes several species that hunt spiders, or species parasitizing hosts with a similar food prey item are usually sister species in the phylogeny of cuckoo wasps, Chapter 3).

10.1.2. Counter-adaptations in the host

A number of counter-adaptations were observed in hosts of cuckoo wasps, including diversification of cuticular profiles with methyl-branched compounds and a shift in chain length in COLw hosts, and an increase in intraspecific variability of profiles in HYMw hosts (Chapter 7). These changes in the CHC profile may allow hosts to detect the presence of their parasites in the nests. All COLw hosts which do not require prey embalming, produce more methyl-branched compounds (Wurdack *et al.*, 2017) and have CHC compounds of higher molecular weight (*i.e.*, profiles with longer mean chain lengths, Chapter 7). Diversifying CHC profile with very specific combinations of methyl-branched compounds may permit the detection of foreign and enemy chemical cues in the nest of hosts more readily. For example, populations/species that are more heavily parasitized tend to diversify their phenotype both in insects (by diversifying their CHC profile via inclusion of methyl-branched alkanes, *e.g.*, Martin *et al.*, 2011, Lorenzi *et al.*, 2014) and in avian brood parasites (by evolving larger phenotypic variation in eggs' apperance, Oien *et al.*, 1995, Spottiswoode & Stevens, 2012, Medina *et al.*, 2016). Moreover, long-chain compounds are less volatile than short-chain compounds, thus, by evolving CHC compounds of rather higher molecular weight, hosts may be able to more easily detect short-chain compounds (cues left by their

parasites). Furthermore, adding one carbon to the chain, increases the number of internal positions at which methyl groups may be added, which in turn, increases the number of possible methyl-branched compounds that theoretically exist (especially for polymethyl-branched compounds, Chapter 9). For example, if two species would evolve randomly the same number of methyl-branched compounds within a similar range of carbons, chances are that less compounds are shared at longer chain lengths. In this sense, increasing chain length can also contribute to the evolution of very species-specific profiles because it is less probable that two species evolve the same combination of methyl-branched compounds by chance. In fact, dimethyl-branched alkanes are highly diverse in ants (Martin & Drifhout, 2009a). In particular, those ranging between C27-C35, are highly diverse, species-specific and considered putative species signals in a monophyletic clade of *Formica* species (Martin *et al.*, 2008a). On the other hand, methyl-branched enriched profiles may have resulted from selection against producing unsaturated compounds, because they may be more easily detected as kairomones by cuckoo wasps. For example, it has been demonstrated that females of *H. rutilans* use not only visual but also chemical cues to locate and identify its host (Kroiss *et al.*, 2008). Nests of *P. triangulum* are covered with cues from their CHC profiles, with at least 34 compounds identified, but unsaturated compounds constitute a great proportion of them, and the two known chemotypes of *P. triangulum* females can be also distinguished in the sand of their nests (Kroiss *et al.*, 2008). I have proposed three ways (*i.e.*, diversification by methyl-branched compounds, shift in chain length and the avoidance of more fluid, and potentially more readily detectable unsaturated CHC cues) by which the changes observed in CHC profiles of COLw species may have contributed to escape parasitism by cuckoo wasps. These remain, however, testable hypotheses to be distinguished with behavioural experiments.

Intraspecific variability of cuticular profiles of females of HYMw and COLw showed very different patterns. CHC profile variability among female individuals of COLw host species was similar or even lower than those of their conspecific males and much lower than those of female HYMw. Whereas a low variability of CHC profile in COLw females may enhance recognition of the presence of a female parasite in their nest, females of HYMw species showed rather variable profiles, which were reflected in a much larger overlap of their CHC profiles in the chemical space (Figure 7.5). This could be a possible adaptation of HYMw to cope with high parasite pressure. For example in highly parasitized populations of the social paper wasp *Polistes biglumis*, intraspecific variation of CHC profiles was larger than in lightly- or non-parasitized populations, and it has been suggested that negative frequency-dependent selection could be causing it (Lorenzi *et al.*, 2014). Similarly, an increased variation in egg appearance (among clutch variation, increase in polymorphism) has been suggested as a possible alternative to developing better discrimination abilities in order to counterrest parasitism in hosts of the African cuckoo finch (Spottiswoode & Stevens, 2011). Given that HYMw hosts can neither diversify their profile through the production of methyl-branched compounds (due to the constraint imposed by prey embalming behaviour, Wurdack *et al.*, 2017), nor increase chain length (possibly associated with shorter-chain compounds allowing a better fluidity of the CHC profile, Herzner & Strohm, 2008; Menzel *et al.*, 2017a), these wasps rather make use of negative frequency-dependent selection in which rare chemotypes may have an advantage. In fact, females of *P. triangulum* belonging to the same population exhibit polymorphic profiles differentiated by the relative amount of pentacosene or heptacosene (Kroiss *et*

al., 2008, 2009a). Females of *H. rutilans* are more similar to the chemotype with high amounts of heptacosene (Strohm *et al.*, 2008). Thus, by evolving profiles varying in the ratio of their compounds, some host individuals may be able to escape parasitism.

