Co-operation and conflict in societies of the ponerine ant genus *Pachycondyla*

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Contents

1. Introduction

> "Kin selection is the key to understanding the evolution of co-operation in insect societies. However, kin selection also predicts potential kin conflicts, and understanding how these conflicts are resolved is a major goal of current research on social insects" (Keller & Chapuisat 1999).

The evolution of co-operation in eusocial insects has for decades been a central topic in evolutionary biology. The key trait of eusocial ("truly social") species is that most of the colony members (workers) give up their own chances of reproduction and help raise the offspring of nestmates which are highly fecund (sexuals or reproductives). Eusociality according to Wilson (1971) is defined by: 1) the partition of reproduction among the colony members, with sterile or subfertile workers and highly fecund sexuals or reproductives, 2) overlapping adult generations, and 3) co-operative brood care. All ants and termites, some bees and wasps, ambrosia beetles (Kent & Simpson 1992), aphids (Aoki 1987; Benton & Foster 1992), thrips (Crespi 1992), shrimps (Duffy 1996), and some naked mole-rats (Sherman *et al*. 1991; Jarvis *et al*. 1994) have been found to fit this definition.

The existence of workers, acting altruistically in refraining from reproduction („reproductive altruism", Trivers 1985) contradicted Darwin's theory of evolution through natural selection (Darwin 1859). Darwin himself and several other authors solved this paradox, by suggesting that altruism could evolve via benefits to relatives (Darwin 1859; Fisher 1930; Haldane 1932). However, Hamilton (1963; 1964a, b) was the first who developed kin selection as a far-reaching important evolutionary principle. His "kin selection theory" states, that individuals can transmit copies of their genes not only

directly through their own reproduction, but also indirectly, by favouring the reproduction of kin. Altruistic behaviour should be favoured if the ratio of the costs (c) accruing to the donor of the altruistic act to the benefits (b) gained by the beneficiary is lower than the relatedness (r) of the recipient to the donor of the altruistic behaviour (Hamilton's rule, $c/b < r$). Here, benefit means enhanced production or survival of offspring by the beneficiary, cost means the number of offspring lost by the altruist.

Kin selection theory also implies the occurrence of potential kin conflicts, because in contrast to the cells of an organism, nestmates are not genetically identical (Ratnieks & Reeve 1992; Keller & Reeve 1999). Hence, kin selection predicts a dynamic equilibrium between co-operation and conflict, depending on, e.g. genetic composition and size of a colony, benefits and costs of group membership, and benefits and costs of selfish behaviour and policing (Keller & Chapuisat 1999).

In the Hymenoptera, males develop from unfertilised haploid eggs, whereas females are diploid and develop from fertilised eggs. This haplodiploid sex determination system generates relatedness asymmetries among nestmates, leading to a considerable genetic heterogeneity. Thus, reproductive interests of nestmates often differ:

In an ant colony with one, single mated queen, conflict may arise between queen and workers over sex allocation and male production: Workers share three-quarters of their genes by descent with their sisters (relatedness coefficient $r = 0.75$, life-for-life relatedness, Hamilton 1972, p.203), but only a quarter of their genes with their brothers ($r = 0.25$). Selection acting on the workers should favour a biased population sex investment ratio of 3:1 (female sexuals : male sexuals). Queens are equally related to both sons and daughters and therefore, invest equally in both sexes (Hamilton 1972; Trivers & Hare 1976). In many ant species, workers can lay haploid eggs and, therefore, compete with queens for the production of males: In a colony with one, single mated queen, workers are more closely related to their own sons ($r = 0.5$) and to their sister's sons ($r = 0.375$) than they are to their brothers ($r = 0.25$). A queen, however, should prefer to produce sons ($r = 0.5$) rather than let her daughters produce males, to which she is related to $r = 0.25$. Hence, a queen should try to prevent workers from laying eggs (e.g. Hamilton 1964b; Trivers & Hare 1976).

Additionally, conflict among workers over the production of males may arise, as a worker is more related to her son than to her nephew and should try to prevent other workers from laying eggs (Bourke 1988).

In multiple-queen colonies, conflict is expected between queens and workers (e.g. over the total number of young mated queens to be adopted), or between the queens themselves (see Bourke & Franks 1995 for a detailed discussion on conflicts in ants).

However, resistance, counter-manipulation, constraints on perception, and manipulation (e.g. multiple mating, sexual deception, pheromonal regulation) might prevent that potential conflicts over reproduction become actual (Ratnieks & Reeve 1992; Reeve & Ratnieks 1993). Indeed, group living is achieved peacefully in the majority of ant species. Overt aggression among group members is restricted to species with small colony sizes with a few dozen to, more rarely, several hundred individuals. Presumably, fighting in large colonies is ineffective and highly costly for an individual (Reeve & Ratnieks 1993). Additionally, aggression occurs in species with a limited queen-worker dimorphism, with workers having high reproductive potentials, e.g. queenless ponerine ants, where workers are able to mate and lay eggs (e.g. Ito & Higashi 1991; Higashi *et al*. 1994). In *Leptothorax* ants, aggressive interactions may be due to strong ecological constraints (Reeve & Ratnieks 1993; Bourke & Heinze 1994).

Individuals compete by fighting or by eating the eggs of other colony members. Aggressive interactions typically result in dominance hierarchies, in which dominance rank and reproductive success are often positively correlated (reviewed by Heinze *et al*. 1994). Physical aggression may occur, e.g. between queens and workers (Franks & Scovell 1983), among workers (Cole 1981; Oliveira & Hölldobler 1990; Heinze *et al*. 1996), between "gamergates" (mated workers) and workers of queenless ant species (Ito & Higashi 1991; Higashi *et al*. 1994), between gamergates and queens (Sommer & Hölldobler 1992), or between workers and dealated virgin queens (Oliveira & Hölldobler 1991).

Overt competition among queens is rare (reviewed by Heinze 1993). Conceivably, queens compete chemically rather than physically, by producing pheromones which inhibit the reproduction of the other females (e.g. Mercier *et al*. 1985). Physical aggression was observed among queens of the ponerine ant *Odontomachus chelifer* (Medeiros *et al*. 1992) and among *Camponotus planatus* queens (Carlin *et al*. 1993). In both species, several queens laid eggs, and the egg-laying rates were correlated to the dominance status of each queen. Fighting between leptothoracine ant queens may lead to the monopolisation of the reproduction by one single queen (functional monogyny; Heinze & Buschinger 1989; Heinze 1993). In many ant species, newly mated queens

associate with other queens for colony founding (pleometrosis). In these foundress associations, aggression between queens starts after the emergence of the first workers. Queens then become intolerant of each other and usually, all but one queen are killed or expelled (secondary monogyny; Hölldobler & Wilson 1990; Heinze 1993; Choe & Perlman 1997; Bernasconi & Strassmann 1999). In a few ant species, however, young queens not only associate temporarily, but co-operation continues even when the colony has become mature and sexuals are produced (primary polygyny), e.g. the leaf-cutter ant *Atta texana* (Mintzer & Vinson 1985), and *Acromyrmex versicolor* (Rissing *et al*. 1989).

Foundress associations show two important traits which makes the study of these species so valuable: Firstly, foundresses in the same association are usually unrelated (e.g. Rissing & Pollock 1986; Hagen *et al*. 1988; Rissing *et al*. 1989). Secondly, all foundresses in a group lay eggs (e.g. Mintzer & Vinson 1985; Rissing & Pollock 1986). The study of unrelated co-operating individuals allows a critical test for predictions made by theories on reproductive conflicts, in which relatedness is a key parameter, e.g. reproductive skew theory which makes predictions on the allocation of reproduction (e.g. Vehrencamp 1983; reviewed by Johnstone 2000; Reeve & Keller 2001), and kin selection theory (Hamilton 1964a,b). In this context, the study of primary polygynous species is especially rewarding, because here, co-operation between unrelated queens continues after the emergence of workers. Thus, unrelated individuals compete, e.g. in the production of sexuals.

One of the few species with primary polygyny is the neotropical ponerine, *Pachycondyla* cf. *inversa* (then referred to as *P. villosa*, Trunzer *et al*. 1998). Foundress associations consisted of two to five co-operating queens. Direct aggressive interactions between the queens, or ritualised dominance behaviour was never observed, neither during the founding stage until the first workers eclosed, nor in mature colonies when sexuals were produced (Trunzer *et al*. 1998). In a previous study with DNA multilocus fingerprinting, most queens from seven polygynous colonies were unrelated to their nestmate queens: Only one pair-wise comparison suggested a closer relatedness between two queens (Heinze *et al*. 2001).

P. cf. *inversa* queens need to leave the nest to forage during the founding phase (semi-claustral founding). In associations with multiple foundresses, one queen specialises in this risky task (Trunzer *et al*. 1998). The study of *P.* cf. *inversa* is therefore

especially well suited to investigate this altruistic behaviour among unrelated individuals which stands in contrast to the fundamental evolutionary concept of kin selection.

The first objective of this study was to clarify the status of morphologically different forms of the species complex *P. villosa*, to which *P.* cf. *inversa* belongs (chapter 2). In the following part (chapter 3), molecular microsatellite markers were applied to analyse the colony and population structure of *P.* cf. *inversa*. This provided further insight in the genetic basis of this species and therefore, helped to test theories on reproductive conflicts. The division of labour among foundresses, particularly the causes of the altruistic behaviour of foraging queens is subject of the fourth chapter. In a next step, cuticular hydrocarbons of queens from foundress associations were analysed by GC-MS to detect cues which convey information of the queens' social or reproductive status (chapter 5). Finally in the last chapter, genetic markers are presented which will be useful for the study of the genetic structure of another ponerine species, *Pachycondyla obscuricornis* (chapter 6).

2. The species complex *Pachycondyla villosa*

2.1. Introduction

The ant *Pachycondyla villosa* FABRICIUS, 1804 belongs to the phylogenetically primitive subfamily Ponerinae which are distributed within tropical and subtropical habitats. Some ponerine species possess highly derived morphological and social characteristics (reviewed in Peeters 1997). However, most species are characterised by ancestral morphological traits: In contrast to more advanced subfamilies, the queen-worker dimorphism is less pronounced. The limited queen fertility usually results in small colony sizes, from a few dozen to several hundred individuals (Peeters 1993). Similar number and size of the ovaries from queens and workers, and the presence of a spermatheca in workers may lead to diverse conflicts over reproduction.

Pachycondyla villosa is a widespread and very common species in the neotropics. Its distribution ranges from Texas to central Brazil and Paraguay (Pérez-Bautista *et al*. 1985). *Pachycondyla villosa* ants are opportunistic cavity breeders which nest in dead wood, dead cavities in live branches, bromeliad bases, and abandoned or peripheral cavities of myrmecophytic *Cecropia* (Longino 1999). Nests occur in the canopy or near ground level. Workers are generalised arboreal predators preying on arthropods. Additionally they collect liquids, e.g. from extra-floral nectaries (Pérez-Bautista *et al*. 1985).

The existence of various taxa which are currently all synonymised with *Pachycondyla villosa* indicates that this "species" is very heterogeneous. The enormous morphological variability of *P. villosa* was already pointed out by Roger (1861), who regarded the species names *bicolor* GUÉRIN-MENÉVILLE, 1844, *pedunculata* F. SMITH, 1858 and *pilosa* F. SMITH, 1858 as junior synonyms of *Pachycondyla villosa*. Emery (1904, 1911) combined *Ponera inversa* F. SMITH, 1858 and *P. villosa* var. *curvinodis* FOREL, 1899 to *Pachycondyla villosa inversa*. Today, two valid subspecies are recognised: the nominal *P. villosa villosa* and *P. villosa inversa* (Bolton 1995).

The aim of this study was to clarify the status of morphologically different forms of *P. villosa* from Bahia, Brazil. Morphological heterogeneity in *Pachycondyla villosa* was previously regarded as an intraspecific polymorphism.

2.2. Materials and methods

Ant collection

Colonies of *Pachycondyla villosa* were collected on the territory of the CEPLAC (Centro de Pesquisas do Cacau) near Itabuna, Bahia, north-eastern Brazil, in March 1998. The CEPLAC is located in the coastal Brazilian Atlantic rain forest in the biome Mata Atlântica. The climate is moist and warm throughout the year with a mean annual precipitation of 1600 mm and a mean temperature of 23.5 °C (maximum mean temperatures per month: January to March with 25 °C, minimum mean temperatures: July and August with 21,3°C to 21,5°C). Maximum rainfall is in March (196 mm) and November (190 mm), figure 1.

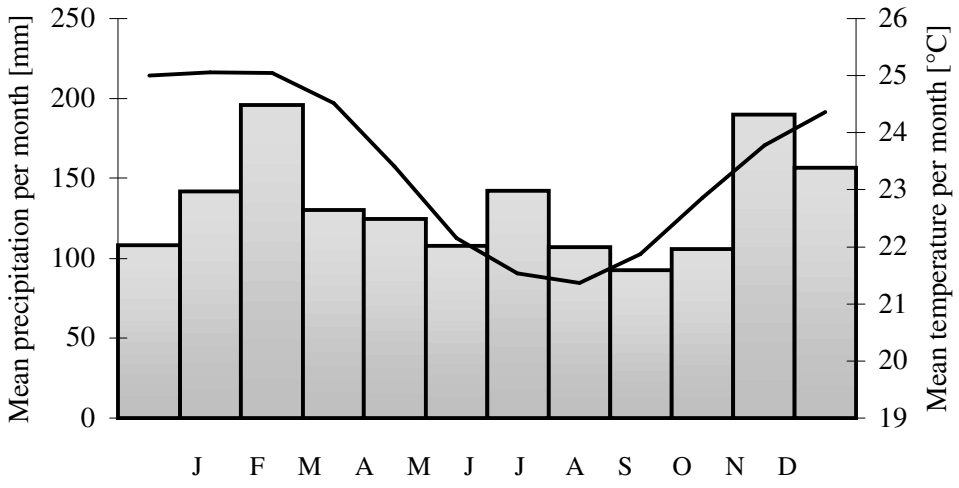

Figure 1. Mean monthly temperature (indicated by line) and mean monthly rainfall (indicated by bars) at the Centro de Pesquisas do Cacau, Brazil (data from 1988-1998).

A total of 112 colonies of *Pachycondyla villosa* were collected from knotholes of cocoa trees and rotten cocoa pods in cultivated, or abandoned cocoa plantations. Whole cocoa pods, mostly containing entire founding colonies were collected from the cocoa trees and put into plastic boxes. Individuals from colonies nesting in knotholes, were caught with forceps and subsequently put into plastic boxes, with one colony per box. The colonies were provided with food (pieces of banana and diluted honey) every two days. After the transfer into the laboratory, colony composition and brood number were immediately examined.

Measurements and morphology

The external morphology of 60 queens and 60 workers was thoroughly examined. Queens and workers were compared to seven workers and two syntypes, one worker and one intermorph, of *P. villosa inversa* obtained from the British Museum of Natural History (BMNH), London. Thorax length (pronotum to meta-epinotal suture in dorsal view), alitrunk width, alitrunk length (frontal profile of the pronotum to the insertion of the petiole in lateral view), head length, and head width (behind the eyes) of 54 queens were measured under a binocular microscope at 50 x magnification. The measurements were repeated three times to determine the measurement error (table 2). Thirteen queens were dissected to check for ovarian condition. All examined queens were mated egg-layers.

Allozyme electrophoresis

Whole ants were crushed in 50µl of distilled water. Two to 14µl of the homogenate were subsequently applied onto cellulose acetate plates which were pre-soaked in the buffer used for electrophoresis: Protein separation was carried out in three different buffer systems (0.025 M Tris-glycine pH 8.6, 0.1 M Tris-citrate pH 8.2, 0.1 M Tris-maleate-EDTA pH 7.4 and pH 8.3), with 1-7 mA per gel for 5-35 min, depending on the enzyme system (table 1). Using slightly modified protocols from Murphy *et al*. (1990), 28 enzymes were stained. Alkaline phosphatase (ALP), esterases (EST), glucose-6-

phosphate isomerase (GPI), isocitrate dehydrogenase (IDH), trehalase (TRE) and xanthine dehydrogenase (XDH) were examined in greater detail.

Table 1. Conditions for allozyme-electrophoresis in the species complex *Pachycondyla villosa*.

Buffer system: 1 (0.1 M Tris-citrate pH 8.2), 2 (0.1 M Tris-maleate-EDTA pH 7.4 or pH 8.3), and 3 (0.025 M Tris-glycine pH 8.6). EC: enzyme code number. The quality of electromorphs is indicated by ++ (resolution/colouration is sufficient for interpretation), + (resolution is not sufficient, but colouration), 0 (no banding patterns visible).

Enzyme	EC	Buffer	Power [mA]	Voltage [V]	Duration [min]	Application frequency	Quality
ACON	4.2.1.3	1	5	200-230	30	4	++
ADH	1.1.1.1	1,2,3	1-5	150-250	5-15	3-7	0
AK	2.7.4.3	1,2,3	1-5	150-250	10-30	3-7	+
ALP	1.2.2.1	1	5	200-230	10-20	4	++
AO	1.2.1.5	1,2,3	1-5	150-250	5-15	3-7	0
ARK	2.7.33	1,2,3	1-5	150-250	10-30	3-7	++
CAP	2.4.5.5	1,2,3	1-5	150-250	5-15	3-7	0
EST	3.1.1.1	1,2,3	3-5	200-230	30-35	3-4	++
FUMH	4.2.1.2	1,2,3	1-5	150-250	5-15	3-7	0
G3PDH	1.1.1.8	1	5	200-230	20	3	++
G6PDH	1.1.1.49	1	5	200-230	30	3	++
GCDH	1.1.1.47	1,2,3	1-5	150-250	5-15	3-7	0
GPI	5.3.1.9	1	5-6	200-230	30	1	++
GTDH (NAD)	1.1.1.46	1,2,3	1-5	150-250	5-15	3-7	0
GTDH(NADPH)	1.1.1.45	1,2,3	1-5	150-250	5-15	3-7	0
HBDH	1.1.1.30	1,2,3	1-5	150-250	10-30	3-7	+
HK	2.7.1.1	1	5	200-230	30	3	++
IDDH	1.1.1.14	1,2,3	1-5	150-250	5-15	3-7	+
IDH	1.1.1.42	2	4	180-230	25	5	++
LDH	1.1.1.27	1,2,3	1-5	150-250	10-30	3-7	+
MDH	1.1.1.37	1,2,3	1-7	150-250	5-20	3-5	+
MDHP	1.1.1.40	1,2,3	1-7	150-250	5-20	3-5	+
PGDH	1.1.1.44	1	2	200-220	5	3	+
PGM	5.4.2.2	1,2,3	1-7	150-250	5-20	3-5	+
PK	2.7.3.3	1	6	200-230	15	3	+
SOD	1.15.1.1	1,2,3	1-5	150-250	5-15	3-7	0
TRE	3.2.1.28	1	5	200-230	20	3-5	++
XDH	1.2.3.2	1	5	200-230	20	5	++

One to six workers from each of 55 colonies, except for ALP (2 colonies) and XDH (12 colonies) were used for allozyme electrophoresis. In addition, four to five workers from each of three different colonies of *P. villosa*, here referred to as "form C", were analysed (provided by C. Lucas). These *P. villosa* colonies also originated from the CEPLAC at Itabuna, but differed in morphology from the 112 colonies collected for this project.

2.3. Results

Measurements and morphology

All five different measurements carried out on 54 queens showed a significantly bimodal distribution, suggesting the existence of two morphologically distinct forms, one larger and the other significantly smaller in all examined characters (Mann-Whitney U tests, $U_1 = 25$, $U_2 = 16$, $U_3 = 14.5$, $U_4 = 17.5$, $U_5 = 19$, $p < 0.0001$, see also table 2). As expected, the different characters were highly correlated with each other (multiple regression $r = 0.96$, $p < 0.0001$), hence the smaller forms appeared to be isometrically reduced.

Small queens (here referred to as "form A") were completely black, their clypeus was conspicuously elongated and their petiole was anteriorly concave (figure 2). The anterior margin and the upper surface of the petiole formed a sharp angle in lateral view. In large queens ("form B"), petiole, parts of the legs, and the base of the gaster were of dark reddish coloration. Their clypeus was comparatively short. The anterior margin of the petiole was not concave and formed a right angle with the upper surface (figure 3). Dissection of the ovaries from 13 queens revealed that they consist of 2 x 3 ovarioles in form A queens and of 2 x 4 ovarioles in form B queens.

Of the two syntypes of the BMNH, the worker clearly belonged to form A, the intermorph to form B. Six of the seven workers of *Pachycondyla villosa inversa* in the BMNH, all from Guyana, resembled form B in morphology. Three of them were labelled "*Neoponera* (s.str.) *villosa* (subsp. *inversa* ?)". One, labelled "*Neoponera* ? *villosa* subsp.

inversa" from Rio de Janeiro, could not be assigned, because its clypeus was more elongated than in the other forms.

Table 2. Measurements [mm] of queens from two different forms of *Pachycondyla villosa* from Itabuna, Brazil.

	Form A		Form B	
	Mean	S.D.	Mean	S.D.
Alitrunk length	4.55	0.13	5.40	0.13
Alitrunk width	2.10	0.08	2.41	0.07
Thorax length	3.35	0.08	4.08	0.15
Head length	2.92	0.08	3.25	0.09
Head width	2.80	0.08	3.22	0.08

Figure 2. Queen of *Pachycondyla villosa*, form A.

Figure 3. Queen of *Pachycondyla villosa*, form B.

Allozyme electrophoresis

Twenty of a total of 28 examined enzyme systems could be reliably visualised (table 1). The banding patterns of five enzymes (ALP, EST, GPI, IDH, TRE) showed clear differences between individuals from 38 colonies of form A, ten colonies of form B and three colonies of form C. Heterozygote individuals were never found. It thus appears that the three taxa are fixed for different electromorphs at these five diagnostic loci. Another enzyme, XDH, differed consistently between form A/C and form C, but had identical electromorphs in form A and form C.

At the respective loci, no intraspecific variation occurred. Banding patterns of four additional enzymes (MDH, MDHP, PGM and PGDH) probably also differed between species, but could not be sufficiently resolved using cellulose acetate electrophoresis.

Individuals from seven colonies were morphologically intermediate to forms A and B which made it impossible to assign them unambiguously to one of the two forms: Both workers and queens were faintly coloured reddish, their petiole was slightly concave but

did not form a sharp angle in lateral view, and their clypeus was intermediate to form A and B. However, using allozyme electrophoresis their status could be clarified: their banding patterns were always identical to those of form A (table 3).

Table 3. Electromorph patterns of six enzymes in three morphologically different forms of *Pachycondyla villosa* from Itabuna, Brazil.

Migration velocity of different electromorphs in the gel is indicated by f (fast), m (medium), s (slow). No: number of colonies.