10.1.3. Females evolve changes (first) in both parasites and hosts

All studied adaptations occurred to a greater extent or exclusively in females. Methyl-branched compounds were more predominant in the CHC profiles of COLw females than in conspecific males. Mean chain length was also longer in females than in males of COLw. Intraspecific variability was only larger in females of HYMw hosts. Moreover, the diversification of CHC profile via methyl-branched compounds in cuckoo wasps parasitizing COLw hosts only happened in females (Chapter 7). The fact that changes were stronger in females suggests that these changes are directly evolving because of selection imposed by the antagonistic interaction between female hosts and their female parasites. Males of these antagonistic solitary species rarely interact, and other selective forces may be affecting the evolution of their CHC profiles (Chapter 6).

While intersexual differences in the hosts arose from quantitative variation of most common CHC compounds, parasites differed strikingly in the CHC profiles, with females and males producing several different compounds. Moreover, since the CHC profile of females resembled those of their female hosts, cladograms constructed using CHC profiles of males better reflected the history of evolution of cuckoo wasps (see Chapter 5).

Since the comparison of CHC profiles of *Hedychrum* and its hosts is mainly correlational, caution should be taken into account because correlation between matching traits in hosts and their parasites does not necesarily imply coevolution (*i.e.*, changes in the profiles arising due to reciprocal selection, Janzen, 1980, Nuismer *et al.*, 2010). The same traits can have arisen in hosts and parasites due to shared history (*e.g.*, evolving adaptations to changes in the environment), or because a matching trait already existed in the parasite before it started parasitizing the host (Janzen, 1980). Nevertheless, in comparison to other morphological traits, the study of CHC profiles of *Hedychrum*, and their hosts provide interesting insights, despite being correlational. First, CHC profiles are composed of not only one but several compounds, many of which are subject to chemical mimicry. In this sense, the matching observed between host and parasite is a matching of several genetically determined CHC compounds. Whereas some of these CHC compounds are correlated because they are produced partly by the same enzymatic pathway (Martin & Drijfhout, 2009), in many cases host and parasite show exactly the same CHC compounds. Second, if shared selection (adaptation to the environment or to other selective process operating on both interacting species) would have shaped CHC profiles of hosts and their parasites, CHC profiles of males would also have evolved these changes. Third, in all cases, adaptations and counteradaptations seem to operate first and mainly in females, the only sex directly being affected by brood parasitism. Last, but not least, this study includes few species pairs, and in all, but one, the observed adaptations and counteradaptations point to changes in CHC profile being driven by coevolution. Nonetheless, it is always recommended to complement a comparative analysis with experimental tests (Weber & Agrawal, 2012). In the future, it would be interesting to study different populations of the commonly studied species of *Hedychrum* and its hosts across a

geographic range, and compare how CHC profiles would change between heavily parasitized and unparasitized populations. For example, parasitized populations of *C. arenaria* and *P. triangulum* are expected to have respectively higher proportions of matching traits (e.g, shared methyl-branched compounds, in the first), whereas populations where parasites are inexistent may show adaptations towards less proportion of methyl-branched compounds (*e.g.*, compounds may exist, but they represent smaller abundances). Similarly, differences between females and males should be stronger in heavily parasitized populations.

10.2. Sexual dimorphism in cuckoo wasps largely influenced by coevolution with their hosts

Sexual dimorphism originates by differences in the strength of selection operating on each sex. Sexual selection can cause extreme modification of a male trait and it has been often credited to generate sexual dimorphism (Andersson, 1994; Allen *et al.*, 2011). However, when selection acting on females is stronger than that acting on males, sexual dimorphism may arise by changes in the female sex (*e.g.*, Kunte, 2008, Krüger *et al.*, 2007, Cooper *et al.*, 2016a). In species of the genus *Hedychrum*, host-parasite interactions can lead to strikingly sexually dimorphic CHC profiles. Could then chemical dimorphism originate as a result of changes on females' CHC profiles driven by brood parasitism? Examples of extreme differences between the sexes are particularly numerous in Hymenoptera and have been observed in many traits including CHC (*e.g.*, size, extreme shapes, coloration, songs, chemical signals, Stubblefield and Seger, 1994). Nevertheless, although a past review on dimorphism of cuticular hydrocarbons in insects, indicated that it is common in insects, and particularly frequent in Hymenoptera (Thomas & Simmons, 2008b), not many studies had compared differences in CHC profiles between sexes across a large number of related species (>12 spp, Chapter 4). While chemical dimorphism was strong in *Hedychrum*, only five species were compared, and it remained to be seen how general this finding was across cuckoo wasps. Chapter 4 shows that dimorphism of CHC profiles in cuckoo wasps is very common and strong, usually caused by the production of very different CHC compounds in both sexes. An overall pattern was observed in which unsaturated compounds occurred more often in females, and methyl-branched compounds (especially dimethyl-branched compounds) in males (Chapter 4). Moreover, females' CHC profiles are evolving at a faster pace and are more divergent between closely related species than those of males (Chapter 6). I suggest that the parasitic lifestyle imposes strong selection on females to overtake their host's recognition abilities and remain undetected after having oviposited inside their host's nests through a plausible evolution of chemical mimicry of their hosts (Chapter 6). In the future, a comparison of the CHC profiles of cuckoo wasps with those of their hosts should be necessary to corroborate whether chemical mimicry is a driver of sexual dimorphism in this group. Chemical mimicry, is particularly expected in species that parasitize open nests (or briefly and slightly temporarily closed), for which provisions by the host are still being brought in (inquilines, sensu Malyshev, 1968). However, species that are considered orthoparasites (*e.g.*, *Chrysis viridula*) which are able to open sealed nests, to oviposit, long after the host larvae has developed into a pupa/prepupa, should not profit from chemical mimicry from their hosts (*e.g.*, Wurdack *et al.*, 2015). Nevertheless, chemi-

cal sexual dimorphism among these species may still occur, if selection pressures on females and males differ.

A greater diversity of methyl-branched compounds was apparent in males, and might serve as species-specific recognition signals. There was also a consistent pattern on the distribution of the double bond position of alkenes between sexes. Alkenes with more internal double bond positions (> 11) abound in males, whereas females contain more often alkenes with double bonds at position 9 (Chapter 4). In many cases, some isomeric forms of these alkenes contributed the most to differences between the sexes, suggesting a potential role as intraspecific signals. In concordance with this suggestion, previous studies have shown that especially (Z)-9 alkenes (among others) are often important pheromonal components in many insects (ants, Martin *et al.*, 2008; *Drosophila*, Carlson *et al.*, 1971; beetles, Ginzel *et al.*, 2003, Ginzel *et al.*, 2006, Chapter 4). Nevertheless, behavioural assays and electroantennograms need to be conducted in the future to test whether the suggested components do elicit a response in antennal receptors of cuckoo wasps. Nonetheless, despite biotic interactions can drive the evolution of strong differences in the profiles of females of cuckoo wasps, sexual selection may affect the profile of males, albeit having a comparatively lesser influence on dimorphism than coevolution with the hosts does on the CHC profiles of their conspecific females (Chapter 6).

10.3. CHC are species-specific

In spite of adaptations evolved by female parasites to resemble the cuticular profiles of their female hosts, CHC profiles of both males and females cuckoo wasps were unique (with few exceptions, Chapter 5). CHC mediate intraspecific recognition processes in many solitary and social species (Singer, 1998). While a number of volatile compounds (*e.g.*, acetates, esters, etc.) are used as long-distance pheromones (Ando *et al.*, 2004; Keeling *et al.*, 2004), intraspecific recognition mediated by CHC compounds requires physical contact or interactions at short range (Singer, 1998). This may especially apply for many species-specific methyl-branched compounds that were occurring in relatively low amounts. CHC compounds that are under selection for chemical mimicry to avoid recognition by the hosts might include relatively abundant compounds, which may passively be transferred into the host's nest. On the other hand, it is possible that scarce CHC compounds may not leave a strong cue in the nest because their small quantity gets diluted with other odors present in the nest. For example, a number of species-specific low-amount methyl-branched compounds were present in female parasites of *Hedychrum rutilans* which are lacking in its female host, but seem to have no detrimental effect on mimicry (Strohm *et al.*, 2008). These CHC compounds may evolve under other selective forces (sexual selection being one of them).