Enzyme	Form A	Form B	Form C	Unknown	No.
ALP	*mm*	*ff*	*ss*	*mm*	5
EST	*ss*	*ff*	*mm*	*ss*	58
GPI	*ff*	*mm*	*ss*	*ff*	58
IDH	*mm*	*ff*	*ss*	*mm*	58
TRE	*mm*	*ss*	-	*mm*	58
XDH	*ss*	*mm*	*mm*	*ss*	15

2.4. Discussion

The banding patterns of five different enzyme systems clearly showed differences between three morphologically different, sympatric forms of the ant *Pachycondyla villosa,* form A, B and C. A sixth locus allowed distinguishing form B from form A/C. The absence of heterozygotes in the sympatric populations at Itabuna suggests that the three taxa are reproductively isolated, and thus, represent three different species and not an intraspecific polymorphism. This is supported by the fact that in approximately 100 examined colonies the different forms never co-occurred. Preliminary observations showed that mating between the forms B/B and B/A resulted in the production of males only in the first case (Lucas *et al*. 2002). Reproductively isolated taxa which are difficult to distinguish by morphological analysis, are quite common in ants. They often are only

detected by detailed morphometric studies of large numbers of individuals or by molecular approaches (e.g. Ward 1980). An unambiguous distinction between the species studied in this investigation is probably also only possible by a more detailed morphological investigation or allozyme electrophoresis: at a first glance, individuals from a small minority of colonies were morphologically more or less intermediate between forms A and B (see chapter 2.3). However, in all five diagnostic allozyme loci, the banding patterns of workers from these colonies were identical to those of form A.

The taxonomic status of the two species is not yet clear. Most early references do not record the morphology of the described ants in sufficient detail to allow the assignment of the forms described here to one of the previously described taxa (e.g. Smith 1858; Guérin-Méneville 1844). Roger (1861) reported on two forms of *Pachycondyla villosa*: one, collected in Columbia, resembles form A, the other, from Mexico and Demarara, resembles form B. From individuals with intermediate morphology he excluded that the two forms are separate species. Unfortunately, Roger did not compare his findings to *Pachycondyla villosa inversa* (then *Ponera inversa*) which he described as "species incertae sedis". According to later descriptions of the morphology of *Pachycondyla villosa inversa* (referred to as *Ponera inversa* SMITH, 1858 and *Pachycondyla villosa* var. *curvinodis*, FOREL, 1899), form A might be identical to this taxon. Nominal *P. villosa villosa* appears to be morphologically similar to form B (Wheeler 1908; Gallardo 1918). A comparison with material from the BMNH, including two syntypes of *P. villosa inversa*, did not clarify the taxonomic status of the forms A and B, as it contained individuals from both. In the following chapters, form B is called provisionally *Pachycondyla villosa* and form A *Pachycondyla* cf. *inversa*. Form C appears to be a new species which is termed *Pachycondyla subversa* (Lucas *et al*. 2002).

Though morphological or chemical analyses are of diagnostic value to separate the three species, allozyme electrophoresis is the most powerful technique (see also Lucas *et al*. 2002). By means of allozyme electrophoresis and/or morphology, 112 colonies of the *Pachycondyla villosa* complex could be distinguished into 77 colonies of *P.* cf. *inversa* (39 one-queen, 21 two-queen, 12 three-queen, 3 four-queen and 2 five-queen founding colonies, with up to ten workers each and 10 colonies with more than 10 workers each), and 35 colonies of *P. villosa* (15 one-queen, 8 two-queen, 9 three-queen, 2 four-queen and one five-queen founding colony, with up to ten workers per colony).

3. Colony and population structure in *Pachycondyla* cf. *inversa*

3.1. Introduction

A significant relatedness among interacting individuals is of fundamental importance for the evolution and maintenance of eusociality (kin selection theory, Hamilton 1964a,b). Social insects are considered as prime examples for the central role of relatedness in evolution. Nevertheless, insect societies exhibit a wide variety of genetic structures, with relatedness estimates for nestmate workers ranging from as low as zero to 0.75 and more. Societies where unrelated individuals co-occur are of special importance, as they allow a critical test of predictions based on theories of sex allocation and reproductive skew in which relatedness is a key parameter.

The co-occurrence of unrelated lineages within an insect society may arise either from intraspecific parasitism (Foitzik & Heinze 1998) or from primary polygyny. In many ant species, freshly mated queens associate with other queens for colony founding regardless of relatedness (pleometrosis; Hagen *et al*. 1988; Sasaki *et al*. 1996). Typically, after the eclosion of the first workers all but one queen are expelled from the nest or killed, resulting in secondary monogyny (Hölldobler & Wilson 1977, 1990; Rissing & Pollock 1988; Heinze 1993; Choe & Perlman 1997). In a few ant species, however, young queens not only associate temporarily, but co-operation continues even when the colony has become mature and sexuals are produced (primary polygyny).

One of the few species with primary polygyny is the neotropical ponerine, *Pachycondyla* cf. *inversa*. The study of ponerine ants is especially rewarding because they are often regarded as "primitive" and their mating, dispersal, founding behaviour and social life might reflect ancestral conditions (Peeters 1993).

P. cf. *inversa* belongs to the *Pachycondyla villosa* group and is presumably identical to a taxon originally described by Smith (1858) as *Ponera inversa* and was later

combined with *Pachycondyla villosa* var. *curvinodis* to *P. villosa inversa* (Emery 1904). Morphological, chemical, and genetic analyses suggest that *P. villosa inversa* and *P. villosa* are indeed two separate species (chapter 1; see also Mariano *et al*. 2000; Lucas *et al*. 2002). Approximately 50% of all founding colonies of *P.* cf. *inversa* collected near Itabuna, Brazil, consisted of two or more founding queens (referred to as *P. villosa*, Trunzer *et al*. 1998). Co-foundresses displayed aggressive interactions and formed dominance hierarchies which predominantly served to force subordinates to forage (see chapter 4.1). Furthermore, dominant queens fed on the eggs laid by the subordinate queen (chapter 4.1). After emergence of some workers, aggression ceased and most co-foundresses still co-existed when the colony was mature and sexuals were produced (Trunzer *et al*. 1998). In a previous study with DNA multilocus fingerprinting, most of the 16 queens from 7 colonies were found to be unrelated to their nestmate queens. However, due to the complex nature of fingerprints it was not possible to exactly determine queen-queen and worker relatedness and the mating frequency of queens (Heinze *et al*. 2001). Therefore, microsatellite primers which were originally developed for other ponerine ants were used in this study to investigate the genetic structures of colonies and populations of this peculiar ant species.

3.2. Materials and methods

Colonies and rearing conditions

Founding colonies of *Pachycondyla* cf. *inversa* were collected in various collecting sites within a 2.5 x 2.2 km² area at the Centro de Pesquisas do Cacau, CEPLAC, Itabuna, Bahia, north-eastern Brazil in March 1998. In the laboratory, the ants were housed in 19 cm x 19 cm x 9 cm plastic boxes with a plaster floor. Two connected chambers in the plaster (3 cm x 3cm x 1cm) covered by a glass plate, served as a nest. The colonies were kept at room temperature and fed with diluted honey and live crickets every one or two days. A permanent water supply was provided near the feeding arena.

The genetic structure of colonies was investigated shortly after the first workers had emerged, i.e. the data reflect the conditions during an early phase of colony life.

Genetic analyses

DNA was extracted from 10-20 mg of thorax tissue of frozen queens and workers using either a phenol/chloroform extraction protocol as described in Sambrook *et al*. (1989), or a Puregene® DNA Isolation Kit (Gentra Systems). For the phenol/chloroform extraction, tissue was homogenised in 18.75 µl 1M DTT, 5µl Proteinase K (20 mg/ml), 140 µl 10% SDS and 536.25 µl TNE buffer (100 mM NaCl, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0) to a total volume of 700 µl. After overnight incubation at 37 °C, DNA was extracted three times with 750 µl phenol, phenol/chloroform-isoamyl alcohol (1:1), and chloroform-isoamyl alcohol. After precipitation, the DNA was dried for one hour, re-suspended in 50-100 µl ddH_2O and stored at –70 °C until use.

For DNA isolation with the Puregene® Isolation Kit, the tissue was ground with 50 µl of Cell Lysis Solution and incubated for 45 min at 65 °C. Subsequently, 17 µl of Protein Precipitation Solution were added and then centrifuged for 5 min at 14000 rpm. The supernatant was put in 65 µl of 100% cold isopropanol. After centrifugation for 5 min at 14000 rpm, the supernatant was discarded and the pellet was washed with 50 µl of 75% ethanol, then dried for one hour and re-suspended in 50 µl of ddH_2O.

Three primer pairs originally established for *Platythyrea punctata* (Schilder *et al*. 1999; primers 4101, 3303, 3302) and five primers originally developed for *Gnamptogenys striatula* (Giraud *et al*. 1999; L2, L4, L6, L19, L20) were used to study the genetic variation in *P*. cf. *inversa* colonies. Therefore, 7 to 13 workers from different colonies were genotyped. Two primer pairs, 4101 and L4, were examined in greater detail. Here, the 25 µl reaction mixture for one polymerase chain reaction contained 1 µl DNA, 2.5 µl 10x *Taq* DNA polymerase buffer (without $MgCl_2$), 1.5-2 mM $MgCl_2$, 100 µM dNTPs, 10 pmol of each primer, and 1 U *Taq* polymerase (MBI). The PCR profile consisted of an initial denaturation step (3 min at 94 °C), followed by 35 cycles of 60 sec at 92 °C, 75 sec at 54 °C and 60 sec at 72 °C. PCR products were separated electrophoretically on 40 cm long non-denaturing 10% acrylamide gels at 25-30 W for 3-4 hours and subsequently stained for 45 min with SYBR®green. DNA bands were

visualised under UV light (280 nm). Alleles were distinguished by their relative mobility on the gel.

Forty-three queens from 14 colonies (4 two-queen, 7 three-queen, 1 four-queen and 2 five-queen colonies) and 129 workers from 18 colonies (4 to 20 workers from each of 2 two-queen, 6 three-queen, 1 four-queen and 2 five-queen colonies, and 2-3 workers each from 6 additional two-queen colonies) were genotyped at locus 4101. In addition, 12 queens from 6 different colonies were genotyped at locus L4. Because L4 did not exhibit sufficient variation, only data obtained from the more variable locus 4101 is analysed in detail.

Population genetic inferences and statistical analysis

The genotypes of queens and workers allowed to infer mother/daughter relationships and also the haplotype of the workers' fathers. Allele frequencies, heterozygote deficiency, and regression relatedness of queens and their progeny were calculated using the computer program RELATEDNESS 4.2, based on algorithms by Queller & Goodnight (1989).

Dissection

Before genetic analysis, the gasters of all queens were dissected to check the ovarian status and the presence of sperm in the spermatheca.

3.3. Results

Two of a total of seven investigated microsatellite loci were found to be polymorphic in an initial screening of 7 to 13 workers in the study population of *P.* cf. *inversa*. Locus L2 did not show any amplification products, the four remaining loci were monomorphic. Locus 4101 showed moderate intraspecific variation with eight alleles (figure 4; for

details see table 4), whereas locus L4 displayed low variability with two alleles (table 6). Only data based on the more variable locus 4104 are shown. In both queens and workers, the observed heterozygosity (H_o) was significantly lower than the expected heterozygosity H_e (table 4; multisample score test by Raymond & Rousset 1995, $p = 0.028$).

Correspondingly, the inbreeding coefficient F_{IT} was significantly above zero in both queens ($F_{IT} = 0.310 \pm$ SE 0.120; two-tailed t-test, d.f. = 13, $t = 2.58$, $p < 0.02$) and workers ($F_{IT} = 0.330 \pm 0.100$, d.f. = 19, $t = 3.30$, $p < 0.01$, table 5). The excess of homozygotes relative to the expectation under random mating did not result from geographic substructuring of the population. F_{ST}-values did not significantly differ from zero (one-tailed t-test, queens: $F_{ST} = -0.026 \pm 0.021$, d.f. = 7, $t = 1.24$, $p > 0.05$; workers: $F_{ST} = -0.027 \pm 0.019$, d.f. = 9, $t = 1.42$, $p > 0.05$, table 5). There was also no significant relationship between the genetic similarity and the geographic distance between pairs of colonies (Mantel test, queens: matrix correlation $R = -0.043$, $p = 0.612$).

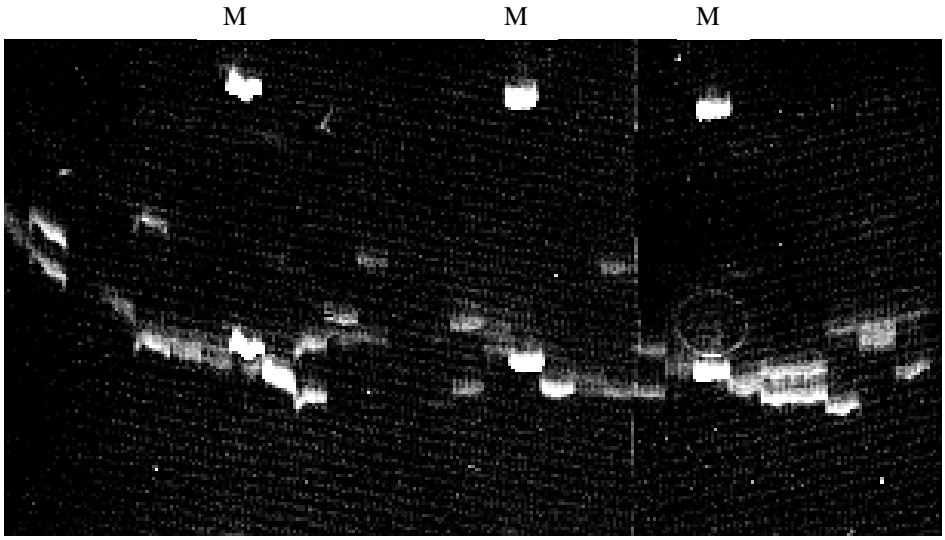

Figure 4. Typical microsatellite pattern from workers of *Pachycondyla* cf. *inversa* at locus 4101. M.: λ *Hind* III marker.

Table 4. Allele frequencies, expected heterozygosity (H_e) and observed heterozygosity (H_o) for workers and queens of *Pachycondyla* cf. *inversa* at microsatellite locus 4101.

	Allele frequencies								H_e	H_o
	a	b	c	d	e	f	g	h		
Queens	0.074	0.091	0.044	0.201	0.227	0.267	0.082	0.015	0.79	0.61
Workers	0.085	0.092	0.051	0.206	0.259	0.210	0.081	0.017	0.815	0.71

Table 5. Estimated nestmate relatedness (r) and F-statistic values with jacknifed standard errors in a study population of *Pachycondyla* cf. *inversa*, calculated from allele frequencies.

	r ± SE	F_{IS} ± SE	F_{IT} ± SE	F_{ST} ± SE
Queens	0.097 ± 0.090	0.316 ± 0.117	0.310 ± 0.120	-0.026 ± 0.021
Workers	0.499 ± 0.108	0.335 ± 0.103	0.330 ± 0.100	-0.027 ± 0.019

The average relatedness among queens from the same colony was r = 0.097 ± SE 0.09 (table 5) which is not significantly different from zero (two-tailed t-test, d.f. = 13, t = 1.08, $p > 0.10$). Dissection showed that all examined queens were mated egg-layers. With only one locus showing considerable variability it was not possible to calculate

meaningful relatedness estimates for individual pairs of queens. However, a closer inspection of queen genotypes showed that in three out of a total of 14 colonies, the alleles of co-foundresses did not overlap (table 6). In six additional colonies, the alleles of at least one queen per colony did not overlap with the alleles of nestmate queens.

Table 6. Genotypes of queens and workers in colonies of *Pachycondyla* cf. *inversa.*

Colony	Genotype queens		No. of workers of a specific genotype
	at locus L4	at locus 4101	at locus 4101
PvII 14		*ac, ac*	
PvII 50		*gg, ee*	
PvII 131		*af, ag*	*aa* 2, *ac* 6, *af* 1, *ag* 9, *ef* 1, *gg* 1
PvII 171	*bb, aa*	*be, bf*	*bd* 1, *bf* 5, *df* 2, *ff* 4
PvII 53		*ah, ee, hh*	*aa* 1, *ch* 1, *ee* 3
PvII 89		*bf, dd, df*	*dd* 1, *df* 2, *dg* 5, *ff* 5
PvII 147	*ab, ab, ab*	*be, ce, ee*	*ac* 3, *ae* 4, *bc* 1, *ee* 2
PvII 146		*be, ff, ff*	*ae* 1, *af* 1, *bb* 3, *be* 1, *bf* 2, *bg* 7
PvII 149		*ac, df, ff*	*ac* 1, *ae* 1, *ag* 1, *dd* 2, *df* 6
PvII 175		*gg, dd, ff*	
PvII 176		*ee, ee, bf*	*ee* 4
PvII 178		*ae, ef, ef, eg*	*be* 1, *bf* 2, *de* 2, *ee* 2, *eg* 3, *gg* 2
PvII 75	*bb, - , ab, ab, aa*	*ac, af, bf, ee, ef*	*be* 3, *ee* 1, *ef* 6
PvII 51	*ab, - , ab, - , ab*	*ae, be, ch, dd, df*	*bf* 1, *cf* 1, *df* 3, *ff* 2, *fh* 2

Nestmate workers were on average closely related (r = 0.5 ± 0.11, table 5). In some cases, queens did apparently not contribute equally to the workers, as suggested for immature colonies based on differential oophagy and domination in foundress associations (chapter 4). For example, in colony PvII 146, one queen (genotype *be*) produced 11 workers, while two queens (genotype *ff*) together produced only 0 to 3 workers. In colony PvII 131, one queen (genotype *ag*) was the mother of 10 to 16 workers, the second queen (genotype *af*) of 0 to 6 workers (table 6).

For six of ten colonies, in which both queens and workers were genotyped, the data were consistent with queens being singly-mated (monandry). In four colonies (PvII 131, PvII 171, PvII 146 and PvII 149), some worker genotypes could only be explained by assuming multiple mating of the mother queens with two to three males (polyandry). For example, the queen with the genotype *af* of colony PvII 131 presumably mated twice (genotype *a* and *e*) and queen *ag* was mated with a *g* male. One of the queens had to be mated additionally with a *c* male (table 6). Additional cases of multiple mating might have remained undetected because different males could have identical alleles or contributed unequally to the progeny. However, more data are needed to justify the estimation of effective mating frequencies in the study population.

3.4. Discussion

This study shows that nestmate queens in founding colonies of the ponerine ant, *Pachycondyla* cf. *inversa* are on average unrelated. These findings corroborate results from a multilocus DNA fingerprint study, in which the band-sharing coefficients of nestmate queens overlapped with the range of background similarity among workers taken from different colonies (Heinze *et al*. 2001). Due to the complexity of multilocus fingerprints, an exact calculation of relatedness was not possible. From variation at a single microsatellite locus, a queen-queen relatedness of r = 0.097 ± SE 0.09 was estimated which was not significantly different from zero relatedness. This matches

results from studies in myrmicine and formicine ants, according to which founding queens do not preferentially co-operate with related foundresses (Hagen *et al*. 1988; Sasaki *et al*. 1996). In contrast to most of these species, in which the queen number is down-regulated to secondary monogyny, however, founding associations of *P.* cf. *inversa* develop into mature polygynous societies. In founding colonies, the dominant queen feeds on the eggs of the subordinate queens. This is reflected in the unequal presentation of the alleles of individual queens in the colony's first workers. In contrast, in mature colonies dominance behaviour among queen and egg-eating becomes rare or stops completely, resulting in a more balanced contribution of queens to the colony's workers and sexuals (Heinze *et al*. 2001).

The analysis of microsatellites also revealed a significant deficiency of heterozygotes leading to positive inbreeding coefficients in both queens and workers. This apparently is not caused by a Wahlund effect, i.e. a geographical substructuring of the study population. F_{ST} values of both queens and workers were not significantly different from zero, and geographical distance and genetic similarity, based on the genotypes of queens, were not significantly correlated. Hence, gene flow seemed not to be restricted in the collecting site of 2.5 x 2.2 km². Heterozygote deficiency can also be caused by the frequent occurrence of null-alleles, though this appears unlikely in this study because DNA amplification rarely failed. Alternatively, the positive inbreeding coefficient might be explained by temporal substructuring of the population, i.e. sexuals from different colonies mating during different times of the year. Winged queens and males were indeed found in *P.* cf. *inversa* colonies in the study population both in March and September, and whereas some founding colonies collected in March contained only eggs, others already had worker pupae or callows. Furthermore, several recent studies have documented that, in contrast to ants from boreal or temperate habitats, nuptial flights of many tropical ant species are not restricted to a short period of the year but may occur almost year-round (Kaspari *et al*. 2001; Torres *et al*. 2001). Little is known on the reproductive biology of *P.* cf. *inversa*. From the lack of a significant geographical substructure and the low relatedness of queens in pleometrotic associations it can be concluded that virgin queens of *P.* cf. *inversa* do not mate near their natal nest and disperse before founding colonies. This is in accordance with Peeters' (1993) assumption that most winged ponerine queens disperse and mate away from their natal nests. Indeed, nuptial flights of two *Pachycondyla* species have been observed (Ortius & Lechner 1997).

In *P.* cf. *inversa*, individual colonies might produce sexuals during different times of the year and mating might occur during several, non-overlapping mating swarms with different allele frequencies. In such a case, a population genetic analysis, in which mating of queens during temporally distinct nuptial flights was disregarded, would reveal an apparent deviation from panmixia despite of random mating within individual mating swarms.

A comparison of the genotypes of queens and workers suggested the rather common occurrence of multiple mating in *P.* cf. *inversa* queens. A previous multilocus DNA fingerprinting analysis could not exclude multiple mating because of the high band sharing similarity among males (Heinze *et al*. 2001). In general, multiple mating appears to be rare in ants. Most cases have been documented from species with very large colony sizes, such as leaf-cutter ants (e.g. Villesen *et al*. 1999; Murakami *et al*. 2000; Strassmann 2001). However, recent genetic studies gave evidence for polyandry being rather common also in some species with small colony size, such as the dinosaur ant, *Nothomyrmecia macrops* (Sanetra & Crozier 2001). The extent of multiple mating in *P.* cf. *inversa* is not yet clear, as the data do not suffice for a detailed analysis of effective mating frequencies taking non-detection probabilities into account.

In most cases, microsatellite data did not allow to determine the origin of workers in polygynous societies. Nevertheless, it appears that queens occasionally contributed unequally to the colony's workers. Because the genotypes were determined shortly after the first workers eclosed, this might reflect differential egg-eating by the dominant queen in founding associations (chapter 4.1). In contrast, all queens contributed more or less equally to the brood in mature colonies (Heinze *et al*. 2001).

4. Co-operation and conflict in foundress associations of *Pachycondyla* cf. *inversa*

Figure 5. Dominance and subordinance behaviour displayed by two *Pachycondyla* cf. *inversa* foundresses.

4.1. Rank orders and division of labour among unrelated co-founding ant queens

4.1.1. Introduction

The elaborate division of labour in insect societies is a particularly impressive example for kin-selected co-operation. Some females forego reproduction and take over risky tasks such as colony defence and foraging. By thus helping a close relative increase the number or survival rate of its offspring they indirectly also maximise their own fitness (Hamilton 1964a,b). Given the importance of relatedness between a helper and the recipient of the help in inclusive fitness theory, it is not surprising that most mature insect societies are characterised by a significant non-zero relatedness among nestmates (e.g. Bourke & Franks 1995; Crozier & Pamilo 1996) with the exception of some unicolonial or highly polygynous species (e.g. Kaufmann *et al.* 1992; Goodisman & Ross 1998). In contrast, relatedness is apparently not a major factor in the evolution of joint colony founding among ant queens (pleometrosis). In many species, unrelated queens work together when excavating a common nest burrow and rearing their first young (Hagen *et al.* 1988; Strassmann 1989; Sasaki *et al.* 1996). Once the first workers have eclosed, the co-foundresses typically become intolerant of each other, begin to fight and only a single queen survives per colony (Hölldobler & Wilson 1990; Heinze 1993; Choe & Perlman 1997; Bernasconi & Strassmann 1999). Jointly founded societies grow faster than founding colonies with a single queen and are thus believed to have an advantage in territorial contests with neighbouring colonies (e.g. Bartz & Hölldobler 1982; Tschinkel & Howard 1983; Rissing & Pollock 1987; Sommer & Hölldobler 1995).

During the founding phase, queens of most species exclusively use tissue from their wing muscles, stored fat and protein reserves in order to rear their first young (claustral

founding). In several species, however, founding queens leave their nests to forage (semi-claustral founding). The case of the desert leaf-cutter ant *Acromyrmex versicolor* is of special interest because here unrelated queens co-operate during semi-claustral founding. Queens have to forage for leaves in order to cultivate a symbiotic fungus, and co-operatively founded colonies appear to be more successful in initiating their fungus garden (Cahan & Julian 1999). One queen specialises in foraging while the other queens stay in the nest (Rissing *et al.* 1989). Once a forager, the queen remains in this task and is punished if she stops foraging (Rissing *et al.* 1996). Task allocation in *A. versicolor* does not appear to be regulated by aggressive interactions and it was suggested that, because the group succeeds or fails as a unit in competition with other such groups, an individual queen should be indifferent as to whether she takes over the more or less risky tasks (Rissing *et al.* 1989). Fighting about task allocation was selected against, as it would decrease the success of the colony as a whole. Group selection has occasionally been invoked in order to explain pleometrosis in general (Dugatkin *et al.* 1992; Mesterton-Gibbons & Dugatkin 1992; but see Nonacs 1993) and the division of labour without social dominance in *A. versicolor* has been interpreted as a particularly strong case of group-selected co-operation (Wilson 1990; Dugatkin 1997; but see Pollock 1994).

Queen co-operation during semi-claustral founding has also been reported from neotropical ponerine ants in the *Pachycondyla villosa* complex (Trunzer *et al.* 1998). As in *A. versicolor*, one queen per group specialises in foraging but, as it is demonstrated below, the division of labour among *Pachycondyla* queens is achieved by aggression. The dominant queen coerces the subordinate into foraging and the frequency of these attacks increases with the time since food was last brought into the nest.

4.1.2. Material and methods

Fifty-two newly founded colonies of *Pachycondyla* cf. *inversa* were collected from knotholes in cocoa trees and rotten cocoa pods at Centro de Pesquisas do Cacau,

CEPLAC, Itabuna, Bahia, north-eastern Brazil in March 1998. The ants were housed in the laboratory in 19 cm x 19 cm x 9 cm plastic boxes with a plaster floor with two connected 3 cm x 3 m x 1 cm large chambers in the plaster covered by a glass plate serving as nest. They were kept at room temperature and fed with live crickets and diluted honey at variable intervals (every one to six days). The plaster was regularly watered to maintain humidity.

All queens in the experimental colonies were individually marked with dots of enamel paint on the gaster for observations of behaviour. Behaviour was observed directly in one hour sessions or after recording on video from April to September 1998. All instances of aggressive interactions (antennal boxing and biting), brood care, foraging, guarding the nest entrance, inactivity on or near the brood pile and nest maintenance were noted. The behaviour of queens from six groups (3 two-queen, and 3 three-queen colonies) was observed in more detail by focal and/or scan sampling (Altmann 1974) with a total observation time of 5-9 h per colony ("focus colonies"). Behavioural observations were ended after the eclosion of the first six workers. Egg-laying rates were determined by time-lapse video recordings over a total observation time of 168 to 336 h per colony, totalling 1440 h. Most queens were killed and dissected after the end of the observations.

For statistical analysis, the total duration of a certain behaviour was calculated for each queen during each hour of observation. The data were then compared by Friedman's ANOVA or Wilcoxon matched-pairs test (Sachs 1992).

4.1.3. Results

Out of a total of 52 founding colonies without workers, 26 had one queen (50 %), 14 had two queens (27 %), ten had three queens (19 %) and two had four queens (4 %). Queens engaged in aggressive interactions in all 26 pleometrotic associations. One individual attacked another by violent antennation, whereas the latter assumed a

crouching posture with retracted antennae (figure 5) and/or attempted to escape, e.g. by leaving the nest. Aggressive interactions between queens in the six focus colonies resulted in clear dominance hierarchies (tables 7 and 8). Approximately 49.9 % (527 of 1057) of all attacks in the three-queen groups and 64.6 % (126 of 195) in the two-queen groups resulted in a clear winner-loser relationship. In the remaining interactions, the attacked queens did not react unmistakably to the attack. In none of the observed 1252 aggressive interactions did a queen that had previously shown clear submissive behaviour towards an attacker retaliate the attack. Aggressive interactions continued after the emergence of workers until the end of this study when approximately seven workers had eclosed. However, during two years of behavioural observations queen antagonism was only rarely observed in mature colonies.

Table 7. Dominance interactions among co-founding queens in two-queen colonies of the ant *Pachycondyla* sp. *inversa.*

Only those interactions which resulted in a recognisable reaction by the ant attacked are included.

Founding colony	Attacking queen	Attacks against queen A	Attacks against queen B	Total observation time (h)
PvII 21	A	-	65	5
	B	0	-	
PvII 129	A	-	44	5
	B	0	-	
PvII 190	A	-	17	7
	B	0	-	

Table 8. Dominance interactions among co-founding queens in three-queen colonies of the ant *Pachycondyla* sp. *inversa*.

Only those interactions which resulted in a recognisable reaction by the ant attacked are included.

Founding colony	Attacking queen	Attacks against queen A	B	C	Total observation time (h)
PvII 49	A	-	131	35	7
	B	0	-	76	
	C	0	0	-	
PvII 174	A	-	67	20	5
	B	0	-	44	
	C	0	0	-	
PvII 175	A	-	92	36	5
	B	0	-	26	
	C	0	0	-	

The frequency of aggressive interactions was significantly higher in three-queen groups (three two-queen groups, medians 6, 9 and 12 attacks per hour, range 3 to 34 and total observation time 17 h and three three-queen groups, medians 26, 45 and 83 attacks per hour, range 15-122 and total observation time 17 h) (Mann-Whitney U-test with medians, $n_1 = n_2 = 3$, $U = 0$, $p = 0.05$). The return of low-ranking queens from the arena (see below) into the nest often initiated aggressive interactions between all three queens until the lowest ranking queen had again left the nest or at least moved to the nest entrance.

Subordinate queens generally performed foraging trips at a much higher frequency than dominant queens, whereas the latter rested with extended legs on or near the brood pile ("brood-guarding"). The queens assuming the middle position in the hierarchy in three-queen groups also had frequencies of foraging between those of the two other queens. Observations in the six focus colonies corroborated these qualitative results. Only the dominant queens exhibited brood-guarding behaviour (Wilcoxon matched-pairs test for two-queen groups, $t = 0.00$ and $p < 0.05$ for each colony and Friedman's ANOVA for three-queen groups, $\chi^2 = 10$, 10 and 8 and $p < 0.02$ for each colony) (table 9). Although all queens of focus colonies were occasionally observed outside of the nest, low-ranking queens left the nest for significantly longer periods than high-ranking queens (two-queen groups, $t = 0$ and $p < 0.02$ in two groups and $t = 4$ and $p = 0.09$ in colony PvII 21 and Friedman's ANOVA for three-queen groups: $\chi^2 = 17.2$, 11.6 and 0.65 and $p < 0.01$ for each colony). In only one out of 48 observation sessions of one hour each did a dominant queen spend more time outside of the nest than a subordinate (PvII 21). The lowest ranking queens typically undertook less brood care than the higher ranking queens (table 9).

A positive correlation was found in the three three-queen groups between the frequency of dominance interactions and the time elapsed since the last addition of food (table 10). The failure to detect such a correlation in two-queen groups was probably due to the much lower aggression rate in these colonies (tables 7 and 8).

Table 9. Time budget (i.e. percentage of time spent in various types of behaviour) of queens in founding colonies in *P.* cf. i*nversa*.

A, B and C denote queens of decreasing dominance status. Note that data for exact time budgets were only collected in part over a shorter period of time than for foraging or for the determination of dominance hierarchies in tables 1 and 2. As some types of behaviour are not included in the table (e.g. resting), the totals do not add up to 1.00.

Founding colony	Queen	Brood guarding	Brood care	Nest maintenance	Guarding nest entrance	Total observation time (h)	Foraging	Total observation time (h)
PvII 21	A	0.284	0.179	0.023	0.002	5	0.069	7
	B	0.000	0.007	0.071	0.044		0.328	
PvII 129	A	0.333	0.100	0.038	0.006	5	0.037	8
	B	0.000	0.000	0.002	0.000		0.434	
PvII 190	A	0.211	0.150	0.000	0.140	6	0.099	8
	B	0.000	0.008	0.138	0.082		0.587	
PvII 49	A	0.435	0.148	0.000	0.000	6	0.001	9
	B	0.000	0.225	0.030	0.111		0.169	
	C	0.000	0.037	0.049	0.098		0.568	
PvII 174	A	0.157	0.086	0.000	0.000	5	0.073	6
	B	0.000	0.103	0.002	0.080		0.298	
	C	0.000	0.013	0.000	0.000		0.682	
PvII 175	A	0.163	0.234	0.000	0.011	5	0.006	7
	B	0.000	0.135	0.020	0.007		0.351	
	C	0.000	0.019	0.027	0.000		0.535	6

Table 10. Correlation (Spearman's rank correlation, r_S) of the frequency of aggressive acts per hour and the duration since last feeding in two- and three-queen groups of *P.* cf. *inversa*.

Colony	Number of queens	Number of observation sessions	r_S	p
PvII 21	2	5	0.154	0.8
PvII 129	2	5	0.102	0.87
PvII 190	2	7	-0.25	0.6
PvII 49	3	7	0.89	0.008
PvII 174	3	5	0.95	0.014
PvII 175	3	5	0.95	0.014

The egg-laying rates were extremely low and, therefore, probably did not differ between subordinate and dominant queens (table 11), (two-queen groups, median dominant queens 0.43 eggs per day, subordinates 0.27 eggs per day and total observation time 600 h, Mann-Whitney U-test, U = 3.5 and p = 0.663 and three-queen groups, median dominants 0.29 eggs per day, subordinates 0.21 eggs per day and total observation time 840 h, Kruskal-Wallis H-test, $\chi^2 = 0.90$, p = 0.637) or between queens in two- and three-queen groups (U = 14.5 and p = 0.141). However, dominant queens regularly attacked egg-laying subordinates and occasionally robbed and ate their eggs, while these behaviours never occurred in the opposite direction (Fisher's exact test, two tailed p = 0.0008 and total observation time 1440 h). In order to escape attacks, subordinates were observed four times laying their eggs outside of the nest after having been attacked by the dominant queen. On one occasion an egg-laying subordinate was chased out of the nest by the dominant and attacked when attempting to return with a newly laid egg. A foraging worker later carried the egg into the nest without being attacked by the dominant queen.

Dissection of some of the queens at the end of the observations (both queens each of the two two-queen colonies and five queens from two of the three-queen colonies) proved that their ovaries were all well developed and their spermathecae contained sperm.

Table 11. Number of eggs laid by individual queens in foundress associations of *P.* cf. *inversa* during the observation period and number of egg-laying events during which a queen was either harassed or attacked by another queen, laid her egg outside of the nest or after which the egg was eaten by another queen ("disturbed egg-laying").

Colony	Number of eggs laid			Disturbed egg-laying			Observation time (h)
	A	B	C	A	B	C	
PvII 21	4	5		0	3		168
PvII 129	3	3		0	1		264
PvII 190	3	1		0	1		168
PvII 49	4	3	0	0	1	-	336
PvII 174	1	3	2	0	2	2	168
PvII 175	5	3	0	0	3	-	336

4.1.4. Discussion

This study provides clear evidence that the division of labour among co-founding queens in *Pachycondyla* cf. *inversa* results from social competition. Co-foundresses of *P.* cf. *inversa* engage in aggressive interactions and the dominant forces the subordinate to leave the nest and forage. Furthermore, the dominant also feeds on the eggs laid by the

subordinate and harasses her during egg laying. The subordinate therefore presumably produces less offspring than the dominant during these early stages of colony ontogeny (see also chapter 3).

Dominance interactions have been suggested as underlying the division of labour in foundress associations in wasps (Pratte 1989; Strassmann 1989) and in small ant societies (Bourke 1988; Ito & Higashi 1991). However, in all these cases, the interacting individuals were typically close relatives. In contrast, co-founding queens of *P.* cf. *inversa* are unrelated (chapter 3; Heinze *et al.* 2001). This is the first observation of dominance interactions among unrelated individuals in eusocial insects leading to a clear-cut division of labour.

Subordinates regularly returned to the nest with prey and, though harassed by the dominant, also attempted to lay their eggs in the nest. Why then did they not instead abscond from the nest and found their own colony solitarily if they were forced to leave and forage anyway? Assuming that subordinate queens behave in an adaptive way, the benefits from joint founding must outweigh the costs of being a subordinate (e.g. Robertson *et al.* 1998).

Optimal skew theory (e.g. Reeve & Ratnieks 1993) provides a framework for understanding the outcome of social interactions between individuals. In particular, the behaviour of unrelated co-founding queens is thought to depend on their respective fighting abilities, the ratio of productivity of a group to that of a solitary individual and the magnitude of ecological constraints. Increased group productivity and strong ecological constraints have previously been suggested to favour pleometrotic associations in social insects, for example faster colony growth (Bartz & Hölldobler 1982; Tschinkel & Howard 1983; Rissing & Pollock 1987; Sommer & Hölldobler 1995; but see Pfennig 1995; Jerome *et al.* 1998), increased protection against predators and usurpation (Gamboa 1978; McCorquodale 1989; Balas & Adams 1996) and a shortage of nest sites (e.g. Pfennig 1995). Though empirical data from the field do not exist for *P.* cf. *inversa*, pleometrosis might be associated with similar advantages (Trunzer *et al.* 1998).

If ecological constraints are large, a queen with low fighting ability might still benefit from joint nesting even if she is forced to specialise in dangerous tasks and some of her eggs are destroyed by the dominant. It is often assumed that foraging is more risky than brood guarding. However, *Pachycondyla* queens have a powerful sting for deterring predators (Schmidt *et al.* 1980; Starr 1985) and the mortality rate of foraging queens is

possibly not very high. Furthermore, *Pachycondyla* feed opportunistically on insect prey (e.g. Fresneau 1994) which often is large enough to be shared with other individuals but which cannot be stored. Foraging costs are therefore probably not much higher for a subordinate in a pleometrotic association than for a solitary queen.

Whether egg eating and harassing during egg laying in this early phase affects the future reproductive success of a subordinate is currently unclear. However, what might tip the scale strongly towards pleometrosis in *P.* cf. *inversa* is that, in striking contrast to most other co-operatively founding ants, the queen number in founding associations is not regulated to monogyny after the eclosion of workers. In most species, queens begin to fight when first workers have eclosed and all but one queen is finally killed or expelled from the nest (Bartz & Hölldobler 1982; Heinze 1993; Choe & Perlman 1997). However, pleometrosis in *P.* cf. *inversa* leads to primary polygyny and co-founding queens still coexist without much aggression once the colony has become mature and sexuals are produced (see also Trunzer *et al.* 1998). As expected from optimal skew theory (Reeve & Ratnieks 1993), reproduction is quite evenly partitioned among the unrelated queens in polygynous societies of *P.* cf. *inversa* (Heinze *et al.* 2001). Interestingly, co-operative founding among unrelated queens in *Acromyrmex versicolor* results similarly in primary polygyny (Rissing *et al.* 1989), suggesting that it pays for a queen to take over foraging only if she does not risk being expelled from the nest after successful founding. Pleometrosis in semi-claustrally founding ants might therefore be a suitable model system for testing optimal skew theory.

This study also fits the hypothesis that energetically costly antagonism among queens is only possible after the colony has changed from a closed- to an open-energy system (Rissing and Pollock 1986). Ant queens which found claustrally typically begin to fight only after the first workers have started to forage and to carry in new resources. Newly founded colonies of *P.* cf. *inversa* are open systems due to foraging by the subordinate and social competition is therefore possible.

The results of this study differed from those of a previous analysis of pleometrosis in the same population of *P.* cf. *inversa* (then referred to as *P. villosa*, Trunzer *et al.* 1998). In this investigation, the division of labour among queens appeared to be achieved without queen antagonism similar to the relationships in *A. versicolor*. Furthermore, egg-laying rates were much higher. Both discrepancies are probably explained by differences in the provisioning of the laboratory colonies with food. Whereas the colonies studied by

Trunzer *et al.* (1998) were fed every two days, here food was added after longer intervals and less regular intervals. In colonies with three queens at least, the frequency of attacks significantly increased with the time since food was added to the arena. Less favourable conditions might therefore lead to more frequent aggression (and, thus, to more intensive foraging by the subordinate queen) and make dominance relationships among queens more obvious for an observer. Similarly, food shortage accentuated the reproductive dominance of one queen in polygynous colonies of *Myrmica rubra* (Sommeijer & Van Veen 1990). The apparent lack of overt aggression among queens in foundress associations or mature polygynous colonies therefore does not necessarily mean that dominance relationships do not exist at all. Division of labour may instead result from subtle and difficult-to-observe interactions among co-foundresses (Bernasconi & Keller 1998; see also Choe & Perlman 1997). Therefore, group selection alone does not always explain apparent altruistic behaviour of unrelated queens.

4.2. Body size affects dominance rank in foundress associations of the ant *Pachycondyla* cf. *inversa*

4.2.1. Introduction

Colonies of social insects are often initiated co-operatively by several founding queens (pleometrosis). In wasps, aggression among foundresses often leads to the early formation of stable dominance hierarchies, in which dominants monopolise egg laying and subordinates forage (e.g. Pardi 1948; West-Eberhard 1969, 1978; Fletcher & Ross 1985; Röseler 1991). In contrast, aggression in foundress associations of ants typically starts only after the emergence of the first workers. Queens then become intolerant of each other and all but one queen are killed or expelled (Hölldobler & Wilson 1990; Heinze 1993; Choe & Perlman 1997; Bernasconi & Strassmann 1999). Founding queens of many ant species do not leave the founding nest and rely on histolysed body tissue to rear their first brood, and it was suggested that fighting among ant queens could begin only after new food sources have become available through worker foraging.

The ant *Pachycondyla* cf. *inversa* is a striking exception. Here, unrelated co-founding queens very quickly begin to fight over dominance, and the dominant queen forces the subordinate to forage for prey (chapter 4.1). Contrasting the reversion to secondary monogyny in many other ants with pleometrotic foundation, aggression among *P.* cf. *inversa* queens ceases after worker emergence and nestmate queens stay together even in mature colonies (Trunzer *et al.* 1998).

Though dominance hierarchies have been described from a number of social insects, it is still unclear for most cases, what proximately affects hierarchy rank. Individual size (Turillazzi & Pardi 1977; Dropkin & Gamboa 1981; Noonan 1981; Sullivan & Strassmann 1984), age (e.g. Strassmann & Meyer 1983; Hughes & Strassmann 1988), and social experience (Hsu & Wolf 2001) have been found to be of importance in some

taxa but not in others (Bourke 1988; Heinze & Oberstadt 1999). Here, data from an experimental study on the importance of asymmetries in size and nest ownership in pleometrotic associations of *P.* cf. *inversa* are presented.

4.2.2. Material and methods

Twenty-six solitarily founding queens of *Pachycondyla* cf. *inversa* were collected in an experimental cocoa plantation at the Centro de Pesquisas do Cacau, CEPLAC, Itabuna, Bahia, in March 1998. In the laboratory, the ants were housed in artificial nests (see chapter 4.1.2). Rearing conditions of the colonies were similar to those described above (chapter 4.1.2).

Behavioural observations were started in April 1998, shortly after the ants had been transferred into the laboratory nests. Sixteen single foundresses were individually marked with dots of Edding paint markers. The head width of live queens was measured under a binocular microscope at 50 x magnification. Measurements were repeated twice at the end of this study to determine the measurement error (table 12). Directly before the start of the observations all queens were weighted on a precision balance (Sartorius Research R 200 D) to the nearest 0.02 mg (table 12).

In the behavioural studies, the queens were paired in eight groups of two individuals which were collected from nests which were at least 20 m apart. One of the two queens was put in an arena of 43 cm x 27 cm x 10 cm which contained a nest and water supply similar to those described above (chapter 4.1.2). The second queen was introduced 4 hrs to 33 days later (PiII 1 and PiII 7, 4 hrs; PiII 2, 1 day; PiII 4, 3 days; PiII 3, 5 days; PiII 6, 8 days; PiII 8, 9 days; PiII 5, 33 days). Even when carefully handled, the introduced queens were sometimes highly aggressive when taken out of the nest with forceps. They were therefore carefully placed into the arena and covered with a plastic box of 12 cm diameter to allow the queens to calm down. The walls of the plastic box were coated with a thin layer of mineral oil so the ant could not climb up the walls. The resident queens, if they were out of the nest, carefully examined the box, but no interactions between the

ants occurred at this time. After 30 min, the box was removed and the observation started. Behaviour was observed directly for one to four hours after the introduction of the second queen and thereafter video recorded for one hour per day at irregular intervals from day 2 to day 10. From day 10 to day 55, behaviour was directly observed in eight to nineteen 10 min sessions and one to three 60 min sessions, depending on the colony. All instances of aggressive interactions were noted (e.g. antennal boxing, biting, pulling, stinging). Observations ended in September 1998 when the first 5 to 10 workers had eclosed.

Table 12. Size and weight of *Pachycondyla* cf. *inversa* queens.

The measurement error of head width was 0.01 mm or less, except in queen 8A, where it was 0.04 mm. A and B denote queens in the order in which they were introduced into the nest. Dominant queens are marked with bold letters.

Pair	Queen	Weight (mg)	Head width (mm)
PiII 1	**A**	53	2.80 ± 0
	B	52	2.72 ± 0.01
PiII 2	**A**	52	2.80 ± 0
	B	48	2.74 ± 0
PiII 3	**A**	49	2.84 ± 0.01
	B	50	2.80 ± 0
PiII 4	A	54	2.78 ± 0
	B	54	2.85 ± 0
PiII 5	**A**	49	2.85 ± 0
	B	50	2.72 .± 0
PiII 6	A	45	2.68 ± 0
	B	56	2.72 ± 0.01
PiII 7	**A**	51	2.82 ± 0
	B	53	2.81 ± 0
PiII 8	A	38	2.60 ± 0.04
	B	52	2.87 ± 0

Seven natural founding associations (four colonies with two queens each, and three colonies with three queens each) were examined to determine the influence of size on hierarchy rank in natural colonies. Dominance relationships were examined by directly observing queen-queen interactions in one hour sessions for a total of 6 to 9 hrs per colony. After the behavioural observations, all foundresses were measured twice on a precision balance (Sartorius Research R 200 D) to the nearest 0.02 mg.