CHC have been suggested to be magic traits or dual traits, because they can potentially be affected by selection to adapt to desiccation resistance and mating. As such, changes evolved by ecological divergent selection (*e.g.*, to evolve desiccation resistance), may have also led to reproductive isolation in two separated populations of the same species (Chung & Carroll, 2015). For example, methyl-branched compounds are both affecting dessication resistance and mate choice in *Drosophila serrata*, and the loss of expression of these compounds in ancestral populations adapting to humid rain-

forests, possibly contributed to reproductive isolation between the two sibling species *D. serrata* and *D. birchii* rendering methyl-branched compounds in the first species a possible dual trait (Chung *et al.*, 2014). Traits simultaneously involved in ecological adaptation and in non-random mating may not be as rare as previously thought, although they are very difficult to demonstrate (Maan & Seehausen, 2011). Moreover, divergent selection affecting sensory systems may contribute to speciation even without geographic isolation (*e.g.*, Seehausen *et al.*, 2008). Differences in ambient light can affect perception of conspicuous coloration in cichlid fish, which in turn, is preferred by females. Adaptation to different light regimes at different depths included changes to long wavelength sensitivity in visual pigments and associated changes in preferences for sexual coloration, probably linked to perceptual biases, thus contributing to reproductive isolation of the two sibling co-occurring species *Pundamilia nyererei* and *P. pundamilia* (Seehausen *et al.*, 2008). The use of chemosensory systems and chemical signals is widespread in animals and these traits are not only involved in processes of mate choice, but also used in other contexts (searching for habitats, searching for hosts, Smadja & Butlin, 2009). Thus, the potential for these traits to be affected by divergent selection and subsequently contribute to speciation is high (Smadja & Butlin, 2009). In fact, CHC compounds are good examples of such traits (*e.g.*, *Drosophila melanogaster* races, Smadja & Butlin, 2009). More interestingly, ecological divergence of the CHC profile may not only be driven by adaptation to environmental conditions but by biotic interactions. Many studies are now showing that diet is one of the most significant environmental factors shaping chemical signals in animals (see Table 1 in Henneken *et al.*, 2017). Insects reared on different hosts (either herbivorous insects on plants, or predatory insects on other insects) can vary quantitative and qualitatively in their CHC composition (*e.g.*, Espelie & Bernays, 1989; Liang & Silverman, 2000). If preferences to mate with insects having been reared on the same plant/host arise, premating isolation between these two groups may occur. Several examples indicate that this is possible (*e.g.*, Stennet & Etges, 1997; Rundle *et al.*, 2005; Kühbandner *et al.*, 2012; Geiselhardt *et al.*, 2012; Xue *et al.*, 2016). Rundle and collaborators showed that *Drosophila serrata* modified its cuticular hydrocarbons in response to being reared on different environments (diets), and that this also affected mating preferences (Rundle *et al.*, 2005). More interestingly, these changes may not only happen after several generations of selection, but also without prior environmental adaptation. The mustard leaf beetle *Phaedon cochleariae* can modify its CHC profile only after two weeks of being fed a different plant (Geiselhardt *et al.*, 2012). At the same time, males show host-plant specific assortative mating (Geiselhardt *et al.*, 2012). Although it is a more complex scenario to imagine, it may be possible that changes in CHC profile of parasites (*e.g.*, in order to chemically mimic the host), could generate changes in the mating preferences (maybe due to pleiotropic effects). In some of the previously mentioned examples (*e.g.*, *Drosophila serrata* and *D. birchii*), changes in CHC profiles seem to have originated by single genes having a large effect (*e.g.*, Chung *et al.*, 2014; Smadja & Butlin, 2009), which would be compatible with a model of evolution by saltational shifts, as suggested for compounds required to be highly species specific (Symonds & Elgar, 2008).

In any case, differences in CHC profiles may evolve fast. Differences in CHC profiles among closely related species may be more evident than those by morphology and/or genes, because of the role of CHC in intraspecific recognition processes. Therefore, the high degree of species specificity in cuticular profiles of cuckoo wasps encourages their

use as a complementary approach in taxonomy (Chapter 5), especially since a number of computational tools are becoming available and allow a more rapid processing of chemical profiles (Chapters 8 and 9).

Whereas I have analyzed CHC profiles in this study, it is not yet known how other cuticular compounds (*e.g.*, esters, alcohols, etc., that do occur in the cuticle of Chrysididae) may vary between females and males and could, for instance, be involved in intraspecific recognition especially for chemically monomorphic species (*e.g.*, several *Hedychridium* species, Chapter 4). It would be interesting to understand how the different sexes recognize each other in the cases where very subtle or absent chemical dimorphism was found. Do males use other signals to recognize females or are they able to distinguish them based on the quantitative variation of their shared compounds? In other solitary hymenopteran parasitoids, the degree of CHC reliability to mediate species recognition can vary across closely related species (Weiss *et al.*, 2015; Buellesbach *et al.*, 2018). For example, Weiss and colleagues have shown that three species of *Leptopilina* differ in the degree to which CHC contribute to mate recognition (in one species, it suffices for a complete discrimination whereas in other iridoids, different chemical compounds, play a more important role, Weiss *et al.*, 2015).