At the end of the study, nine queens from the experimental colonies, and 11 individuals from the natural founding associations were dissected to check for ovarian condition. All examined queens were mated egg-layers.

4.2.3. Results

Aggressive interactions were observed almost immediately after queens of *Pachycondyla* cf. *inversa* were placed together. In all eight experimentally assembled pairs, the dominant individual could be determined within the first 20 min after introduction of the second queen. The individual initiating a contest always won the interaction and became dominant.

In three colonies, PiII 2, PiII 5 and PiII 6, one queen dominated her opponent immediately after the first contact: both queens engaged in a severe fight with violent antennation, biting, stinging attempts and opening of the mandibles (figure 6). The queen initiating the fight was more aggressive, seized and pulled the mandibles of the opponent and dragged it through the nest. In each case, the opponent reacted submissively by crouching within 1 to 4 min after first contact. The frequencies of aggressive behaviour during the first 30 min are shown in table 13.

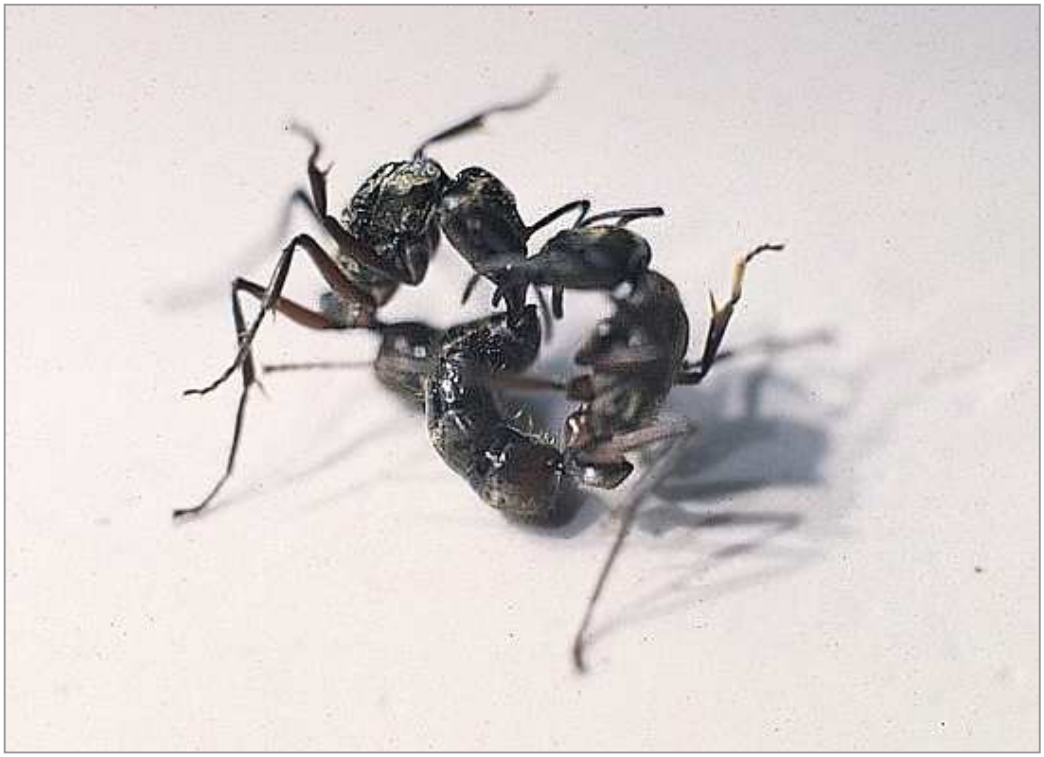

Figure 6. Severe fighting of *Pachycondyla* cf. *inversa* foundresses.

In the five remaining colonies, the winner of the contest became apparent after 10 to 20 min. After the first contact, one or both of the queens tried to escape. Meeting again, they examined each other with their antennae for 1 to 5 min. One queen, mostly the resident, then lowered the body to the ground and tried to push the opponent out of the nest. The attacked queen retreated, antennated and, in some cases, tried to escape. After 10 to 20 min, one individual started violent antennation attacks with biting or stinging which always resulted in a submissive behaviour of the opponent (table 13).

The pushing (shoving) behaviour which was observed in six out of a total of eight colonies was not detected in natural founding associations of *P.* cf. *inversa* (chapter 4.1) and thus, appears to be typical for territorial contests among freshly assembled queens.

Table 13. Total number of aggressive interactions or avoidance behaviour exhibited during the first 30 min by queens of *Pachycondyla* cf. *inversa*. Dominant queens are marked with bold letters.

Pair	Queen	Territorial behaviour		Submissive behaviour	Dominance behaviour				
		Pushes the other queen	Retreats	Crouches after antennation	Drags the other queen	Opens mandibles	Bites	Bends gaster	Steps on opponent
PiII 1	**A**	7	0	0	0	3	7	0	4
	B	0	14	15	0	4	0	0	0
PiII 2	**A**	1	0	0	19	0	3	2	0
	B	0	0	21	0	0	1	1	0
PiII 3	**A**	7	0	0	0	2	3	0	0
	B	0	2	7	0	2	0	0	0
PiII 4	A	17	0	7	0	6	7	2	0
	B	0	10	0	2	5	11	0	0
PiII 5	**A**	0	0	0	19	0	5	2	0
	B	0	0	8	0	0	0	0	0
PiII 6	A	0	0	72	0	0	2	1	0
	B	0	0	0	51	0	18	1	0
PiII 7	**A**	12	0	0	3	4	3	1	0
	B	1	11	5	0	4	0	1	0
PiII 8	A	21	2	2	0	0	0	0	0
	B	2	24	0	0	5	2	0	0

On the subsequent days of the observation, aggressive interactions became less frequent in all eight colonies (day 1 vs. day 2 to 10, Wilcoxon matched pairs test, t = 2, p = 0.025). The dominance relationships which were established during the first 20 min, remained stable over the whole observation period in all eight experimental pairs (table 14). The subordinate queen A of colony PiII 4 was laying two eggs from day 26 to 31, and therefore became very aggressive and repeatedly expelled B from the nest. Nevertheless, queen A did not became the dominant individual in the subsequent observation period.

Table 14. Dominance interactions in eight pairs of two *Pachycondyla* cf. *inversa* queens.

A and B denote queens in the order in which they were introduced into the nest. Only those aggressive interactions which resulted in recognisable reaction by the ant attacked are included.

Colony	Queen	Attacks against queen		Total observation time (min)
		A	B	
PiII 1	A	-	67	1210
	B	18	-	
PiII 2	A	-	78	800
	B	2	-	
PiII 3	A	-	19	600
	B	0	-	
PiII 4	B	83	-	660
	A	-	12	
PiII 5	A	-	66	590
	B	1	-	
PiII 6	B	138	-	320
	A	-	0	
PiII 7	A	-	36	440
	B	1	-	
PiII 8	B	101	-	540
	A	-	0	

The resident queen became dominant in five of eight trials, the new queen in three trials (table 14). Nest-ownership therefore did not influence the outcome of dominance interactions (chi-square test, $\chi^2 = 0.5$, $p > 0.4$). Additionally, the outcome of the interactions was not influenced by the time elapsed until the second queen was introduced (social rank vs. order in which individuals were placed into the arena, Mann-Whitney U-test, $n_1 = 5$, $n_2 = 3$, $U = 4$, $p = 0.293$).

In all eight trials, the queen with the larger head-width became dominant (sign test, $z = 3.75$, $p < 0.001$). Hence, queen size apparently plays an important role in the establishment of dominance hierarchies. However, the head widths of dominant and subordinate queens from seven natural founding associations did not differ significantly (Wilcoxon matched pairs test, $T = 11$, $p = 0.612$), table 15.

Table 15. Measurements (mm) of head widths of queens from seven naturally occurring polygynous colonies.

A, B and C denote queens of decreasing dominance status. Note that queen B of colony PvII 174 died before the end of this study.

Colony	Queen	Head width
PvII 190	A	2.73 ± 0
	B	2.83 ± 0
PvII 129	A	2.94 ± 0
	B	2.97 ± 0.04
PvII 21	A	2.88 ± 0
	B	2.90 ± 0.02
PvII 189	A	2.88 ± 0
	B	2.84 ± 0
PvII 49	A	2.85 ± 0
	B	2.94 ± 0
	C	2.88 ± 0.07
PvII 175	A	3.29 ±0.07
	B	2.97 ± 0
	C	2.72 ± 0
PvII 174	A	2.77 ± 0
	C	2.80 ± 0.02

4.2.4. Discussion

Foundresses of *Pachycondyla* cf. *inversa* displayed highly aggressive fights, when forced to associate in the laboratory. Queen behaviour during hierarchy establishment is thus more overtly aggressive than later, when the hierarchy is stabilised mostly by ritualised antennation. The resident queens attempted to push the other queen out of the nest. This shoving behaviour has previously not been observed in dominance interactions in *P.* cf. *inversa* (chapter 4.1) and appears to be typical for territorial contests among freshly assembled queens.

Clear dominance relationships were established within 1-20 min and remained stable over the whole observation period. The individual starting the first contest was more aggressive than the opponent and always won the interaction. In contrast to eusocial wasps, in which late joiners in a founding nest became subordinate (e.g. Samuel 1987; Pratte & Gervet 1992), the order of placing *P.* cf. *inversa* queens into the nest site did not affect the rank relationships. Nest ownership at least for a couple of days therefore appeared to be unimportant in our experiment.

Body size appeared to play an important role in the establishment of dominance hierarchies in the experimental encounters: in all trials, the queen with a larger head width became dominant. Body size has previously been found to be positively associated with the dominance rank also in colonies of several *Polistes* wasps (Turillazzi & Pardi 1977; Dropkin & Gamboa 1981; Noonan 1981; Sullivan & Strassmann 1984), the stenogastrine wasp *Liostenogaster flavolineata* (Strassmann *et al*. 1994), and bumble bees (van Doorn 1989). Head width might be related to the size and power of mandibular muscles and therefore important in mandible fighting (Pabalan *et al.* 2000). In contrast, body size did not differ between subordinates and dominants in *Parischnogaster alternata* (Strassmann *et al.* 1994), and the ants *Harpagoxenus sublaevis* (Bourke 1988) and *Leptothorax* sp. A (Heinze & Smith 1990). Data were ambiguous in worker hierarchies of *Leptothorax gredleri* (Heinze & Oberstadt 1999).

However, dominant queens from natural foundress associations were on average not larger than subordinates, and in several colonies, large queens were dominated by considerably smaller nest mates.

How can this discrepancy between laboratory and field observations be resolved? In the field, resident biases might override size asymmetries only after a more prolonged period of nest ownership. In ant species from temperate and boreal biomes, mating flights and the establishment of founding colonies occur during a rather short period of time and queens therefore are quite likely to gather in the same nest site within a few days. Mating is less synchronised in many tropical ants (e.g. Torres *et al*. 2001), and male and female sexuals of *P.* cf. *inversa* have been found both in March and November. Young queens might therefore attempt to join other queens that already inhabited a nest site for several weeks. Well-established nest owners might be more successful in dominating even large newcomers, because a nest containing eggs and larvae is even more valuable to the nest-owner than to the usurper.

5. Chemical profiles, division of labour and social status in *Pachycondyla* cf. *inversa* queens

5.1. Introduction

Cuticular hydrocarbons have long been assumed to play a fundamental role in nestmate recognition in social insects (Hölldobler & Wilson 1990; Singer 1998; Lahav *et al.* 1999). Individuals living in the same society share a bouquet of chemicals on their cuticles which serves as a "colony odour" and enables them to discriminate between nestmates and strangers. Additional intracolonial variation in hydrocarbon patterns is associated with differences in sex, caste, and developmental stage (Mintzer *et al.* 1987; Bonavita-Cougourdan *et al.* 1988, 1990, 1993; Morel & Blum 1988; Liu *et al.* 1998), and also with the role an individual performs in the society (Bonavita-Cougourdan *et al.* 1993; Wagner *et al.* 1998).

Cuticular cues apparently also convey information about the social and reproductive status of an individual. For example, in a number of ant species, workers and/or queens form hierarchies, in which only the highest ranking individuals reproduce (Heinze *et al.* 1994). From the course of dominance interactions it is evident that differences in social status are recognised, suggesting the occurrence of chemical "status labels". Indeed, the socially dominant, egg-laying α-workers in colonies of the queenless ponerine ant, *Dinoponera quadriceps,* have a much larger quantity of 9-hentriacontene on their cuticle than their nestmates do (Monnin *et al.* 1998). The amount of this substance on the cuticle of the ß-worker increases significantly after the α-worker has been removed from the nest (Peeters *et al.* 1999). Differences in the pattern of cuticular hydrocarbons associated with dominance rank and/or reproductive status have also been demonstrated in *Harpegnathos saltator* (Liebig *et al.* 2000) and *Diacamma ceylonense* (Cuvillier-Hot *et al.* 2001).

Young queens of the neotropical ponerine *Pachycondyla* cf. *inversa* may co-operate during colony founding (pleometrosis). About half of all founding colonies contain two, three or more queens (chapter 2). Co-foundresses engage in aggressive antennation, and by this the dominant queen forces the subordinate to forage. Social status is apparently not associated with reproductive status: all foundresses lay eggs at similar rates, though the subordinate may be harassed during egg laying and some of her eggs may be eaten by the dominant (chapter 4.1). Observations suggested that social status might be recognised without direct contact at a distance of a few millimetres. Therefore, cuticular hydrocarbons were extracted from live founding queens by solid-phase microextraction (SPME), and were subsequently subjected to a GC/MS analysis to investigate whether different social status is associated with differences in the chemical bouquet.

5.2. Methods

Ant Culture and Behavioural Observations

In March 1998, 26 foundress associations of *Pachycondyla* cf. *inversa* were collected from their nests in rotting cocoa pods and knotholes in an experimental cocoa plantation (CEPLAC) near Itabuna in north-eastern Brazil. In the laboratory, the ants were housed in artificial nests (see 4.1.2). Rearing conditions of the colonies were similar to those described in chapter 4.1.2.

The behaviour of the individually marked queens in three founding associations with two, and three associations with three queens each was recorded for a total observation time of 5 to 9 h per colony. Workers had not yet eclosed during this period. The social relations among individual queens were determined from their behaviour in dyadic encounters (for details, see chapter 4.1). In addition, aggressive interactions between two queens of another colony (PvII 177) were sporadically observed. Because of the short observation time, this colony was not included in chapter 4.1. However, as in the other

foundress associations one queen appeared to be clearly dominant over the other. Dominance relations among queens remained stable over the whole study period.

SPME and Gas Chromatography

Cuticular hydrocarbons were sampled with a 30µm polydimethylsiloxane fibre. Live ants (16 queens from six experimental colonies and 26 workers) were immobilised by gently pushing them head down into a slit in rubber foam. The fibre was then carefully rubbed against the 3rd, 4th and 5th sclerites of the abdomen for 15 min. Because of the flexible structure of the fibre, the ants were not injured and it was possible to repeat the extraction between two to seven times after waiting for at least 12 h to leave enough time for the replenishment of cuticular hydrocarbons.

Substances were desorbed from the fibre in the injection port of a gas chromatograph in the splitless mode for 5 min. As in this case the SPME technique did not allow the usage of an internal standard, two external standards (n-C_{15} and n-C_{27}) were used to estimate the quantity of substances from peak areas.

For the analysis of Dufour and poison glands, workers which had eclosed in the study colonies after the behavioural and chemical analyses of queens were killed by freezing. Glands were dissected out of the abdomen, sealed in small boron silicate capillaries and injected into the gas chromatograph using the solid sample injector method (Morgan & Wadhams 1972).

Gas chromatography analyses were performed in a HP 5890 chromatograph with a split-splitless injector, heated to 290 °C, and a SE-52 fused-silica capillary column (25 m x 0.22 mm i.d., 0.25 µm film thickness; effluent split 300:1). A nitrogen flow of 2 ml/min was used as carrier gas. The oven temperature was kept at 60 °C for 4 min, increased at a rate of 8 °C/min, to 280 °C, and thereafter kept at 280 °C for 25 min.

For combined gas chromatography – mass spectrometry analysis (GC/MS), a Varian 3400 gas chromatograph linked to a Finnigan MAT90 sector field mass analyser was used. Substances were eluted with helium carrier gas at 1 ml/min, using the same temperature profile as above. The 70 eV EI spectra were recorded at a rate of 1 scan/s scanning from 38 to 550 atomic mass units. To estimate the similarity of the hydrocarbon

profiles obtained from different GC analyses, Kováts indices were calculated for each peak and the variation of the coefficients was compared within and among individuals. Methyl-branched alkanes were identified by comparison of Kováts indices and diagnostic ion fragmentation.

At the end of the study, 15 queens were dissected to check the condition of their spermatheca and ovarian activity (all queens from three colonies with two queens and five queens from two three-queen colonies, chapter 4.1). Unfortunately, the Dufour glands of queens were not analysed.

5.3. Results

In all seven founding colonies, queens showed aggressive interactions and formed clear dominance hierarchies, with the dominant queen specialising in brood guarding and the subordinate queen in foraging. The frequency of egg-laying did not differ significantly between individual queens despite different social status. However, dominant queens were observed harassing subordinates during egg laying and feeding on their eggs. The ovaries of all dissected queens were developed and their spermathecae contained sperm. Details on behavioural observations are reported in chapter 4.1.

In the chromatograms obtained by SPME/GC, 17 substances - alkanes, alkenes, and methyl-branched alkanes ranging from C_{15} to C_{29} - were identified (figure 7). The most abundant components, 3-Me-C_{27} and 3,11-Di-Me-C_{27}, made up approximately 20% and 35%, respectively, of the cuticular hydrocarbons.

In each study colony, only the socially dominant queen or queens consistently showed considerable amounts of n-C_{15} and n-C_{17}:1 (150 to 450 ng) on the dorsal surface of their abdomen (figure 8), constituting approximately 7% each of the total amount of cuticular hydrocarbons extracted by SPME. "Chemical hierarchies" based on the presence of these two substances matched the rank orders constructed from behavioural observations (table 16).

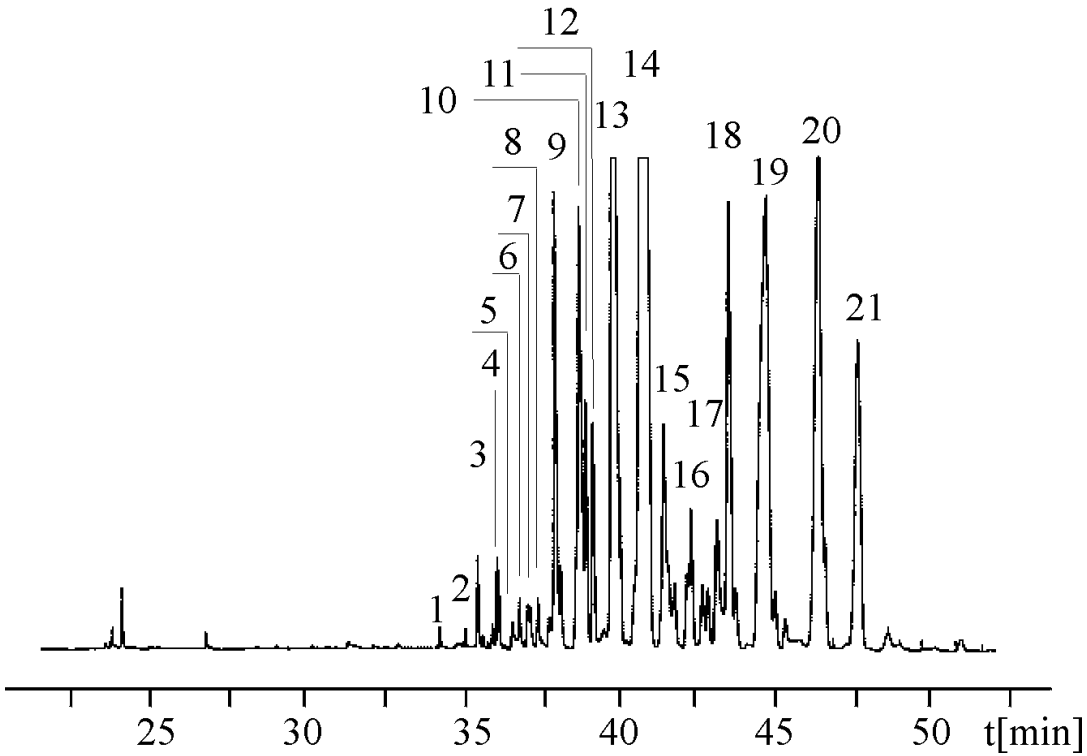

Figure 7. Gas chromatogram of hydrocarbons obtained by SPME from the cuticle of a low-ranking founding queen of *Pachycondyla* cf. i*nversa*. Substances: 1) n-C_{25}, 2) 11-Me-C_{25}, 3) 3-Me-C_{25}, 4), 3,9-di-Me-C_{25}, 5) 10-Me-C_{26}, 12-Me-C_{26}, 6) 6-Me-C_{26}, 7) 4-Me-C_{26}, 8) 2-Me-C_{26}, 9) n-C_{27}, 10) 9-Me-C_{27}, 11-Me-C_{27}, 13-Me-C_{27}, 11) 7-Me-C_{27}, 12) 5-Me-C_{27}, 13) 3-Me-C_{27}, 14) 3,11-di-Me-C_{27}, 15) 12-Me-C_{28}, 16) C_{29}:1, 17) 4,12-di-Me-C_{28}, 18) n-C_{29}, 19) 11-Me-C_{29}, 13-Me-C_{29}, 20) 3-Me-C_{29}, 21) 3,11-di-Me-C_{29}.