10.4. Hosts explain evolutionary history of cuckoo wasps

Coevolutionary interactions can rapidly drive the evolution of phenotypic diversity. The phylogenetic inferences of Chapter 3 have challenged previous relationships of cuckoo wasps based on cladistic analyses of morphological characters (Kimsey & Bohart, 1991), and revealed that host associations may be more important in explaining the evolutionary history of cuckoo wasps. Species parasitizing similar hosts (either hosts that are phylogenetically related or hosts phylogenetically distinct but whose larvae are fed the same type of food) were often occurring close in the phylogeny, despite having been geographically separated for a long time (*e.g.*, *Chrysurissa* and *Spintharina*, Chapter 3). The phylogenetic analysis also showed that all cuckoo wasps that are parasitoids of bee-collecting hosts grouped into a single monophyletic clade. CHC profiles of some of the species belonging to bee-parasitizing groups (*Chrysura, Chrysis comparata* species group), could be well defined and characterized by a number of clade-specific CHC compounds (Chapter 5). In addition, the comparison of CHC profiles showed that species of *Chrysura* tended to have a different overall pattern of evolution than other cuckoo wasps, showing a large diversity of species-specific CHC compounds (Chapter 4). CHC similarity and CHC diversity across these species may then reflect similar selection pressures (associated with parasitizing pollen-feeding species).

CHC profiles of males contained more phylogenetic signal than those of females, nevertheless, close phylogenetic relationships only rarely matched cladograms built using CHC profiles of males (Chapter 5). While I have argued that selection for chemically deceiving their female hosts has shaped CHC profiles of female cuckoo wasps, it would be too simplifying to expect that female CHC profile similarity always indicates similarities in their hosts. Whereas, some of the CHC compounds of the profiles may indeed be selected by convergence on chemical mimicry for parasitizing closely related hosts, there are other CHC compounds that respond to a number of locally

adapted and species-specific selective processes that have nothing to do with host's type. Therefore, the selection of CHC compounds defining clades of species by CHC, neither grouped females by phylogeny nor by host's type (Chapter 5). Nevertheless, these analyses set the basis for further explorations on what selection pressures drive the evolution of CHC profiles by suggesting putative functions for some CHC compounds (Chapter 4). Moreover, none of the analyses on this thesis would have been possible without having a reliable phylogeny. Despite many relationships among genera were not well resolved, and additional phylogenomic approaches would need to be undertaken in the future; the current most complete phylogenetic hypothesis on cuckoo wasps (Pauli *et al.*, accepted, Chapter 3), has already allowed to confirm the importance of hosts in determining patterns of diversification of cuckoo wasps.

10.5. What function do compound classes play in communication?

One of the remaining challenges in chemical ecology is to understand how the different compound classes are perceived by insects and what role they play. It is clear from many studies, that linear alkanes play a less important role in communication (due to their commonness, their relative lack of defining features conferring advantages for communication and their lack of response to aggression bioassays in a number of insects, Dani *et al.*, 2001; Dani *et al.*, 2005; Châline *et al.*, 2005; Jongepier & Foitzik, 2016). In cuckoo wasps, the analyses of the pattern of evolution of CHC compounds belonging to the three main compound classes also indicated that alkanes may play little role in communication, especially in males (Chapter 6). Alkanes in males evolve under a Brownian Motion model of evolution (suggesting evolution under neutral processes, Chapter 6). On the contrary, alkanes in females indicate some selection acting on them in the recent history. Whereas the evolution of linear alkanes is more or less understood by not being commonly subject to selection for communication, unsaturated compounds and methyl-branched compounds are being selected but in complex manners. Comparisons of CHC profiles of different related species (which are unfortunately rare for more than 20 closely related species, but see Pokorny *et al.*, 2015), have shown that putative sex pheromones can both be unsaturated compounds (alkenes, alkadienes) or methyl-branched compounds and that there is a large variation across species (Chapter 4). Nevertheless, the finding that a different double bond position seemed to be prominent in the different sexes, may give some hint to their roles. In fact, (Z)-9 alkenes are often associated with female sex pheromones in a large array of insects (see Chapter 4), either reflecting an ancestral trait or some kind of overarching benefit in signalling "female presence/receptivity" efficiently. Similarly, the finding that alkenes with internal double bonds occurred frequently and reflecting phylogenetic signal in males, seems not random. Several questions need to be investigated: Are alkenes preferred over methyl-branched compounds for signaling attractiveness? Are (poly)methyl-branched compounds more often selected as species-specific cues? What are the differences in biosynthesis costs between compound classes that could eventually help to speculate on which selective forces may be affecting their evolution? Whereas it has been suggested that methyl-branched compounds may be both more costly to produce (their synthesis requires the availability of essential aminoacids such as valine, leucine and methionine, that need to be obtained from (a proteinaceous)