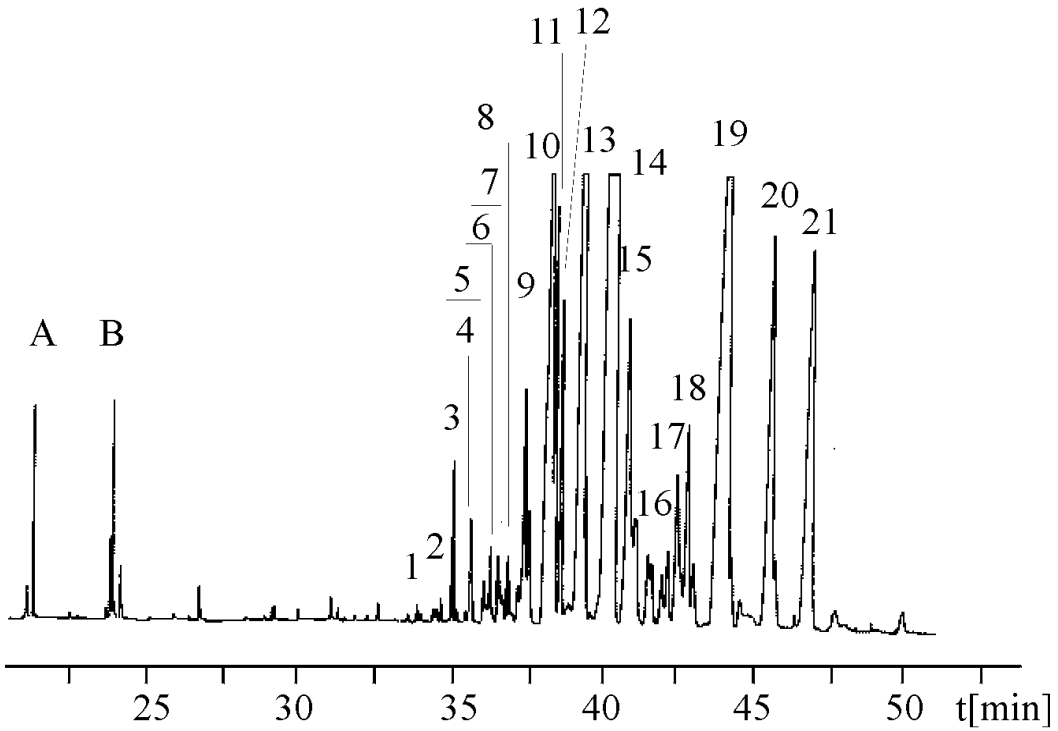

Figure 8. Gas chromatogram of cuticular hydrocarbons obtained from a socially dominant founding queen of Pachycondyla cf. inversa. The substances are numbered as in figure 7. Two substances (A: n-C15, B: n-C17:1) are present in large quantities only on the cuticle of dominant queens.

Table 16. Rank orders based on behavioural observations (see chapter 4.1) and the relative amount of cuticular n-C_{15} and n-C_{17}:1 of co-founding queens of the ant, *Pachycondyla c*f. *inversa.* Cuticular substances were extracted from each queen at least three times by SPME. In each colony, the two substances were almost absent in the lowest ranking queen.

Colony	Relative amount (%) of C_{15} Mean ± S.D.			Relative amount (%) of C_{17}:1 Mean and coefficient of variation		
	α-queen	β-queen	γ-queen	α-queen	β-queen	γ-queen
PvII 21	2.97 ± 0.29	0.00 ± 0.00	-	3.15 ± 0.23	0.01 ± 0.00	
PvII 49	5.58 ± 0.17	1.42 ± 0.13	0.01 ± 0.00	4.21 ± 0.16	1.42 ± 0.11	0.00 ± 0.00
PvII 129	2.30 ± 0.12	0.00 ± 0.00	-	2.65 ± 0.16	0.00 ± 0.00	-
PvII 174	5.76 ± 0.72	4.46 ± 0.50	0.02 ± 0.00	6.18 ± 0.91	5.16 ± 0.42	0.03 ± 0.01
PvII 175	6.99 ± 0.45	1.84 ± 0.08	0.04 ± 0.01	5.36 ± 0.11	2.29 ± 0.14	0.04 ± 0.01
PvII 177	3.86 ± 0.15	0.01 ± 0.00	-	3.73 ± 0.09	0.02 ± 0.00	-
PvII 190	3.65 ± 0.09	0.00 ± 0.00	-	4.60 ± 0.15	0.00 ± 0.00	-

Interestingly, n-C_{15} and n-C_{17}:1 were the major components of the Dufour glands at least of workers (figure 9). Poison gland secretions did not contain considerable quantities of these two substances but consisted mostly of fatty acids. n-C_{15} and n-C_{17}:1 were also not found on the cuticles of larvae (only male larvae investigated). Instead, here the major components were unbranched alkanes, especially C_{27}, C_{28} and C_{29}.

Dominant queens frequently first rubbed the tip and subsequently the back of their abdomen with their hind legs. This behaviour might serve to distribute Dufour gland secretions on the cuticle of the abdomen; however, the frequency of this behaviour has not been quantified.

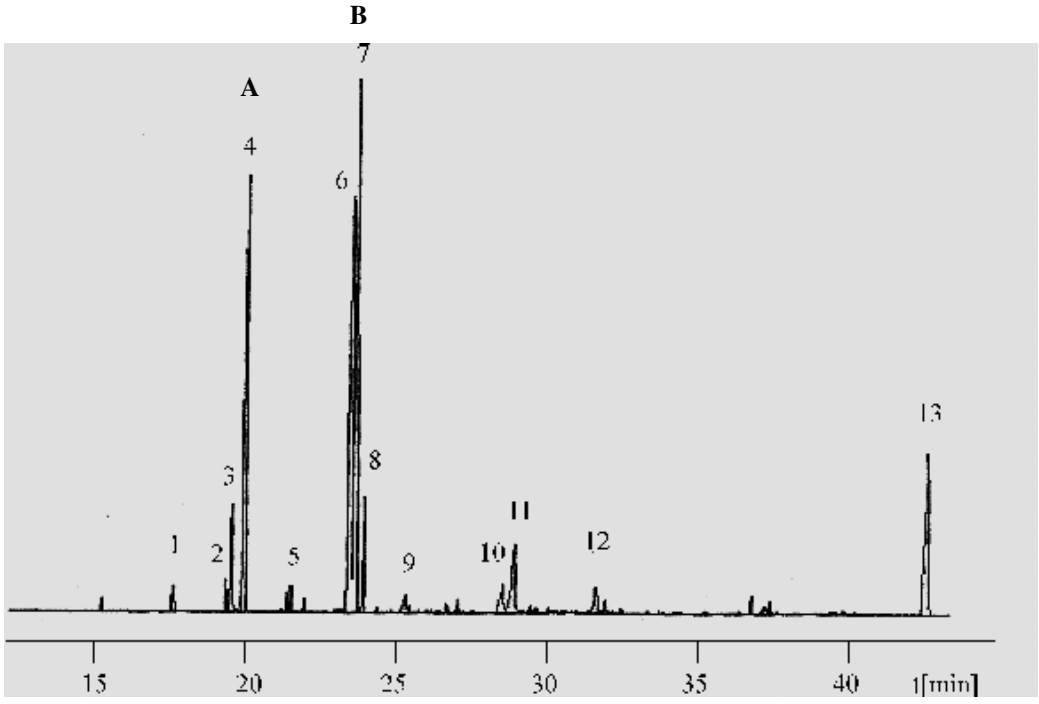

Figure 9. Reconstructed chromatogram of the GC/MS-analysis of three Dufour glands of *P.* cf. *inversa* workers, investigated using the solid sample technique. Substances: 1) n-C_{14}, 2) C_{15}:2, 3) C_{15}:1, 4) n-C_{15} (***A***), 5) n-C_{16}:1, 6) n-C_{17}:2, 7) n-C_{17}:1 (***B***), 8) n-C_{17}, 9) C_{14}:0-acid, 10) cis-9-C_{16}:1-acid, 11) C_{16}:0-acid, 12) cis-9-C_{18}:1-acid, 13) unidentified substance.

5.4. Discussion

Division of labour between queens in foundress associations of the ant *Pachycondyla* cf. *inversa* appears to be associated with differences in their cuticular hydrocarbon pattern. The profiles of dominant queens which engaged in brood care were characterised by the presence of two substances, n-C_{15} and n-C_{17}:1, which were absent on the cuticle of subordinate foraging queens. Because the chemical results closely matched the observed social hierarchies in all seven studied colonies, it appears unlikely that the amounts of n-C_{15} and n-C_{17}:1 vary randomly between queens regardless of their social status. Queens with medium rank in three-queen colonies both showed intermediate behaviour and had amounts of C_{15} and C_{17}:1 between those of the α- and the γ-queen. It was not possible to determine why the β-queen in PvII 174 was chemically more similar to the α- than the γ-queen, in contrast to the β-queens in PvII 49 and PvII 175.

n-C_{15} and n-C_{17}:1 were found in the Dufour glands of workers of *P.* cf. *inversa*, but not in their poison glands. It is likely that they are present also in the Dufour gland of queens (which were not studied) and from there are distributed onto the cuticle by dominant queens rubbing first the tip and subsequently the back of the abdomen with their hind legs.

Intracolonial variability in cuticular hydrocarbon profiles has previously been shown to be associated with differences in task (Bonavita-Cougourdan *et al.* 1993; Wagner *et al.* 1998) and reproductive status (Peeters *et al.* 1999; Liebig *et al.* 2000; Cuvillier-Hot *et al.* 2001). In founding associations of *P.* cf. *inversa*, task and social status are tightly correlated. However, egg-laying rates are not significantly different between individual queens, though the dominant may eat the eggs laid by the subordinate (chapter 4.1). Hence, in contrast to the changes in cuticular hydrocarbons reported from other ponerine ants (Peeters *et al.* 1999; Liebig *et al.* 2000; Cuvillier-Hot *et al.* 2001), the two substances found only on dominant queens do not seem to be closely linked to ovarian development. It is also unlikely that the differences are proximately caused by the two queens taking over different tasks e.g., by the dominant queen acquiring the substances during more

intensive brood care, because they were absent from the cuticle of larvae. It therefore appears reasonable to assume that the presence of the two substances is somehow associated with social dominance alone. Chemical "badges of status" have been identified in male cockroaches, *Nauphoeta cinerea*, where the quantities of certain constituents of the sex pheromone vary with social status (Moore *et al.* 1997). In *P.* cf. *inversa*, the distribution of n-C_{15} and n-C_{17}:1 might either be an epiphenomenon of high social status without signalling function or may serve to communicate high social status. In the latter case, the comparatively high volatility of n-C_{15} and n-C_{17}:1 might explain why subordinates react to approaching dominants before having physical contact.

Further studies

In an initial experiment, it was investigated if the two substances, n-C_{15} and n-C_{17}:1, were present on the cuticle of workers. Therefore, colony PvII 49 (see above) was examined a second time when the colony consisted of more workers. Twenty workers, and three queens were analysed by GC/MS (see above). Subsequently, the ovaries of all workers were dissected. The substances n-C_{15} and n-C_{17}:1 were not only found on the cuticle of the two dominant queens (table 16), but also on the cuticle of four workers. The ovarian status of the workers was not correlated with the presence of the two substances.

Additionally, two queenless worker colonies were studied, as the division of labour in colony PvII 49 has not been investigated: Division of labour in the queenless colonies was correlated with the dominance rank of the workers, similar to observations made by Heinze *et al*. (1996). However, similar to colony PvII 49, the substances n-C_{15} and n-C_{17}:1 which were detected on the cuticle of some workers, were not correlated with the dominance rank. Apparently, rank and fertility were not associated with n-C_{15} and n-C_{17}:1 in workers.

6. Molecular markers in the ponerine ant, *Pachycondyla obscuricornis*

6.1. Introduction

Relatedness among interacting individuals plays a central role for predictions made by theories on reproductive conflicts, e.g. theories over reproductive skew (reviewed by Johnstone 2000, or Reeve & Keller 2001), and kin selection theory (Hamilton 1964a,b). Testing these predictions requires good genetic markers which provide sufficient information on kinship. In this chapter, diverse molecular markers are described which will help to investigate the genetic structures from colonies and populations of the neotropical ponerine ant, *Pachycondyla obscuricornis*.

In this species, freshly inseminated females are presumably adopted by mature colonies after the mating flight; secondary polygyny (pers. com. J.H.C. Delabie, J. Heinze). In more advanced species, females tend to join their natal nests as new reproductives and therefore, queens often are related in secondarily polygynous colonies (Keller 1995; Crozier & Pamilo 1996; Seppä 1996). If this is also true for the more "primitive" ponerines, relatedness among queens of *P. obscuricornis* would be higher than among queens from foundress associations of *P.* cf. *inversa*. Therefore, a comparison between these two species may give valuable information to test predictions made by reproductive skew models: A high skew (one individual produces most offspring) should be associated with high relatedness, whereas a low skew (more equitable reproduction) should be associated with low, or an absence of relatedness (Reeve & Ratnieks 1993).

Five classes of markers were used in this study: allozyme and microsatellite loci, non-functional tandem-repeats (minisatellites), and a segment of the mitochondrial DNA investigated by using restriction digests, and sequencing. Many studies on the genetic structure of colonies and populations of social insect species have been based on neutral protein markers (allozyme electrophoresis; Crozier & Pamilo 1996). Allozyme loci

encode functional enzymes, and the variability results from point mutations in exons that cause charged amino acid substitutions. Individual genes can be scored at particular loci and provide good Mendelian markers. Multilocus DNA fingerprinting is based on individual variation in the number of tandem repeated sequences (in this study: minisatellites from non-coding regions). The use of this method is restricted because individual genotypes cannot be defined. In contrast, microsatellites provide genotypic data when PCR-amplified using locus specific primers. Microsatellite loci are segments of DNA containing variable numbers of short repeat units from non-coding DNA regions. Variability results from polymerase slippage during replication. Mitochondrial DNA is maternally inherited and can be used to identify matrilines, or to estimate the dispersal of females. Haplotypes can be identified by different point mutations from restriction fragments (restriction fragment length polymorphisms, RFLPs), or directly from sequences (for more details on molecular markers in population genetics, see e.g. Loxdale & Lushai 1998).

The aim of this study was to detect intra- and intercolonial variability among individuals of *P. obscuricornis*. Furthermore, seven additional *Pachycondyla* species (including *P. apicalis*) were examined by means of allozyme electrophoresis to clearly separate *P. obscuricornis* from those species. Especially *P. apicalis* is almost identical to *P. obscuricornis* in morphology, to which it occurs sympatrically. In contrast to *P. obscuricornis*, *P. apicalis* has bright yellow antenna tips and the petiole is slightly more inflated with less distinct lateral margins posteriorly (Longino 1999).

6.2. Material and methods

Ant collection

For the analysis of allozymes, microsatellites, and mitochondrial DNA, colonies of *Pachycondyla obscuricornis* were collected on the territory of the La Selva Biological Station, La Selva, Costa Rica, in October 1999. Additionally, allozymes were investigated using workers from eight *Pachycondyla* species (*P.* cf. *inversa, P. villosa*, *P.*

crenata, *P. apicalis*, *P. harpax*, *P. unidentata*, *P. crassinoda*, and *P. obscuricornis*) from a study population near Itabuna, Bahia, Brazil (see also chapter 2). For multilocus DNA fingerprinting, *P. obscuricornis* colonies were collected in March/April 1995 from the same population.

After the transfer into the laboratory, colony composition and brood number was immediately examined: *P. obscuricornis* colonies from Costa Rica consisted of 12 one-queen, 4 two-queen, 3 three-queen, 1 four-queen, 3 five-queen, and four colonies, with seven, nine, eleven and twelve queens, respectively. The number of workers ranged from 15 to 250 individuals per colony (mean number 51 ± SE 49). Additionally, two foundress associations were collected, each containing two queens, some eggs and up to six larvae. Two *P. obscuricornis* colonies from Brazil (1998) consisted of two singly founding queens, with up to two workers per colony. Six *P. obscuricornis* colonies collected in 1995 from the same population, contained at the beginning of this study (in 1998) a mean number of 30 dealated queens, 100 workers and numerous alated females and males.

Each *P. obscuricornis* colony was housed in two plastic boxes of 19 cm x 19 cm x 9 cm with a plaster floor, which were connected with a plastic tube. In one box, five connected chambers (3 cm x 3 cm x 0.5 cm) in the plaster covered by a glass plate, served as a nest. The second plastic box contained a permanent water supply and served as a feeding arena. The colonies were fed with diluted honey and live crickets every one or two days and were kept at room temperature. Rearing conditions for other *Pachycondyla* species are as described in chapter 3.1.

Allozyme electrophoresis

Whole ants were crushed in 100 µl of distilled water. Six to 14µl of the homogenate were subsequently applied onto pre-soaked cellulose acetate plates. Protein separation was carried out in three different buffer systems (0.025 M Tris-glycine pH 8.6, 0.1 M Tris-citrate pH 8.2, 0.1 M Tris-maleate-EDTA pH 7.4 and pH 8.3), with 1-9 mA per gel for 5-35 min, depending on the enzyme system (table 17). Using slightly modified protocols from Murphy *et al*. (1990), 27 enzymes were stained. One worker each from 33 colonies of *P. obscuricornis* from Costa Rica, two colonies from Brazil, one colony of

each *P.* cf. *inversa*, *P. subversa*, *P. villosa*, *P. crenata*, *P. harpax*, *P. unidentata*, and *P. crassinoda* were analysed. Additionally, three colonies of *P. apicalis* (with three workers each) were analysed. Individuals from two of these colonies were designated as *P. apicalis*, because of the typical curvature of the petiole, though their antennae were coloured black.

Multilocus DNA Fingerprinting

Multilocus DNA fingerprints were investigated by using the protocols of Heinze *et al.* (2001). Individuals were homogenised in 250 µl extraction buffer A (10 mM Tris/HCl pH 7.5, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermine, 0.15 mM spermidine) and 250 µl extraction buffer B (0.2 M Tris/HCl ph 9.0, 30 mM EDTA, 2 % SDS). The homogenate was incubated with 10 µl of proteinase K (10mg/ml) at 37° overnight. DNA was extracted twice with 250 µl phenol and once with 500 µl chloroform-isoamyl alcohol (25:1). After precipitation, the DNA was dried for 1 h, re-suspended in ddH_2O, and digested with 20 units of *Pal I*, or *Sau3A I*. Products were subsequently separated on 25 cm long 1 % agarose gels at 60 V for 20 h and transferred to uncharged nylon membranes by vacuum blotting. DNA was immobilised by UV irradiation and hybridised with one of the following probes: $(GGAT)_4$, $(GATA)_4$, $(GTG)_5$, or $(GACA)_4$. Fragments were endlabeled with digoxigenin and visualised by chemo luminescence on a Fuji-RX X-ray film. Two to 5 workers from 2 to 4 colonies of one *P. obscuricornis* population were investigated. Additionally, the DNA of two individuals from different colonies was analysed with each of the following restriction endonucleases: *Cfo I*, *Alu I*, *Hinf I*, *EcoR I*, *Pst I*, *Rsa I*, *BamH I*, *Hind III*, *Mbo I*. The product was subsequently hybridised with $(GGAT)_4$.

Intra- and intercolonial band-sharing coefficients (S) of the individuals were calculated as $S = 2\ N_{AB}/(N_A+N_B)$, where N_{AB} is the number of scorable bands of similar intensity and similar electrophoretic mobility in individuals A and B, and N_A and N_B are the total number of scorable bands in individual A and B, respectively (Lynch 1990; Bruford *et al*. 1992).

Table 17. Conditions for allozyme-electrophoresis in the ant, *Pachycondyla obscuricornis*.

Buffer system: 1 (0.1 M Tris-citrate pH 8.2), 2 (0.1 M Tris-maleate-EDTA pH 7.4 or pH 8.3), and 3 (0.025 M Tris-glycine pH 8.6). EC: enzyme code number. The quality of electromorphs is indicated by ++ (resolution/colouration is sufficient for interpretation), + (resolution is not sufficient, but colouration), 0 (no banding patterns visible).

Enzyme	EC	Buffer	Power [mA]	Voltage [V]	Duration [min]	Application frequency	Quality
ACON	4.2.1.3	1	5	200-230	30	4	++
ADH	1.1.1.1	1,2,3	1-5	150-250	5-15	3-7	0
AK	2.7.4.3	1,2,3	1-5	150-250	10-30	3-7	+
ALP	1.2.2.1	2	3	200-230	20	3	++
AO	1.2.1.5	1,2,3	1-5	150-250	5-15	3-7	0
ARK	2.7.33	1,2,3	1-5	150-250	10-30	3-7	+
EST	3.1.1.1	1,2	1-5	200-220	15	3-4	+
FUMH	4.2.1.2	1,2,3	1-5	150-250	5-15	3-7	0
G3PDH	1.1.1.8	1	6	200-240	20	3	++
G6PDH	1.1.1.49	1	5	200-230	30	3	++
GCDH	1.1.1.47	1,2,3	1-5	150-250	10-30	3-7	+
GPI	5.3.1.9	1	9	200-230	15	3	+
GTDH (NAD)	1.1.1.46	1,2,3	1-5	150-250	5-15	3-7	0
GTDH(NADPH)	1.1.1.45	1,2,3	1-5	150-250	5-15	3-7	0
HBDH	1.1.1.30	1,2,3	1-5	150-250	10-30	3-7	+
HK	2.7.1.1	1	5	200-230	30	4	++
IDDH	1.1.1.14	1,2,3	1-5	150-250	5-15	3-7	0
IDH	1.1.1.42	1	5	200-230	25	5	++
LDH	1.1.1.27	1	1-5	200-220	20	5	+
MDH	1.1.1.37	1	4	200-230	10	3	++
MDHP	1.1.1.40	1	6	200-230	15	3	++
PGDH	1.1.1.44	1	5	200-230	30	5	++
PGM	5.4.2.2	1	5	200-230	30	4	++
PK	2.7.3.3	2	3	200-230	20	3	++
SOD	1.15.1.1	1,2,3	1-5	150-250	5-15	3-7	0
TRE	3.2.1.28	1	5	200-230	20	3	++
XDH	1.2.3.2	1	6	200-230	20	3	++

DNA extraction for microsatellites and mtDNA

For the analysis of microsatellites and mtDNA, DNA was extracted from 10-20 mg of thorax tissue of frozen workers using either a phenol/chloroform extraction protocol as described in Sambrook *et al*. (1989), or a Puregene® DNA Isolation Kit (Gentra Systems). For the phenol/chloroform extraction, 10-20 mg tissue was homogenised in 18.75 µl 1M DTT, 5µl proteinase K (20 mg/ml), 140 µl 10% SDS and 536.25 µl TNE buffer (100 mM NaCl, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0) to a total volume of 700 µl. After overnight incubation at 37 °C, DNA was extracted three times with 750 µl phenol, phenol/chloroform-isoamyl alcohol (1:1), and chloroform-isoamyl alcohol. After precipitation, the DNA was dried for one hour, re-suspended in 50 µl ddH_2O and stored at -70 °C until use.

For DNA isolation with the Puregene® Isolation Kit, 10-20 mg tissue was ground with 50 µl of Cell Lysis Solution and incubated for 45 min at 65 °C. Subsequently, 17 µl of Protein Precipitation Solution were added and then centrifuged for 5 min at 14000 rpm. The supernatant was transferred to a new tube, and the DNA was precipitated with 65 µl of 100 % cold isopropanol. After centrifugation for 5 min at 14000 rpm, the supernatant was discarded and the pellet was washed with 50 µl of 75 % ethanol, dried for one hour and re-suspended in 30 µl ddH_2O.

Microsatellite analysis

Eight primer pairs originally established for *Platythyrea punctata* (Schilder *et al*. 1999; primers 2001, 2701, 2801, 3302, 3303, 3401, 3506, 4101) and seven primers originally developed for *Gnamptogenys striatula* (Giraud *et al*. 1999; L2, L4, L6, L7, L16, L19, L20) were used to study the genetic variation in *P. obscuricornis* colonies. Therefore, 10 to 15 workers each from different colonies were genotyped. To optimise microsatellite banding patterns, various protocols were used. The protocol described here gave the best results: The 25 µl reaction mixture for one polymerase chain reaction contained 1 µl DNA, 2.5 µl 10x *Taq* DNA polymerase buffer (without $MgCl_2$), 1.5-2 mM $MgCl_2$, 100 µM dNTPs, 10 pmol of each primer, and 1 U *Taq* polymerase (MBI). The PCR profile consisted of an initial denaturation step (3 min at 94 °C), followed by 35

cycles of 60 sec at 92 °C, 75 sec at 50-52 °C and 60 sec at 72 °C. PCR products were separated electrophoretically on 40 cm long non-denaturing 10% acrylamide gels at 25-30W for 3 to 4 h. DNA bands were stained for 45 min with SYBR®green and visualised under UV light (280 nm). Alleles were distinguished by their relative mobility on the gel.