diet, Blomquist, 2010; Kather & Martin, 2015) and maintain (methyl-branched hydrocarbons increase insect vulnerability to desiccation because of their lower melting point so that they could be acting as honest signals, Le Conte & Hefetz, 2008), no conclusive evidence exists for this hypothesis so far.

Therefore, a future avenue of research should include understanding which signals may be more biochemically costly to produce. Whereas with visual signals it is relatively easy to understand what becomes the exaggeration of a trait (*e.g.*, the brighter the coloration pattern, the longer the tail feather, etc.), it remains more difficult to understand which or which type of signals take the role of this exaggeration in CHC. Here, both alkenes with internal bond positions and polymethyl-branched compounds could be involved in this. However, more insight would be gained if one compound class is more costly to be produced, and hence be condition-dependent (*e.g.*, reliable signals of high-quality individuals). Although it has not been often shown, Hymenoptera females mate often only once, and invest comparatively much energy on rearing their offspring in comparison to males, thus they are expected to be choosy when it comes to selecting a male (Stubblefield and Seger, 1994), capable of exerting strong sexual selection.

Studying differences in intraspecific variation of (signaling) traits may provide useful insights into what evolutionary forces may be causing the observed variation in a trait. A consistent difference between intraspecific variability of CHC profiles of males and females was found and I have provided several reasons for this (see Chapter 5). However, much more interesting would be to evaluate the degree by which different CHC compound classes and other commonly occurring CHC compounds across species of cuckoo wasps varied intraspecifically. Visual signals have been studied much longer than chemical signals and a number of predictions based on three properties of variability of coloration patterns of birds have been proposed (Dale, 2006). These may allow researchers to study variability of signals in order to infer what type of selection is acting on it and what kind of information it could be signaling (Table 2 in Dale, 2006). For example, intraspecific variability of plumage colours of birds is larger in sexually selected traits (Delhey & Peters, 2008) and this variability correlates with the level of conspicuousness of these traits (Delhey *et al.*, 2017). In coloration patterns, hypotheses on what role the signal may be playing can more easily be inferred than in CHC compounds, where we still lack much knowledge on how insects perceive the different compound classes. Therefore, it could be beneficial to extend these ideas from coloration patterns in birds to CHC compounds of insects. For example, variability in CHC compounds can help to understand differences in the use of these compounds by insects. Martin and Drifjhout (Martin & Drifjhout, 2009b) compared CHC profiles of foragers and non-foragers of the ant *Formica exsecta*. They found that alkanes were much abundant in foragers and were environmentally influenced depending on the task the ant worker performs. The proportion of alkanes in the CHC profile was significantly different between both groups of ant workers, whereas alkenes variability (in relative amounts) was more stable as alkenes have been implicated as nestmate recognition cues in this species (Martin & Drifjhout, 2009b). By comparing variability of CHC compound classes and CHC compounds across cues, insights may be gained as to what type of function the trait may have. For instance, methyl-branched compoundswere much less intraspecifically variable than other compound classes (Chapter 4). Evaluating these patterns of variability in a much detailed manner would maybe allow us to infer more insights into their role.

10.6. Diversity and evolution of CHC profiles

This dissertation has shown that CHC profiles of cuckoo wasps are very diverse. This diversity is evident not only among species but also within species, with males and females showing often high levels of sexual dimorphism in their profiles. Several mechanisms and selective forces may interact to originate and maintain this diversity. Being obligate brood parasites of other (mainly hymenopteran) hosts has most probably played a dominant role in selecting for rapid changes in the CHC profiles of female cuckoo wasps, the sex directly engaging in an evolutionary arms race with the female hosts. Nevertheless, CHC profiles can also vary due to a number of other reasons and probably more insights into the most important selective forces could be gained by conducting behavioral experiments using few of the most commonly occurring species. The antagonistic interaction between parasites and hosts may result in a number of trade-offs (*e.g.*, changing to produce more of one compound class, may result in a correlated shift of the chain length, Chapter 7) and differences in the strength of interaction and dependence on a single/several hosts may result in a variety of outcomes across species. Thus, disentangling the exact mechanisms that have caused the observed CHC profile variation requires additional studies. In all cases, a combination of studying on one hand many species to gain insights into the general patterns, and on the other hand, studying few cases for which their biology is better known, provides a good approach to better understand how CHC profiles may evolve. The work presented here allows for the future testing of for example the putative signaling function of some CHC compounds in commonly occurring species (Chapter 4). In addition, the two methodological tools introduced here may facilitate the analyses of CHC profiles (Chapter 8 and 9), hopefully reducing the amount of time required for their analyses and thus enhancing their application in a large number of studies.

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A. Appendix

Due to space contraints, the Appendix information is provided in a CD. It contains one folder for each chapter. The files contained in each chapter are briefly described in the following sections.

A.1. Chapter 2: Cuckoo wasps

- Table 1- Species: This table contains taxonomic information, classification, host species and the number of specimens used for each species (59 Chrysididae and 7 Crabronidae) for which CHC chromatograms were analyzed in this thesis.

- Table 2 - Specimens: This document contains three spreadsheet, each containing information for each specimen for which chromatograms were analyzed (species, gender, locality of collection).

A.2. Chapter 3: Phylogeny

- Figure S1. Gene models of all ten investigated nuclear protein-coding genes. Gene identifies refer to those given by Hartig et al. (2012) and the gene name in parenthesis designates the most similar homolog in *Drosophila melanogaster* Meigen 1830. Depicted are coding exons (blue bars) and introns (black bars) of each gene. Exon and intron boundaries were annotated by aligning transcripts of *Chrysis terminata* to the genomic nucleotide sequence of the corresponding gene of this species. Numbers specify the length (in nucleotides) of all sequenced exonic sections of a given gene (numbers in parentheses include also the length of the introns), using the *Chrysis terminata* nucleotide sequence as reference. An asterisk above an intron indicates that the intron was absent in some of the investigated species (indicated in Fig. 1).

- Figure S2. Phylogenetic relationships between and within major cuckoo wasp lineages (continued in Fig. S2) inferred in a Bayesian framework with the software MrBayes and applying the same supermatrix and the same substitution models as in the analysis with the software IQ-TREE (see Figs 1 and 2). Shown is the 50 % majority rule consensus tree. Posterior probability values were inferred from 56,000 sampled trees and are indicated in the tree by colour codes (percent values were rounded to the first digit before the decimal point). *Cephalonomia tarsalis* (Bethylidae) and *Anteon* sp. (Dryinidae) served as outgroups for rooting of the tree.

- Figure S3. Continuation of Fig. S2.

- Table S1. Sample information (taxonomic information, locality of collection for all species used in the phylogenetic analyses).
- Table S2. Oligonucleotide primers used for the amplification of ten nuclear genes and COI in cuckoo wasp and selected outgroup species.
- Table S3. List of Genbank accession numbers.
- Table S4: GC content by gene in percent (%).
- File S1. Supermatrix with the multiple nucleotide sequence alignment (4,946 sites, 189 sequences referring to a total of 188 species) of the concatenated nucleotide sequences of ten nuclear-encoded genes and of one mitochondrial gene, all protein-coding.
- File S2. Table indicating gene boundaries in the supermatrix given in File S1.
- File S3. Supermatrix partition table in Nexus format.
- File S4. Compilation of host associations.
- File S5. Phylogenetic tree (phylogram) with bootstrap support values as depicted in Figs 1 and 2 in Newick format.
- File S6. Phylogenetic tree (phylogram) with posterior probability values as depicted in Figs S2 and S3 in Nexus format.

A.3. Chapter 4: Sexual dimorphism of CHC

- Sample information in Appendix of Chapter 2.
- Table CHC-Females: mean CHC composition for females of each species used in this thesis. CHC compounds are labeled by their class, retention index and their identification. Alkadienes remain unidentified, only separated by retention index. Values are relative amounts.
- Table CHC-Males: A table containing mean CHC composition for males of each species used in this thesis. Same as above.
- Table CHC-sex differences: 67 CHC compounds contribute to 75% of sex differences. These are indicated here. Order refers to the order of importance the CHC compound has in each species and ranges from 1 (the compound contributing to the most differences between sexes) to 16.
- Table Anosim Values: Result of the ANOSIM analysis for each species. P values have been also corrected by Bonferroni. N refers to the sample size.
- File Plot Anosim: graphs showing the results of ANOSIM analysis.
- Figure Species-specific: The first three compounds contributing to major differences between sexes in all species.