Restriction Fragment Length Polymorphisms (RFLPs)

Mitochondrial haplotypes were analysed using a slightly modified protocol from Foitzik & Herbers (2001). A 1550 bp fragment, which combines part of the COI and COII region of the mitochondrial DNA was investigated by using the primers C1-J-2195 (alias COI-RLR) and C2-N-3661 (alias Barbara), Simon *et al*. 1994. The experimental conditions which gave the best results were described here: The 25 µl reaction mixture of one polymerase chain reaction contained 1 µl DNA, 2.5 µl 10x *Taq* polymerase buffer, 2.8 mM $MgCl_2$, 0.24 mM of each dNTP, 1.4 µM of each primer, and 0.8 U *Taq* polymerase (MBI). The PCR profile consisted of an initial denaturation step (5 min at 95 °C), followed by 35 cycles of 60 sec at 94 °C, 60 s at 44 °C and 5 min at 68 °C. Subsequently, the amplification product was cut with *Msp I*, *Hind III*, *Taq I*, *Cla I* and *Hinf I*. The 16 µl reaction mixture for one restriction digest consisted of 8 µl PCR product, 1.6 µl 10x *Taq* DNA polymerase buffer, 0.16 µl BSA, 5.9 µl ddH_2O, and 0.36 µl of the respective restriction endonuclease. After incubation at 37 °C overnight, products were separated electrophoretically on 0.8 % agarose gels (100 mA) and stained with SYBR®green for 15 min. The DNA bands were visualised under UV light (280 nm). Eight workers, each from different colonies of La Selva, Costa Rica, were investigated.

Sequencing of mtDNA

A 1550 bp fragment which combines part of the COI and COII region of the mitochondrial DNA was investigated (see above). PCR conditions were similar to those described for RFLPs. Products were separated on 0.8 % agarose gels for 15 min at 100

mA, stained with SYBR®green and visualised under UV light (280 nm). DNA fragments were excised from the gel and purified using the NucleoSpin® Extract 2 in 1 kit supplied by Macherey and Nagel. The cycle sequencing consisted of 25 cycles of 10 sec at 96 °C, 10 sec at 44°C and 4 min at 60 °C. The 20 µl reaction mixture for one reaction contained 10 µl DNA, 2 µl DNA Sequencing Kit Ready Mix (PE Biosystems), 6 µl 2.5x buffer and 10 pmol of one primer (C1-J-2195 or C2-N-3661). Subsequently, 20 µl of the product was precipitated with ethanol, re-suspended in 20 µl TSR buffer, denatured at 90°C and then sequenced with an ABI Prism™ 310 Genetic Analyser (Applied Biosystems). Eight workers, each from different colonies of La Selva, Costa Rica, were investigated.

6.3. Results

Allozyme electrophoresis

Twenty of a total of 27 examined enzyme systems could be reliably visualised (table 17). In 35 colonies of P*achycondyla obscuricornis*, neither intra- nor intercolonial variability could be detected. Thirty-three colonies from Costa Rica and two colonies from Brazil did not differ in their electromorph patterns.

The banding patterns of eight enzymes showed clear differences between individuals from 35 colonies of *P. obscuricornis* and three colonies of *P. apicalis*: ACON, GPI, HBDH, IDH, PGM, HK, locus 2 of MDH, and locus 1 and 2 of MDHP (table 18). In both species, heterozygote individuals were not detected. Therefore, the taxa are fixed for different electromorphs at these eight diagnostic loci. The curvature of the petiole of individuals from two colonies was typical for *P. apicalis*. Thus these two colonies were designated as *P. apicalis*, though the antennae tips of the workers were not yellow coloured. The allozyme pattern showed, that these species indeed belonged to *P. apicalis*. In comparison with eight other *Pachycondyla* species, individuals of *P. obscuricornis* differed consistently in four diagnostic loci: ACON, HBDH, MDHP-1 and MDHP-2 (table 18).

Table 18. Electromorph patterns of 18 enzymes in eight different species of the ant genus *Pachycondyla*.

Migration velocity of different electromorphs in the gel is indicated by *a* (slow) to *f* (fast). No.: number of colonies.

Enzyme	*P.* cf. *inversa*	*P. villosa*	*P. subversa*	*P. obscuricornis*	*P. apicalis*	*P. crenata*	*P. harpax*	*P.unidentata*	*P. crassinoda*
ACON	*bb*	*bb*		*aa*	*bb*	*bb*	*bb*		*cc*
ALP	*bb*	*cc*	*aa*						
EST	*aa*	*cc*	*bb*						
GPI	*cc*	*bb*	*aa*	*ee*	*ff*	*dd*	*dd*	*dd*	*ee*
HBDH	*bb*	*bb*		*aa*	*bb*	*bb*	*bb*	*bb*	*cc*
HK				*aa*	*bb*				
IDH	*bb*	*cc*	*aa*	*cc*	*bb*	*bb*	*bb*	*bb*	
LDH	*aa*	*aa*			*aa*	*aa*	*aa*	*bb*	*bb*
MDH-1	*aa*	*aa*		*aa*	*aa*	*bb*		*cc*	
MDH-2	*bb*			*bb*	*aa*	*aa*		*aa*	
MDHP-1	*bb*	*bb*		*aa*	*bb*	*bb*		*bb*	
MDHP-2	*bb*			*aa*	*cc*	*bb*		*bb*	
PGDH-1	*aa*	*aa*	*aa*		*aa*	*aa*		*bb*	*bb*
PGDH-2	*aa*	*aa*			*aa*	*aa*		*cc*	*bb*
PGM	*aa*	*aa*		*cc*	*dd*	*aa*	*dd*	*bb*	*cc*
PK				*aa*	*aa*				
TRE	*bb*	*aa*							
XDH	*aa*	*bb*	*bb*	*bb*	*bb*	*bb*	*bb*		*cc*
No.	1	1	1	34	3	1	1	1	1

Multilocus DNA Fingerprinting

The digestion of DNA with *Pal 1* and hybridisation with $(GATA)_4$ or $(GTG)_5$ did not reveal any variability in three workers from two *P. obscuricornis* colonies, and two workers from one colony. The combination *Pal I*/$(GACA)_4$ resulted in banding patterns with two to five scorable bands per individual worker. The variability was very low with a mean intercolonial band-sharing coefficient of S = 0.9 ± SD 0.1 and a mean intracolonial bandsharing-coefficient of S = 0.97 ± 0.03 (to colonies with three and four workers, respectively). Hybridisation with $(GGAT)_4$ made it possible to resolve three to seven clear bands per individual in two colonies each with three, and two colonies each with five workers (figure 10). The intracolonial variability obtained from banding patterns of four colonies with three workers each, was very low with a mean band-sharing coefficient of S = 0.9 ± 0.22, whereas the intercolonial variation was higher with a mean band-sharing coefficient of S = 0.58 ± 0.26 (range: 0.125 to 0.89).

Digestion of DNA with *SAU 3A I* and hybridisation with $(GATA)_4$ or $(GTG)_5$ did not lead to scorable banding patterns in eight workers from four colonies. With the combination *SAU 3A I* and $(GACA)_4$, eight to nine scorable bands in two colonies each with three and four workers, respectively could be distinguished. The intercolonial band-sharing coefficient was 0.9 ± 0.07 and the intracolonial bandsharing-coefficient was 0.7 ± 0.13. Similar results were obtained by using $(GGAT)_4$, with a band-sharing coefficient S of 0.9 ± 0.09 (intercolonial) and 0.66 ± 0.29 (intracolonial). Three to five scorable bands in two colonies each with three, and in two colonies each with four workers were detected.

In an initial screening of DNA probes which were cut with different restriction endonucleases and hybridised with $(GGAT)_4$, six out of nine restriction endonucleases led to scorable banding patterns. Two to five scorable bands were obtained per individual worker. The bands obtained by the combination *Cfo I*/$(GGAT)_4$ did not differ among two workers from different colonies. In the banding patterns obtained by *Rsa I* and *BamH I* three bands differed between two workers from different colonies, in *Alu I* and *Mbo I* two bands differed, and in *Pst 1* one band. *Hinf I*, *EcoR I*, and *Hind III* were not visualised.

Most banding patterns obtained from workers of *P. obscuricornis* were weakly coloured, as a consequence of DNA limitation due to the small size of individuals. The banding patterns obtained by hybridisation with $(GACA)_4$ resulted in bands which were hardly distinguishable.

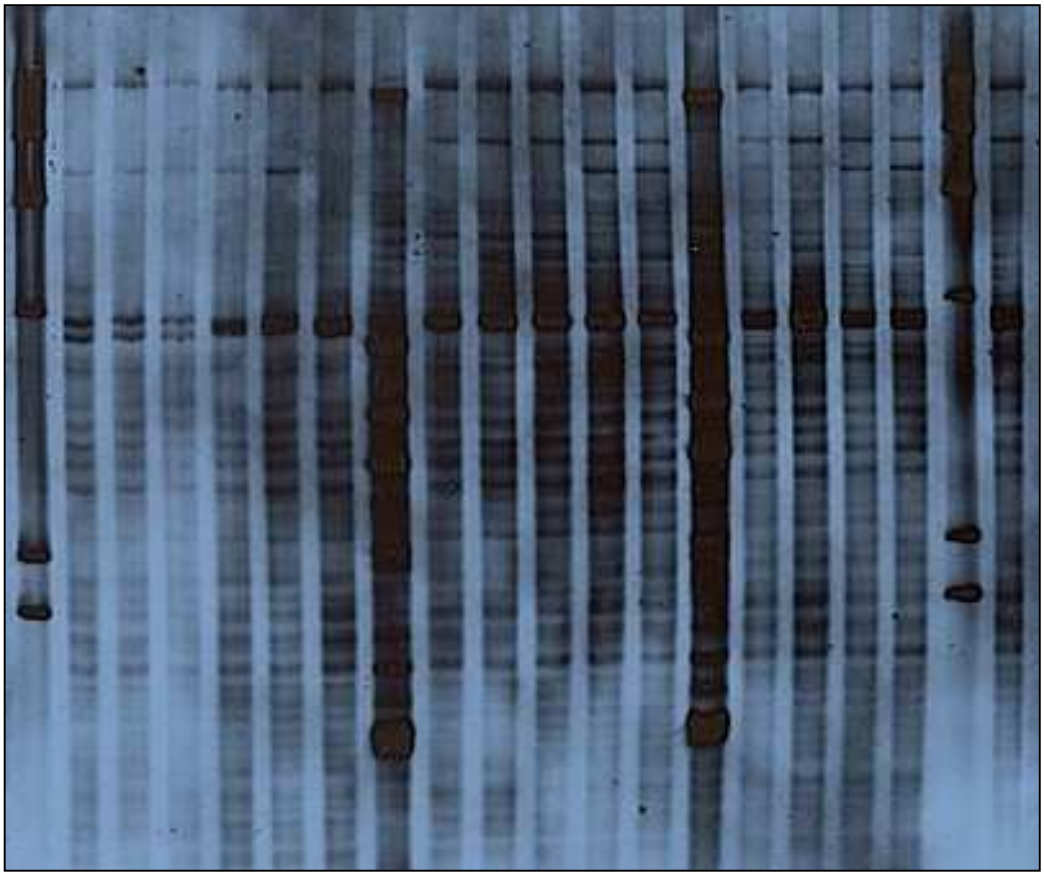

Figure 9. Multilocus DNA fingerprints (*Pal I*/$(GGAT)_4$) from four colonies of the ant *Pachycondyla obscuricornis*.

Microsatellite Analysis

Two of a total of 15 investigated microsatellite loci were found to be polymorphic in an initial screening of 10 to 15 workers each from different colonies from one *P.*

obscuricornis population in Costa Rica. Locus 3302 and L6 displayed low variability with two different alleles in 14 and 10 workers, respectively. The loci L7, L16, and L19 did not show any amplification products, the ten remaining loci were monomorphic.

Restriction Fragment Length Polymorphisms (RFLPs)

In an initial screening of eight *P. obscuricornis* workers from different colonies from one population, polymorphic banding patterns were obtained when cutting with *Taq I* or *Hind III*: Two haplotypes could be distinguished among seven and eight workers, respectively. No variability was detected by using *Cla I. Msp I* and *Hinf I* did not cut mtDNA under the chosen conditions. Though various protocols were used, PCR conditions and the digestion of mtDNA did not lead to a good quality of banding patterns. In approximately 70 % of all experiments, restriction endonucleases did not cut at all.

Sequencing of mtDNA

In an initial sequencing of the COI/COII region of the mitochondrial DNA, six, respectively seven workers, each from different colonies of the study population in Costa Rica were sequenced successfully (tables 19 and 20). Using the primer C1-J-2195, five different haplotypes could be distinguished, table 19 (only differences in basepairs which were marked in blue were counted). The individuals from colonies CR-52 and BG 99-151 showed the same haplotypes. However, these two individuals differed when sequenced using the primer C2-N-3661 (table 20).

The significance of differing basepairs which were marked in red in tables 19 and 20 may be due to real mutations, reading errors, or, particularly in experiments using primer C2-N-3661, by contamination.

Table 19. Sequence of mtDNA obtained from the primer C1-J-2195 (alias COI-RLR).

Significant differences among individuals were marked in blue. Differences among individuals marked in red are presumably due to artefacts, e.g. at the beginning, or ending of the sequencing read, or due to contamination)

Colony	10	20	30	40	50	60	70	80
BG 99-126	TTATA–CT	TCTTATTCTT	CCAGGATTTG	GTTTAATTTC	CCACATTATT	ATAAATGAA– –A	GAGGAAAAAA	AGAAACTTTT
BG 99-141	TTTNATATNN	TCTTATTCTT	CC–GGATTNG	–TTTAATTTC	CCACATTATT	ATAAATGAA– –A	GAGGAAAAAA	AGAAACTTTT
CR 52	NTATN– –T	—CTTATTCTT	CC–GNATTTG	GTTTAATTTC	CC–CATTATT	ATAAATGAA– –A	GAGGAAAAAA	AGAAACTTTT
BG 99-116	TTAT– –T	TCTTAT–CTT	CCAa GATTTG	GTTTAATTTC	CCACATTATT	ATAAATGAA CGA	GAG–AAAAAA	AGAAACTTTT
BG 99-151	TTNATATATAT	TCTTATTCTT	CCaGGATT–G	GTTTAATTTC	CC–CATTATT	ATAAATGAA– –A	GAGGAAAAAA	AGAAACTTTT
BG 99-104	– – A–TATAT	TCTAATTCTN	CCNG–ATT–G	GTTTAATTTC	CC–CATTATT	ATAAATGAA– –A	GAGGAAAAAA	AGAAACTTTT

	90	100	110	120	130	140	150	160
BG 99-126	GGATCTTTAG	GAATAATTTA	TGCTATATTA	TCAATTGGAT	TCCTTGGATT	TATTGTTTGA	GCCCATCATA	TATTTACTGT
BG 99-141	GGATCTTTAG	GAATAATTTA	TGCTATATTA	TCAATTGGAT	TCCTTGGATT	TATTGTTTGA	GCCCATCATA	TATTTACTGT
CR 52	GGATCTTTAG	GAATAATTTA	TGCTATATTA	TCAATTGGAT	TCCTTGGATT	TATTGTTTGA	GCCCATCATA	TATTTACTGT
BG 99-116	GGATCTTTAG	GAATAATTTA	TGCTATATTA	TCAATTGGAT	TCCTTGGATT	TATTGTTTGA	GCCCATCATA	TATTTACTGT
BG 99-151	GGATCTTTAG	GAATAATTTA	TGCTATATTA	TCAATTGGAT	TCCTTGGATT	TATTGTTTGA	GCCCATCATA	TATTTACTGT
BG 99-104	GGATCTTTAG	GAATAATTTA	TGCTATATTA	TCAATTGGAT	TCCTTGGATT	TATTGTTTGA	GCCCATCATA	TATTTACTGT

	170	180	190	200	210	220	230	240
BG 99-126	AGGTATAGAC	ATTGATACAC	GTGCATATTT	TACATCTGCA	ACCATAATTA	TTGCTATTCC	AACTGGAATT	AAAATTTTTA
BG 99-141	AGGTATAGAC	ATTGATACAC	GTGCATATTT	TACATCTGCA	ACCATAATTA	TTGCTATTCC	AACTGGAATT	AAAATTTTTA
CR 52	AGGTATAGAC	ATTGATACAC	GTGCATATTT	TACATCTGCA	ACCATAATTA	TTGCTATTCC	AACTGGAATT	AAAATTTTTA
BG 99-116	AGGTATAGAC	ATTGATACAC	GTGCATATTT	TACATCTGCA	ACCATAATTA	TTGCTATTCC	GACTGGAATT	AAAATTTTTA
BG 99-151	AGGTATAGAC	ATTGATACAC	GTGCATATTT	TACATCTGCA	ACCATAATTA	TTGCTATTCC	AACTGGAATT	AAAATTTTTA
BG 99-104	AGGTATAGAC	ATTGATACAC	GTGCATATTT	TACATCTGCA	ACCATAATTA	TTGCTATTCC	AACTGGAATT	AAAATTTTTA

	250	260	270	280	290	300	310	320
BG 99-126	GATGAATTTC	TACCCTTCAT	GGAATAAAAA	TTTCTTATAA	TCCAACTCTT	TGATGAACAA	TAGGATTTAT	TTTTTTTATT
BG 99-141	GATGAATTTC	TACCCTTCAT	GGAATAAAAA	TTTCTTATAA	TCCAACTCTT	TGATGAACAA	TAGGATTTAT	TTTTTT–ATT
CR 52	GATGAATTTC	TACCCTTCAT	GGAATAAAAA	TTTCTTATAA	TCCAACTCTT	TGATGAACAA	TAGGATTTAT	TTTTTTTATT
BG 99-116	GATGAATTTC	TACCCTTCAT	GGAATAAAAA	TTTCTTATAA	TCCAACTCTT	TGATGAACAA	TAGGATTTAT	TTTTTTTATT
BG 99-151	GATGAATTTC	TACCCTTCAT	GGAATAAAAA	TTTCTTATAA	TCCAACTCTT	TGATGAACAA	TAGGATTTAT	TTTTTTTATT
BG 99-104	GATGAATTTC	TACCCTTCAT	GGAATAAANN	TT–CTTATAA	TCCAACTCTT	TGATGAACAA	TAGGATTTAT	TTTTTTTATT

	330	340	350	360	370	380	390	400
BG 99-126	TACCATAGGA	GGTTTAACTG	GAATTATATT	ATCAAATTCC	TCTATTGATA	TCATTCTTCA	TGATACTTAT	TATGTAGTAG
BG 99-141	TACTATAGGA	GGTTTAACTG	GAATTATATT	ATCAAATTCC	TCTATTGATA	TCATTCTTCA	TGATACTTAT	TATGTAGTAG
CR 52	TACTATAGGA	GGTTTAACTG	GAATTATATT	ATCAAATTCC	TCTATTGATA	TCATTCTTCA	TGATACTTAT	TATGTAGTAG
BG 99-116	TACTATAGGA	GGTTTAACTG	GAATTATATT	ATCAAATTCC	TCTATTGATA	TCATTCTTCA	TGATACTTAT	TATGTAGTAG
BG 99-151	TACTATAGGA	GGTTTAACTG	GAATTATATT	ATCAAATTCC	TCTATTGATA	TCATTCTTCA	TGATACTTAT	TATGTAGTAG
BG 99-104	TACTATAGGA	GGTTTAACTG	GAATTATATT	ATCAAATTCC	TCTATTGATA	TCATTCTTCA	TGATACTTAT	TATGTAGTAG

	410	420	430	440	450	460	470	480
BG 99-126	GTCATTTCCA	TTATGTTTTA	TCAATAGGAG	CAGTTTTTGC	CATTATTGCT	AGATTTATCC	ACTGATTTCC	TTTAATTTTT
BG 99-141	GTCATTTCCA	TTATGTTTTA	TCAATAGGAG	CAGTTTTTGC	CATTATTGCT	AGATTTATCC	ACTGATTTCC	TTTAATTTTT
CR 52	GTCATTTCCA	TTATGTTTTA	TCAATAGGAG	CAGTTTTTGC	CATTATTGCT	AGATTTATCC	ACTGATTTCC	TTTAATTTTT
BG 99-116	GTCATTTCCA	TTNTGTTTTA	TCAATNGGAG	CAGNTTTTGC	CATTATTGCT	AGATTTATCC	ACTGATTTCC	N
BG 99-151	GTCATTTCCA	TTATGTTTTA	TCAATAGGAG	CAGTTTTTGC	CATTATTGCT	AGATTTATCC	ACTGATTTC–	TTTAATTTTT
BG 99-104	GTCATTTCCA	TTATGTTTTA	TCAATAGGAG	CAGTTTTTGC	CATTATTGCT	ANATTTATCC	ACTGATTTCC	TTTAATTTTT

	490	500	510	520	530	540	550	560
BG 99-126	GGTTTTTCTC	TTAATAATTT	TTATTTAAAT	ATTC–AAT–T	TTTTTCTATA	TTTATTAGAG	– TTAATT T AAC	TTTTTTTCCT
BG 99-141	GGTTTTTCTC	TTAATAATTT	TTATTTAAAT	ATTC–AATCT	TTTTTCTATA	TTTATTAGAN	– TTAATT T AAC	TTTTTTTCCT
CR 52	GGTTTTTCTC	TTAATAATTT	TTATTTAAAT	ATTC–AATCT	TTTTTCTATA	TTTATTACAG	C TTAATT– ANC	TTTTTTTCCT
BG 99-116	-	-	-	-	-	-	-	-
BG 99-151	GGTTTTTCTC	TTAATAATTT	TTATTTAAAT	ATTC–AAT–T	TTTTTCTATA	TTTATTANAG	– TTAATT T AAC	TTTTTTTCCT
BG 99-104	GGTTTTTCTC	TTAATAATTT	TTATTTAAAT	ATTCCAAT–N	TTTTTCTATA	TTTATTACAG	– TTAATT T AAC	TTTTTTTCCT

	570	580	590	600	610	620	630	640
BG 99-126	CAACATA– TA	C– TA– GGTTT	–AAGAGGAATA	CCT–CGACGTT	ATTCTGA–TTA	TCCTGATT–AT	TTTTTTATCA	TGAAAATTTA
BG 99-141	CAACATA a TA	CC TAC G–TTT	–AANAGGAATA	CC–CN	-	-	-	-
CR 52	CAACATA– TA	CC TA– G–TTT	–AA–AGGAATA	CCT–CGACGTT	CTTCTGA–TTN	-	-	-
BG 99-116	-	-	-	-	-	-	-	-
BG 99-151	CAACATA a TA	CCTA – GGTTT	AAANAGGAATA	CCTCGCACGTT	ATTCTGAATTA	TCCTGATT–AT	TTTTTATCA	TGAAAATTTA
BG 99-104	CAACATA– TA	C– Ta a GGTTT	T AAGAGGAATA	CCTCCg ACGTT	ATTCTGA–TTA	TCCTGATTCAT	TTTT–ATCA	TGAAAATTTN

	660	670	680	690	700	710	720	730
BG 99-126	TTTCTTCTAT	TTGGAT–CAAT	AATTTCAATT	ATTAAGAATA	ATTATTTTAA–	TTTTTATTAT	TTGAgAAgCC	TTATCTTcTA
BG 99-141	-	-	-	-	-	-	-	-
CR 52	-	-	-	-	-	-	-	-
BG 99-116	-	-	-	-	-	-	-	-
BG 99-151	TTTCTTcT –T	TTGGATCCAAT	AATTTCAATT	ATTAA—TTA	ATTATTTTAAA	TTTTTTTTa T	TTGAAAACC	CTTTaTTTTA
BG 99-104	-	-	-	-	-	-	-	-

	740	750	760	770	780
BG 99-126	AACAATTTAT	TATTTCaAT–A	TTcTCCCCAA	aTttTTTTTA	AAATgAAAC
BG 99-141	-	-	-	-	-
CR 52	-	-	-	-	-
BG 99-116	-	-	-	-	-
BG 99-151	AACCcATTAT	T–TTTCAATT–	—TTCCC—A	ATTTTTTTA	AaaTN
BG 99-104	-	-	-	-	-

Table 20. Sequence of mtDNA obtained from the primer C2-N-3661 (alias Barbara).