A.4. Chapter 5: Species specificity of CHC

- Sample information in Appendix of Chapter 2 and CHC profile composition in Appendix of Chapter 4.
- Table HedychrumLocalityCHCProfiles: Mean CHC profile composition for each species in each locality for the five species of *Hedychrum* used.
- Table Localities: information on the different localities used in the analysis by geographic ranges.
- Table IndVal Analysis. Results of the Indval Analysis conducted for the two sexes.
- Figures NDMS3dFem and NMDS3dMal. Tridimensional NMDS using all species in females and males respectively.
- Figures AnosimF and AnosimM: results of the Anosim analysis in females and males respectively.
- Figures ClusterAnalysis: results of different cluster analysis using different clustering methods, and different grouping options (see Methods of the chapter and explanation in the corresponding file).
- Figure Intraspecific-Variation: Bray-Curtis dissimilarities between specimens within species in females and males.
- Figures BoxplotsRelativeVariation: Boxplots showing intraspecific variation of CHC compounds separated by compound class in females and males.

A.5. Chapter 6: Effect of natural selection on CHC evolution

- Sample and species information in Appendix of Chapter 2.
- Table Phylogenetic Signal: Results of the Phylogenetic signal calculated for each CHC compound and for different metrics.
- Figure ChemicalDistances: Pairwise comparisons between all specimens of all species in females and in males. This includes more than 180000 comparisons.

A.6. Chapter 7: Evolutionary arms race between Hedychrum and their hosts

- Hosts parasite relationships in File S4 of Chapter 3.
- Sample information in Appendix of Chapter 2.
- Table Provenance: Number of samples, provenance and year of collection for females and males of the species in this study.

- Table Primers: Characteristics of the primers used and number of nucleotides in the exonic regions of the nuclear genes used for the phylogenetic analyses.
- Table SubModels: Substitution models chosen by Modelfinder for the respective phylogenetic analysis in IQTree and MrBayes (in File Suppl)
- Table CHCFemandMales: Mean relative abundance ± standard deviation for each peak or mixture of CHC compounds included in the NMDS of all species analyzed (females and males respectively).
- Table Methyl-BranchedCHC: Mean number of CHC and proportion of methyl-branched compounds, alkenes and alkanes in the different analyzed groups.
- File Supplementary. Intra- and interspecific variability of cuticular hydrocarbon profiles in female and male individuals of all host species using only five specimens in each species for the calculation.

A.7. Chapter 8: A protocol for analyzing CHC profiles

- File Visualization. Graphic visualization of the same identifications in each chromatograms.

A.8. Chapter 9: Tool for the rapid identification of methyl-branched compounds

- R Script. File containing the two functions explained in this chapter. List of ions that need to be provided.

B. Publication List

Chapter 3:

Pauli, Thomas; Castillo-Cajas, Ruth F.; Rosa, Paolo; Kukowka, Sandra; Berg, Alexander; van den Berghe, Eric; Fornoff, Felix; Hopfenmüller, Sebastian; Niehuis, Manfred; Peters, Ralph S.; Staab, Michael; Strumia, Franco; Tischendorf, Stefan; Schmitt, Thomas & Niehuis, Oliver. "Phylogenetic analysis of cuckoo wasps (Hymenoptera: Chrysididae) reveals a partially artificial classification at the genus level and a species-rich clade of bee parasitoids". Accepted for publication in *Systematic Entomology*.

* The two first authors (underlined above) contributed equally to this work.

Author contribution:

The study was conceived by ON, RFCC, TP, and TS. Samples were collected in the field by AB, EvdB, FF, FS, MN, MS, ON, PR, RFCC, RSP, SH, ST, and TS. The laboratory work was performed by SK. All phylogenetic analyses were conducted by ON, RFCC, and TP. Novel information on host associations were provided by FF, MS, and SH. The host association list was compiled by AB, ON, PR, ST, and SH, with PR taking the lead. The manuscript was drafted by ON, RFCC, and TP. All authors read, commented on, and approved the final manuscript.

Participated in	Authors Initials (Decreasing responsibility from left to right)				
Study design	ON	TP	RFCC	TS	
Data collection	ON	TS	MN	PR	AB, EvdB, FF, FS, MS, RSP, RFCC, SH, ST
Host association compilation	PR	ON	TS	FF	AB, ST, SH,MS
Lab work	SK				
Data analysis	TP	RFCC	ON		
Writing of the first manuscript	TP	RFCC	ON		
Editing the manuscript	TP	ON	RFCC	TS	

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