Significant differences among individuals were marked in blue. Differences among individuals marked in red are presumably due to artefacts, e.g. at the beginning, or ending of the sequencing read, or due to contamination)

	10	20	30	40	50	60	70	80
BG 99-126	TNNNNNA	tTT−−ATA−TC	CATGCCTGGT	CGATATATAA	ATAATATAGA	TTGATTTAAT	CGTCCAGGGG	TTGAATCTAT
BG 99-141	-	-	GCCTGGT	CGATATATAA	ATAATATAGA	TTGATTTAAT	CGTCCAGGGG	TTGAATCTAT
CR 52	-	TTC−ATA−TC	−ATGCCTGGT	CGATATATAA	ATAATATAGA	TTGATTTAAT	CGTCCAGGGG	TTGAATCTAT
BG 99-116	T−	TTT−−ATAATN	C−TGCCTGGT	CGATATATAA	ATAATATAGA	TTGATTTAAT	CGTCCAGGGG	TTGAATCTAT
BG 99-151	NA	TTTC TATA−−C	−A−GCCTGGT	CGATATATAA	ATAATATAGA	TTGATTTAAT	CGTCCAGGGG	TTGAATCTAT
BG 99-104	NA	tTT−− ATATTN	NA−GCCTGNT	CGATATATAA	ATAATATAGA	TTGATTTAAT	CGTCCAGGGG	TTGAATCTAT
CR 54	NCNNNNTTTC	TANA−A−−	−ATGCCTGNT	CGATATATAA	ATAATATAGA	TTGATTTAAT	CGTCCAGGGG	TTGAATCTAT

	90	100	110	120	130	140	150	160
BG 99-126	TTTAATTCCT	AGAGAAGGTA	CAGTTCATGC	ATGAATTACA	TCTATAGATG	TT−GTAATGAT	TCG−−AATTGGA	TAGTTGAAAG
BG 99-141	TTTAATTCCT	AGAGAAGGTA	CAGTTCATGC	ATGAATTACA	TCTATAGATG	TT−GTAATGAT	TCG−−AATTGGA	TAGTTGAAAG
CR 52	TTTAATTCCT	AGAGAAGGTA	CAGTTCATCGC	ATGAATTACA	TCTATAGATG	TT−GTAATGAT	TCGCCAATTGGA	TAGTTGAAAG
BG 99-116	TTTAATTCCT	AGAGAAGGTA	CAGTTCATGC	ATGAATTACA	TCTATAGATG	TT−GTAATGAT	TCG−−AATTGGA	TAGTTGAAAG
BG 99-151	TTTAATTCCT	AGAGAAGGTA	CAGTTCATGC	ATGAATTACA	TCTATAGATG	TT−GTAATGAT	TCG−−AATTGGA	TAGTTGAAAG
BG 99-104	TTTAATTCCT	AGAGAAGGTA	CAGTTCATGC	ATGAATTACA	TCTATAGATG	TTCGTAATGAT	TCG−−AATTGGA	TAGTTGAAAG
CR 54	TTTAATTCCT	AGAGAAGGTA	CAGTTCATGC	ATGAATTACA	TCTATAGATG	TT−GTAATGAT	TCG−−AATTGGA	TAGTTGAAAG

	170	180	190	200	210	220	230	240
BG 99-126	GAATAACACA	ACGATTATCT	ACATCAAGAA	GACGAAATTC	ATTTAAATTT	A−GTTCATTAT	AAGGAATTAT	ATATGAATTA
BG 99-141	GAATAACACA	ACGATTATCT	ACATCAAGAA	GACGAAATTC	ATTTAAATTT	A−GTTCATTAT	AAGGAATTAT	ATATGAATTA
CR 52	GAATAACACA	ACGATTATCT	ACATCAAGAA	GACGAAATTC	ATTTAAATTT	ACGTTCATTAT	AAGGAATTAT	ATATGAATTA
BG 99-116	GAATAACACA	ACGATTATCT	ACATCAAGAA	GACGAAATTC	ATTTAAATTT	A−GTTCATTAT	AAGGAATTAT	ATATGAATTA
BG 99-151	GAATAACACA	ACGATTATCT	ACATCAAGAA	GACGAAATTC	ATTTAAATTT	A−GTTCATTAT	AAGGAATTAT	ATATGAATTA
BG 99-104	GAATAACACA	ACGATTATCT	ACATCAAGAA	GACGAAATTC	ATTTAAATTT	A−GTTCATTAT	AAGGAATTAT	ATATGAATTA
CR 54	GAATAACACA	ACGATTATCT	ACATCAAGAA	GACGAAATTC	ATTTAAATTT	A−GTTCATTAT	AAGGAATTAT	ATATGAATTA

	250	260	270	280	290	300	310	320
BG 99-126	AATTCAATAT	TAAAAAAATT	TGTATATTCA	TATGATCAAT	ATCATTGATG	ACCAATAGAT	TTAATTGATA	AGTTAcGATG
BG 99-141	AATTCAATAT	TAAAAAAATT	TGTATATTCA	TATGATCAAT	ATCATTGATG	ACCAATAGAT	TTAATTGATA	AGTTAC–ATG
CR 52	AATTCAATAT	TAAAAAAATT	TGTATATTCA	TATGATCAAT	ATCATTGATG	-	-	-
BG 99-116	AATTCAATAT	TAAAAAAATT	TGTATATTCA	TATGATCAAT	ATCATTGATG	ACCAATAGAT	TTAATTGATA	AGTTAcGATG
BG 99-151	AATTCAATAT	TAAAAAAATT	TGTATATTCA	TATGATCAAT	ATCATTGATG	ACCAATAGAT	TTAATTGATA	AGTTA–GATG
BG 99-104	AATTCAATAT	TAAAAAAATT	TGTATATTCA	TATGATCAAT	ATCATTGATG	ACCAATAGAT	TTAATTGATA	AGTTAcGATG
CR 54	AATTCAATAT	TAAAAAAATT	TGTATATTCA	TATGATCAAT	ATCATTGATG	ACCAATAGAT	TTAATTGATA	AGTTA–GATG

	330	340	350	360	370	380	390	400
BG 99-126	TATTAATTTC	ATCCCTTAAA	TATAAAATTT	TAATTGATGG	AATAGCAATA	AAAATTAAAA	TTACTATA–GG	TACAAT–TGTT
BG 99-141	TATTAATTTC	ATCCCTTAAA	TATAAAATTT	TAATTGATGG	AATAGCAATA	AAAATTAAAA	TTACTATAC–G	TACAATCTGTT
CR 52	-	-	-	-	-	-	-	-
BG 99-116	TATTAATTTC	ATCCCTTAAA	TATAAAATTT	TAATTGATGG	AATAGCAATA	AAAATTAAAA	TTACTATA–GG	TACAAT–TGTT
BG 99-151	TATTAATTTC	ATCCCTTAAA	TATAAAATTT	TAATTGATGG	AATAGCAATA	AAAATTAAAA	TTACTATA–GG	TACAAT–TGTT
BG 99-104	TATTAATTTC	ATCCCTTAAA	TATAAAATTT	TAATTGATGG	AATAGCAATA	AAAATTAAAA	TTACTATACGG	TACAAT–TGTT
CR 54	TATTAATTTC	ATCCCTTAAA	TATAAAATTT	TAATTGATGG	AATAGCAATA	AAAATTAAAA	TTACTATA–GG	TACAAT–TGTT

	410	420	430	440	450	460	470	480
BG 99-126	CAAATTAATT	CAATA–GAATG	ACCTTGAAGT	AAATTTC–GAT	TAATAAATTT	ATTGTTAGTT	AAAGAAATTA	TAATATAAAA
BG 99-141	NAAATNN–TT	CA	-	-	-	-	-	-
CR 52	-	-	-	-	-	-	-	-
BG 99-116	CAAATTAATT	CAATA–GAATG	ACCTTGAAGT	AAATTTCCGAT	TAATAAATTT	ATTGTTAGTT	AAAGAAATTA	TAATATAAAA
BG 99-151	CAAATTAATT	CAATA–GAATG	ACCTTGAAGT	AAATTTC–GAT	TAATAAATTT	ATTGTTAGTT	AAAGAAATTA	TAATATAAAA
BG 99-104	CAAATTAATT	CAATACGAATG	ACCTTGAAGT	AAATTTCCCAT	TAATAN	-	-	-
CR 54	CAAATTAATT	CAATA–GAATG	ACCTTGAAGT	–AAATTTC–GAT	TAATAAATTT	ATTGTTAGTT	AAAGAAATTA	TAATATAAAA

	490	500	510	520	530	540	550	560
BG 99-126	AATTATTATA	ATAATTAgAA	TTATAATAAT	TATGGTAAAA	–TCAT––GAA	AAAATATTAT	TATATCATAA	ATTTGgAg–AA
BG 99-141	-	-	-	-	-	-	-	-
CR 52	-	-	-	-	-	-	-	-
BG 99-116	AATTATTATA	ATAATTANAA	TTATAATAAT	TATGGTAAAA	ATCATCCGAA	AAA–TATTAT	TATATCATAA	ATTTGgAg –AA
BG 99-151	AATTATTATA	ATAATTAgAA	TTATAATAAT	TATGGTAAAA	–TCAT––GAA	AAAATATTAT	TATATCATAA	AT–TGgAGGAA
BG 99-104	-	-	-	-	-	-	-	-
CR 54	AATTATTATA	ATAATTAgAA	TTATAATAAT	TATGGTAAAA	–TCAT––GAA	AAAATATTAT	TATATCATAA	ATNTGGAg–AA

	570	580	590	600
BG 99-126	TTTgAAATTT	TT g AA– –gAgTt	AAATAACC– –	TCCAAN
BG 99-141	-	-	-	-
CR 52	-	-	-	-
BG 99-116	TTTgAAATTT	TT ––AAACA–Tt	-	-
BG 99-151	TTTCAA–TTT	– TCGAA––A– –	-	-
BG 99-104	-	-	-	-
CR 54	TTTgAA–TTT	TT –gAA––AgT–	AA–TAACCAA	N

6.4. Discussion

Two founding colonies of *Pachycondyla obscuricornis* from the study population in Costa Rica, consisted each of two foundresses with some brood. Foundresses displayed aggressive interactions in the laboratory (pers. com. B. Gobin). It is currently unclear, if the number of queens in founding associations of *P. obscuricornis* is down-regulated after the eclosion of the first workers, as it has been reported for most ant species (secondary monogyny; Hölldobler & Wilson 1990; Heinze 1993; Choe & Perlman 1997; Bernasconi & Strassmann 1999), or not (primary polygyny, similar to *P.* cf. *inversa*). However, mature colonies of the study population of *P. obscuricornis* contained up to 12 queens. Colonies containing such high numbers of queens presumably emerged from adoption of freshly inseminated females (secondary polygyny).

The investigation of the mitochondrial DNA will be particularly rewarding in this species, e.g. to study the mode of colony founding (the range of nuptial flights of females, or the occurrence of re-adoption of freshly mated females in their natal colonies), or to detect matrilines among the progeny of queens from multiple-queen colonies: In an initial sequencing of the COI/COII region, variability among female workers was relatively high, with at least six, respectively one point mutation, depending on the primer used. Six different haplotypes could be distinguished among six workers of different colonies from the study population in Costa Rica. Further experiments are needed to clarify the significance of additional sequence variations among individuals, which may be due to real mutations, or represent artefacts. The variability of mitochondrial DNA by analysing RFLPs displayed only low variability: two haplotypes in seven, and eight workers, respectively, from different colonies (cutting with *Taq I* or *Hind III*).

Multilocus DNA fingerprinting of individuals from the study population in Brazil displayed very low intracolonial variability with a bandsharing-coefficient of approximately 0.9 (combinations *Pal I*, *SAU 3A I* each with $(GACA)_4$, $(GGAT)_4$). The intercolonial variability was moderate in the combinations *Pal I*/$(GGAT)_4$, *SAU 3A I*/$(GACA)_4$, and *SAU 3A I*/$(GGAT)_4$, with band-sharing coefficients ranging from 0.58 to

0.7. This variation is sufficient to study aspects of the population and colony structure of *P. obscuricornis*, e.g. the mating frequency of queens. As a consequence of the moderate variability, it will be necessary to analyse the DNA of one individual with a combination of multiple probes, and restriction endonucleases, respectively. Unfortunately, workers of *P. obscuricornis* are hardly large enough for this purpose. Another disadvantage is that multilocus DNA fingerprints, similar to RFLPs, are dominant markers, i.e. individual genes cannot be scored at particular loci. In contrast, microsatellites provide genotypic data, and only small amounts of DNA are necessary for the investigation. In this study, two of 15 loci displayed very low variability with two alleles per locus. It will be more promising to establish additional primers which were originally designed for other ponerine ants, e.g. for the genus *Rhytidoponera* (Tay & Crozier 2000).

Banding patterns obtained by 20 enzyme systems did not reveal any intra- or intercolonial variation in 35 colonies of *P. obscuricornis*. Colonies from Brazil and Costa Rica did not differ at all. However, allozyme electrophoresis is a powerful tool to distinguish *P. obscuricornis* from other sympatrically living *Pachycondyla* species: In comparison with eight other *Pachycondyla* species, individuals of *P. obscuricornis* differed consistently in four diagnostic loci. Especially *P. apicalis* may be confounded with *P. obscuricornis*: At a first glance, individuals from *P. apicalis* colonies are recognised by yellow antennae tips. However, using allozyme electrophoresis, two colonies clearly belonged to *P. apicalis* though their antennae were coloured black.

7. General discussion

Fundamental evolutionary theories, such as the theory of kin selection (Hamilton 1964a,b, 1972), reproductive skew theory (Vehrencamp 1983; reviewed by Reeve & Keller 2001), and sex ratio theory (Fisher 1930; Trivers & Hare 1976) make predictions on the occurrence and the extent of various conflict situations in colonies of social insects. All these hypotheses rest on the genetic relationship among interacting individuals. Therefore, the study of co-operating individuals which are unrelated provides a unique opportunity to critically test predictions based on these theories.

The species complex Pachycondyla villosa

A hundred and twelve colonies of the study species *Pachycondyla villosa* consisted of three morphologically different, sympatric forms which were collected on the territory of CEPLAC (Centro de Pesquisas do Cacau), Itabuna, Bahia, Brazil. Using allozyme electrophoresis, banding patterns were obtained which differed consistently in five diagnostic loci among the three forms (chapter 2). The absence of heterozygotes suggested that the three taxa are reproductively isolated, and thus, represent three different species. This was supported by the fact that in approximately 100 of the examined colonies the different forms never co-occurred in one colony. Furthermore, observations showed that mating between the forms B/B and B/A resulted only in the first case in the production of males (Lucas *et al.* 2002). The taxonomic status of the species is not yet clear (for details, see chapter 2).

Co-operation and conflict in P. cf. inversa

In this study, one of the three species that was termed provisionally *Pachycondyla* cf. *inversa* was examined in greater detail. Newly mated queens of this ponerine ant found their colonies either alone (haplometrosis), or co-operatively with one to several other queens (pleometrosis). In a previous study with DNA multilocus fingerprinting, 14 out of 16 queens from seven polygynous colonies were found to be unrelated to their nestmate queens. Only one pair-wise comparison suggested a closer relatedness between two queens (Heinze *et al.* 2001). Due to the complexity of multilocus fingerprints, an exact calculation of relatedness was not possible. Furthermore, the sample size was quite low in this study. Therefore, microsatellite primers, which were originally developed for other ponerine ants, were investigated here (chapter 3). From variation at a single microsatellite locus, queen-queen relatedness of 43 queens from 14 colonies was estimated to be r = 0.097 ± SE 0.09, i.e. not significantly different from zero relatedness. Thus, co-foundresses in founding colonies were indeed on average unrelated. This matches results from studies in myrmicine and formicine ants, according to which founding queens do not preferentially co-operate with related foundresses (Hagen *et al.* 1988, Sasaki *et al.* 1996).

During the founding phase, co-foundresses of *P.* cf. *inversa* displayed a clear division of labour, with one queen specialising in foraging, "semi-claustral founding" (then referred to as *P. villosa*; Trunzer *et al.* 1998). Foragers of social insects are exposed to greater risks, such as predation, and parasitation, and are also exposed to greater physical and thermal stresses than their nestmates that stay in the nest (Michener 1974; Gamboa *et al.* 1978; Traniello *et al.* 1984). It is currently unknown, if foraging queens of P. cf. *inversa* are exposed to greater risks than their nestmates. However, in queen associations of this species, helpers (the foraging queens) perform potentially risky tasks and aid recipients (the queens that stay in the nest) who do not share their genes. Foraging queens therefore act altruistically in favour of co-foundresses which are not related to them. This behaviour represents a potential challenge to the fundamental evolutionary concept of kin selection.

Only one other ant species displays a similar pattern in behaviour: Similarly to *P.* cf. *inversa*, one queen of the desert leaf-cutter ant *Acromyrmex versicolor* specialises in foraging, whereas the others stay in the nest. Task allocation was not regulated by aggressive interactions. It was suggested that a queen should be indifferent as to whether she takes over the more or less risky tasks, because the group succeeds or fails as a unit in competition with other such groups (Rissing *et al.* 1989). Fighting about task allocation should be selected against as it decreases the success of the colony as a whole. The division of labour without social dominance in *A. versicolor* has been interpreted as a particularly strong case of group-selected co-operation (Wilson 1990; Dugatkin 1997). The foraging queen was viewed as a "weak altruist", meaning that the foragers own personal fitness increases, but the fitness of the group mates increases even more, in contrast to "strong altruists" which entails an absolute negative effect on the actor's personal fitness (Wilson 1979, 1990). However, queen selfishness in foundress associations, though constrained by its effects on the group as a whole, may not be totally absent (Bourke & Franks 1995).

In contrast to these suggestions, this study provided clear evidence that the division of labour among co-foundresses of *P.* cf. *inversa* results from social competition: Approximately 50% of all founding colonies collected near Itabuna, Brazil, consisted of queen associations with two to five queens per colony (chapter 2). Co-foundresses displayed aggressive interactions and formed linear dominance hierarchies. The dominant individual forced the subordinate to leave the nest and forage (chapter 4.1). Usually, in foundress associations of other ant species, aggression between queens starts only after the newly emerged workers start to forage and carry in new resources (Hölldobler & Wilson 1990; Heinze 1993; Choe & Perlman 1997; Bernasconi & Strassmann 1999). In these species, co-foundresses raise their first brood without collecting food outside the nest (claustral founding), and it was suggested that energetically costly antagonism is only possible, after the colony has changed from a closed energy system to an open energy system when the first workers are produced (Rissing & Pollock 1986). Foundress associations of *P.* cf. *inversa* represent open energy systems due to the foraging activity of the subordinates. Thus, energetically costly antagonism, and therefore social competition is possible.

In *P.* cf. *inversa*, the social status of foundresses apparently was not, or only weakly associated with the reproductive status: All co-foundresses laid eggs at more or less

similar rates. However, significant differences may have remained undetected, because egg-laying rates were extremely low. The dominant foundress harassed the subordinate during egg-laying and occasionally, fed on the eggs laid by the subordinate (chapter 4.1). This differential oophagy presumably is also reflected in a microsatellite study of foundress associations which was conducted shortly after the first workers emerged, where the co-foundresses occasionally contributed unequally to the colony's workers: In two colonies, one queen was mother of most of the worker offspring. In seven other colonies, the microsatellite data did not allow to determine the origin of workers (chapter 3). In mature colonies, egg-eating and aggression among the co-foundresses ceased completely, and the co-foundresses were observed staying near each other (observations B. Stengl, B. Trunzer, K. Kolmer). A multilocus fingerprint study by Heinze *et al.* (2001) showed, that the reproduction was quite evenly partitioned among queens of mature colonies, i.e. the reproduction was not, or very weakly skewed, which fits predictions made by reproductive skew theory: reproduction should be equally distributed among unrelated individuals (Reeve & Ratnieks 1993). However, in some colonies, queens contributed unequally to the colony's workers, or sexuals, and the sample size of the investigated individuals was quite low in this study.

In both founding and mature colonies, reproduction seems to be quite evenly partitioned among queens, except for some queens which occasionally reproduce more than others. Nevertheless, many details on the allocation of reproduction among co-foundresses of *P.* cf. *inversa* remain to be investigated in the future. The significance of an unequal reproduction of some queens during the founding phase remains unclear. One possible reason may be the limitation of food in this study (see below) so that hungry dominant queens may eat occasionally subordinates eggs. Resource availability has long been recognised to influence reproduction (e.g. Aron *et al.* 2001). However, egg-eating also occurred in some colonies which were sufficiently provisioned with food (Trunzer *et al.* 1998).

Similarly to *P.* cf. *inversa*, dominance hierarchies in queen associations of wasps (Pratte 1989; Strassmann 1989), and small ant colonies (Bourke 1988; Ito & Higashi 1991) are known to underlie the division of labour. The interacting individuals were typically close relatives in all these cases, except for the social wasp *Polistes dominulus*. In this species, 35 % of the queens from foundress associations were found to be unrelated to their nestmates (Queller *et al.* 2000). Unlike in *P.* cf. *inversa*, the dominant

individual has nearly the complete reproductive dominance over subordinates: The dominant individual lays most of the eggs (93,9 % in early foundresses, and 99,6 % in late foundresses), whereas the subordinates take on most of the risky foraging task. Thus, the study of *P.* cf. *inversa* is the first observation of dominance interactions among unrelated individuals in eusocial insects, where dominance serves predominantly to establish a clear-cut division of labour, but has only minor, or no effects on the allocation of reproduction.

Why do subordinates not abscond from the nest and found their own colony, if they are forced to leave and forage anyway? Foundress associations have a very high survivorship in contrast to solitary founding queens: Pleometrotic associations produce larger initial brood and worker forces in shorter periods (Bartz & Hölldobler 1982; Tschinkel & Howard 1983; Rissing & Pollock 1987; Sommer & Hölldobler 1995; but see Pfennig 1995; Jerome *et al.* 1998). The protection against predators and usurpation is increased (McCorquodale 1989; Balas & Adams 1996). Furthermore, ecological constraints may favour pleometrosis, e.g. shortage of nest sites (e.g. Pfennig 1995). Hence, even a queen with low fighting abilities may still benefit from joint nesting instead of founding solitarily. Though empirical data from the field do not exist for *P.* cf. *inversa*, pleometrosis might be associated with similar advantages (Trunzer *et al.* 1998). Furthermore, staying as a subordinate may be highly advantageous in this species, as queen number in foundress associations is not down-regulated after the eclosion of the first workers, in contrast to most other ant species (Hölldobler & Wilson 1990; Heinze 1993; Choe & Perlman 1997; Bernasconi & Strassmann 1999). In mature colonies of *P.* cf. *inversa*, egg-eating and aggression among the co-foundresses ceased, and the reproduction was quite evenly partitioned among the queens. Thus, it may pay for a queen to become forager if she does not risk being expelled from the nest after successful founding, and may produce sexual offspring. This assumption is corroborated by the finding that the queen number is also not down-regulated in multiple-queen colonies of *Acromyrmex versicolor*.

In contrast to this study, Trunzer *et al.* (1998) did not observe direct aggressive interactions, or ritualised dominance behaviour among queens from founding associations or mature colonies from the same population of *P.* cf. *inversa*. Furthermore, egg-laying rates were much higher in comparison to the egg-laying rates shown here. These differences are presumably due to different provisioning of the laboratory colonies with

food: The colonies studied by Trunzer *et al.* (1998) were fed every two days, whereas here, food was added after longer, and less regular intervals (every one to six days), chapter 4.1. In colonies with three queens at least, the frequency of aggressive interactions significantly increased with the time since food was last added into the arena. Ecological constraints, such as limited food availability, might therefore lead to more frequent aggression and thus, to more intensive foraging by the subordinate queen. Hungry animals indeed exhibit a heightened motivation towards antagonism (e.g Stocker & Huber 2001). Similarly, food shortage accentuated the reproductive dominance of one queen in *Myrmica rubra* (Sommeijer & Van Veen 1990). The apparent lack of aggression among queens in foundress associations or mature polygynous colonies therefore does not mean that dominance relationships do not exist at all. Division of labour may instead result from subtle and difficult-to-observe interactions among co-foundresses (Bernasconi & Keller 1998; Choe & Perlman 1997). Therefore, group selection alone does not always explain the apparent altruistic behaviour of unrelated queens.

Which factors proximately influence the outcome of aggressive interactions during the early formation of dominance hierarchies? Here, the influence of body size and nest ownership on social rank in experimentally assembled associations of founding queens of *P.* cf. *inversa* was studied. Queens engaged in severe fights which resulted in clear dominance relationships within 1 to 20 min (chapter 4.2). The queen initiating the fight always became the dominant queen. It could not be distinguished if certain behaviours (antennation, biting, dragging, mandible spreading) determined the outcome of the contest, e.g. in crickets, antennal fencing served to assess the fighting readiness of the opponent, whereas mandible spreading indicated fighting ability (Hofmann & Schildberger 2001). During the encounters, a behaviour that has not been observed in colonies with an established hierarchy (see chapter 4.1) appears to be typical for territorial contests among freshly assembled queens: Resident queens tried to push new queens out of the nest. In all trials, dominance relations among the queens were stable over the whole observation period, until the first workers eclosed. The queen behaviour was more overtly aggressive during hierarchy establishment than later, when the hierarchy was stabilised mostly by ritualised antennation.

Nest ownership at least for a couple of days did not influence the outcome of dominance interactions in the laboratory experiments, whereas queen body size

apparently played an important role: In all eight trials, the larger queen became dominant. However, dominant queens from natural foundress associations were on average not larger than subordinates, suggesting that in the field, resident asymmetries might override size asymmetries only after a more prolonged period of nest ownership: In contrast to species from temperate or boreal habitats, mating is less synchronised in tropical ants (Kaspari *et al.* 2001; Torres *et al.* 2001). This may also be true for *P.* cf. *inversa*, because male and female sexuals have been found both in March and November. Young queens might attempt to join other queens that already inhabited a nest site for several weeks. Well-established nest owners might be more successful in dominating even large newcomers, because a nest containing eggs and larvae is more valuable to the nest-owner than to the usurper. Differences in the motivational state which influence the outcome of dominance interactions has been reported for hermit crabs and crickets (Elwood *et al.* 1998; Hofmann & Schildberger 2001). Alternatively, differences in ovarian development may influence aggressiveness, because the ovaries from older queens of *P.* cf. *inversa* are more developed than those of younger queens. Dominant foundresses in foundress associations of the wasp *Polistes dominulus* had either more developed ovaries or larger corpora allata than subordinates (Röseler *et al.* 1984).

How can queens in natural founding associations of *P.* cf. *inversa* recognise the social rank of a co-foundress? Cuticular hydrocarbons of queens from young foundress associations were analysed to investigate whether the chemical bouquet of foundresses reflects their social status. The profiles of dominant queens which engaged in brood care were characterised by the presence of two substances, n-C_{15} and n-C_{17}:1, which were absent on the cuticle of subordinate foraging queens (chapter 5). Queens with medium rank in three-queen colonies both showed intermediate behaviour and had amounts of C_{15} and C_{17}:1 between those of the α- and the γ-queen. The two substances, n-C_{15} and n-C_{17}:1, were found in the Dufour glands of workers of *P.* cf. *inversa*, but not in their poison glands. It is likely that they are present also in the Dufour gland of queens (which were not studied) and from there are distributed onto the cuticle by dominant queens rubbing first the tip and subsequently the back of the abdomen with their hind legs. In honeybees, Dufour gland secretions ooze out and spread over the cuticle around the genital chamber (Katzav-Gozansky *et al.* 2001).

Cuticular hydrocarbon profiles have previously been shown to be associated with differences in task (Bonavita-Cougourdan *et al.* 1993; Wagner *et al.* 1998) and reproductive status (Peeters *et al.* 1999; Liebig *et al.* 2000; Cuvillier-Hot *et al.* 2001; Sledge *et al.* 2001). In founding associations of *P.* cf. *inversa*, the social status is tightly correlated with task, but not, or only weakly with the reproductive status. Hence, in contrast to the changes in cuticular hydrocarbons reported from other ponerine ants (Peeters *et al.* 1999; Liebig *et al.* 2000; Cuvillier-Hot *et al.* 2001), the two substances found only in dominant queens are not closely linked to ovarian development. It is also unlikely that the differences are proximately caused by the two queens taking over different tasks, e.g. by the dominant queen acquiring the substances during more intensive brood care, because they were absent from the cuticle of larvae. The diet may also modify cuticular hydrocarbons (Liang & Silverman 2000), but the substances were not present on the food of *P.* cf. *inversa* (crickets and cockroaches). Hence, the presence of the two substances is somehow associated with social dominance alone. Chemical 'badges of status" have been identified in male cockroaches, *Nauphoeta cinerea*, where the quantities of certain constituents of the sex pheromone vary with social status (Moore *et al.* 1997). In *P.* cf. *inversa*, the distribution of n-C_{15} and n-C_{17}:1 might either be an epiphenomenon of high social status without signalling function or may serve to communicate high social status. In the latter case, the comparatively high volatility of n-C_{15} and n-C_{17}:1 might explain why subordinates react to approaching dominants before having physical contact. In three colonies of *P.* cf. *inversa* (one colony containing 20 workers and three queens, and two queenless colonies), the substances n-C_{15} and n-C_{17}:1 were also present on the cuticle of several workers. Social rank and fertility of workers were not associated with the substances n-C_{15} and n-C_{17}:1 (chapter 5; see also Heinze *et al.* in press). Therefore, bioassays are urgently needed to clarify the role of n-C_{15} and n-C_{17}:1, e.g. spreading the two substances onto the cuticle of subordinate queens of young foundress associations.

Co-operation and conflict in mature colonies of P. cf. inversa

When relatedness within the colony decreases, the benefits of co-operation to the workers decrease. Thus, increased selfishness of workers might be favoured which may

strongly influence conflicts among workers, or between workers and queens, e.g. over the division of labour or the pattern of sex allocation. The relatedness among workers, and between workers and queens, decreases considerably either when 1) multiple queens reproduce in the same colony, 2) queen-queen relatedness in multiple queen colonies is very low, or 3) queens mate multiply (Queller 1993). Furthermore, the study of the mating behaviour and the dispersal of freshly mated queens is of importance, as it also determines the genetic population structure, and influence the intracolonial genetic diversity. Relatedness among nestmate workers in colonies of *P.* cf. *inversa* which contain two to five unrelated reproducing queens, is expected to be fairly low. However, nestmate workers were on average closely related ($r = 0.5 \pm 0.11$) in colonies each with two, and three queens, respectively (chapter 3). Additional data obtained by more than one microsatellite locus are needed to examine the exact relatedness coefficients per colony, and thus, clarify the genetic relationships among workers in polygynous colonies.

Multiple mating seemed to be rather common in *P.* cf. *inversa* queens: Polyandrous queens were detected in four colonies out of a total of ten (chapter 3). The extent of multiple mating is not yet clear, as the data do not suffice for a detailed analysis. A previous multilocus DNA fingerprinting analysis could not exclude multiple mating due to the high band sharing similarity among males (Heinze *et al.* 2001).

The study of microsatellites also revealed positive inbreeding coefficients in both queens and workers of *P.* cf. *inversa*, which were not caused by a Wahlund effect, or a substructuring of the studied population (chapter 3). The presence of null-alleles was also very unlikely. The positive inbreeding coefficient is best explained by a temporal substructuring of the population, i.e. individual colonies produce sexuals during different times of the year and mating occurs during several, non-overlapping mating swarms with different allele frequencies. This assumption is corroborated by studies of tropical ants, where mating was less synchronised than in ants from boreal or temperate habitats (Kaspari *et al.* 2001; Torres *et al.* 2001). Furthermore, male and female sexuals of *P.* cf. *inversa* have been found both in March and November. Local inbreeding in small nuptial flights has also been detected in other species of ants, e.g. in *Messor aciculatus* and *Leptothorax nylanderi* (Hasegawa & Yamaguchi 1995; Foitzik & Heinze 2001).

Little is known on the reproductive biology of winged ponerine queens. The presence of winged queens does not necessarily mean that these disperse and mate away from their natal nests, as suggested by Peeters (1993): A microsatellite study of the

ponerine *Gnamptogenys striatula* revealed that female sexuals were mostly produced by one or a few queens, and these groups of full-sisters were recruited back in their original nest after mating (Giraud *et al.* 2001). In *P.* cf. *inversa*, the lack of a significant geographical substructure and the low relatedness of queens in pleometrotic associations indicate that virgin queens do not mate near their natal nest and disperse before founding colonies.

Molecular markers in Pachycondyla obscuricornis

The comparison between *P.* cf. *inversa*, and another ponerine species, *P. obscuricornis*, may give valuable information to test predictions made by reproductive skew models. Here, molecular markers were established to investigate the genetic structures of *P. obscuricornis* colonies and populations for the future. The investigation of the mitochondrial DNA of *P. obscuricornis* will be particularly rewarding: In an initial sequencing of the COI/COII region, variability among female workers was relatively high, with at least seven point mutations. Six different haplotypes could be distinguished among six workers of different colonies from one study population in Costa Rica.

Other methods which were established were not well suited for the study of this species: The variation in RFLPs, microsatellites, and allozymes was too low to study colony and population structure, whereas multilocus DNA fingerprinting displayed moderate variability. For the study of multilocus fingerprints it will be necessary to analyse the DNA of one individual with a combination of multiple probes and restriction endonucleases. However, workers of *P. obscuricornis* hardly contain enough DNA for this purpose. Therefore, it may be more promising to establish additional microsatellite primers, which were originally designed for other ponerine ants, e.g. for the genus *Rhytidoponera* (Chapuisat *et al.* 2000; Tay & Crozier 2000). Allozyme electrophoresis turned out to be a powerful tool to distinguish *P. obscuricornis* from other sympatrically living *Pachycondyla* species, especially the morphologically very similar *P. apicalis*: In comparison with eight other *Pachycondyla* species individuals of *P. obscuricornis* differed consistently in four diagnostic loci.

8. Summary

A significant relatedness is of fundamental importance for the evolution and maintenance of social life (kin selection theory, Hamilton 1964a,b). Not only kin selection itself, but also more complex evolutionary theories make predictions on the occurrence of conflict and co-operation in animal societies. They all depend on the genetic relationships among individuals. Therefore, the study of unrelated, co-operating individuals provides a unique opportunity to critically test predictions based on these evolutionary theories.

Using allozyme electrophoresis, the study species *Pachycondyla villosa* was found to represent three different species. Young queens in one of these species, provisionally called *Pachycondyla* cf. *inversa*, may co-operate during colony founding (pleometrosis). Approximately 50% of all founding colonies collected near Itabuna, Brazil, consisted of two to five founding queens. Queens of *P.* cf. *inversa* have to forage for food (semi-claustral founding), and in founding associations only one queen specialised for this risky task. A microsatellite study showed that nestmate queens were typically not related. How can a division of labour be achieved, where one individual performs risky tasks to the favour of another individual to which it is not related? In contrast to the predictions made by group selectionists, this study provided clear evidence that the division of labour among co-foundresses of *P.* cf. *inversa* results from social competition: Co-foundresses displayed aggressive interactions and formed dominance hierarchies which predominantly served to force subordinates to forage. The frequency of queen antagonism increased with the duration since food was last added to the foraging arena. The social status was not, or only weakly associated with the reproductive status: As predicted by the reproductive skew theory, all foundresses laid eggs at similar rates, though the subordinate may be harassed during egg laying and occasionally, some of her eggs may be eaten by the dominant. The differential oophagy presumably was also reflected in a microsatellite study of foundress associations, which was conducted shortly after the first workers emerged: Here, the co-foundresses occasionally contributed unequally to the colony's workers. Conflicts among workers or between workers and queens, e.g. over the

division of labour or sex ratio, strongly depend on the genetic relationships among members of a colony. The number of two to five co-founding queens in polygynous colonies of *P.* cf. *inversa*, and the lack of relatedness among them, should lead to a decrease in the relatedness of workers. However, nestmate workers were closely related. Furthermore, worker relatedness may decrease as several queens were found to be multiply inseminated. Inbreeding coefficients were significantly different from zero in both queens and workers. No evidence for a geographical substructuring of the population was found. The deviation from random mating presumably was probably due to small, localised nuptial flights. Virgin queens do not mate near their natal nest and disperse before founding colonies.

The analysis of cuticular hydrocarbons obtained from live queens revealed consistent differences between the patterns of cuticular hydrocarbons of queens with high vs. low rank: only high-ranking queens showed considerable amounts of cuticular pentadecane (n-C_{15}) and heptadecene (n-C_{17}:1). The presence of the two substances apparently was not associated with reproductive status. It is not yet known, if the two substances indeed serve to communicate high social status in *P.* cf. *inversa*.

In experimentally assembled associations of two founding queens, queens engaged in aggressive interactions which already within one to twenty minutes resulted in stable dominance hierarchies. The queens attacking first usually won the contest and became dominant. Nest ownership at least for a couple of days did not influence the outcome of dominance interactions in the laboratory experiments, whereas queen body size apparently played an important role: In all eight trials, the larger queen became dominant. However, dominant queens from natural foundress associations were on average not larger than subordinates, suggesting that in the field, resident asymmetries might override size asymmetries only after a more prolonged period of nest ownership.

Sequencing of the COI/COII region of mitochondrial DNA displayed sufficient variability for the study of the sociogenetic structure of the secondarily polygynous ant *Pachycondyla obscuricornis*: Six different haplotypes could be distinguished among six workers of different colonies from one study population in Costa Rica. The variability of other methods which were established (RFLPs, microsatellites, allozymes, and multilocus DNA fingerprinting) was too low for a further study on the genetic structure in *P. obscuricornis*.

9. Zusammenfassung

Die Verwandtschaft zwischen Individuen ist von fundamentaler Bedeutung für die Entstehung und Erhaltung sozialen Lebens (Verwandtenselektionstheorie, Hamilton 1964a,b). Nicht nur die Verwandtenselektionstheorie, sondern auch darauf aufbauende Modelle, die Vorhersagen über das Auftreten von Kooperation und Konflikten treffen, basieren auf den genetischen Beziehungen zwischen Individuen. Die Untersuchung von unverwandten, kooperierenden Individuen stellt somit eine einzigartige Möglichkeit dar, Vorhersagen dieser grundlegenden evolutionsbiologischen Modelle kritisch zu überprüfen.

Mit Hilfe der Allozym-Elektrophorese wurde die neotropische Ameise *Pachycondyla villosa* in drei verschiedene Arten aufgeteilt. Bei einer dieser Arten, vorläufig als *Pachycondyla* cf. *inversa* bezeichnet, können Jungköniginnen nach dem Hochzeitsflug bei der Koloniegründung kooperieren. Die Hälfte aller Gründungskolonien, die in der Nähe von Itabuna, Bahia, in Brasilien gesammelt wurden, enthielten zwischen zwei und fünf Königinnen. *P.* cf. *inversa* Königinnen müssen in der Koloniegründungsphase auf Futtersuche gehen, wobei sich in Gründungsassoziationen immer eine Königin auf diese gefährliche Tätigkeit spezialisierte. Eine genetische Analyse von kooperierenden Königinnen mittels Mikrosatelliten konnte zeigen, dass diese nicht miteinander verwandt sind. Wie kann es zu einer Arbeitsteilung zwischen unverwandten Tieren kommen, bei denen ein Individuum sich zum Vorteil eines anderen verhält, mit dem es nicht verwandt ist? Im Unterschied zu Vorhersagen von Gruppenselektionisten, konnte in dieser Studie gezeigt werden, dass die Arbeitsteilung bei kooperierenden Königinnen auf Konkurrenz basiert: Aggressive Interaktionen führten zu der Ausbildung von Dominanzhierarchien, die vor allem die Arbeitsteilung beeinflussten. Dominante Individuen zwangen unterlegene, auf Futtersuche zu gehen. Der soziale Status eines Individuums war nicht, bzw. nur geringfügig mit dem reproduktiven Status assoziiert: Wie von der „reproductive skew“ Theorie postuliert, legten in den einzelnen Kolonien alle Gründungsköniginnen zu gleichen Anteilen Eier. Allerdings wurden unterlegene Tiere auch während der Eiablage attackiert, und in einigen Fällen

wurden die Eier der unterlegenen Königin gefressen. Dieser selektive Eifraß spiegelte sich auch in einer Analyse der Genotypen von Arbeiterinnen und Königinnen mittels Mikrosatelliten wieder, die kurz nach dem Schlüpfen der ersten Arbeiterinnen durchgeführt wurde: In einigen Fällen produzierten kooperierende Königinnen eine unterschiedliche Anzahl von Nachkommen (Arbeiterinnen). Konflikte zwischen Arbeiterinnen oder zwischen Arbeiterinnen und Königinnen, z.B. über Arbeitsteilung bei den Arbeiterinnen oder die sex ratio, basieren auf den genetischen Beziehungen zwischen den einzelnen Individuen einer Kolonie. In *P.* cf. *inversa* müsste es durch die Anzahl von zwei bis fünf Königinnen in Gründungsassoziationen und deren fehlender Verwandtschaft zu einer ausgeprägten Reduktion des Verwandtschaftsgrades zwischen Arbeiterinnen kommen. Allerdings waren Arbeiterinnen recht eng miteinander verwandt. Zu einer Reduktion des Verwandtschaftsgrades zwischen Arbeiterinnen (in diesem Fall sogar innerhalb einzelner Matrilinien) führte außerdem, dass einzelne Königinnen mehrfach verpaart waren. In dieser Studie konnte ebenfalls gezeigt werden, dass die Inzuchtkoeffizienten (berechnet aus den Allelfrequenzen aus Königinnen und Arbeiterinnen) signifikant von Null unterschiedlich waren, wobei eine geographische Substrukturierung, Wahlund Effekte, oder Null-Allele als mögliche Ursachen ausgeschlossen wurden. Die positiven Inzuchtkoeffizienten sind wahrscheinlich eine Konsequenz von kleinen, örtlich begrenzten Paarungsflügen. Königinnen verpaaren sich dabei nicht in der Nähe des Mutternestes.

Die Analyse kutikulärer Kohlenwasserstoffe lebender Königinnen zeigte eindeutige Unterschiede zwischen dominanten und unterlegenen Königinnen aus Gründungs-assoziationen von *P.* cf. *inversa*. Nur die Kutikula hochrangiger Königinnen wies größere Mengen an zwei Substanzen, Pentadecan (n-C_{15}) und Heptadecen (n-C_{17}:1), auf. Das Vorhandensein dieser Substanzen war dabei nicht vom reproduktiven Status des Tieres abhängig. Es konnte bislang noch nicht geklärt werden, ob die beiden Substanzen tatsächlich einen hohen sozialen Status mitteilen.

In Kolonien, bei denen experimentell zwei Königinnen von *P.* cf. *inversa* zusammengesetzt wurden, kam es zu heftigen aggressiven Interaktionen. Innerhalb von 1 bis 20 Minuten waren stabile Dominanzverhältnisse erkennbar. Die Königin, die mit der ersten Attacke begonnen hatte, wurde das dominante Tier. Für die Ausbildung der Dominanzhierarchie spielte es keine Rolle, ob ein Individuum schon einige Tage länger in dem Nest war als das andere. Vielmehr war die Größe der Königinnen wichtig: in allen

acht Versuchen wurde immer die größere dominant. Allerdings waren dominante Königinnen aus natürlichen Kolonien nicht signifikant größer als unterlegene. Im Freiland ist wahrscheinlich der Besitz eines Nestes für den Ausgang von Dominanzinteraktionen wichtiger als die Körpergröße der Königinnen. So könnten Königinnen, die bereits über einen längeren Zeitraum ein Nest bewohnen, dominant über neuankommende, frisch vermählte Weibchen werden.

Für die Untersuchung der soziogenetischen Struktur einer sekundär polygynen Ameisenart, *Pachycondyla obscuricornis*, erwiesen sich Sequenzen mitochondrialer DNA (COI/COII) als ausreichend variabel: Sechs unterschiedliche Haplotypen konnten bei sechs Arbeiterinnen aus unterschiedlichen Kolonien einer Population unterschieden werden. Alle anderen Methoden, die für diese Art innerhalb dieser Doktorarbeit etabliert wurden (RFLPs, Mikrosatelliten-Analysen, Multilocus DNA Fingerprinting und Allozym-Elektrophorese) waren für eine weitere Untersuchung nicht ausreichend variabel.

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10. Publications

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Kolmer, K., and J. Heinze. 2000. Comparison between two species in the *Pachycondyla villosa* complex (Hymenoptera: Formicidae). *Entomologica basiliensia* 22: 219-222.

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Conference participation

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Kolmer, K., J. Tentschert, J. Heinze, and H.J. Bestmann. 1999. Dominance interactions, division of labour and rank recognition in the neotropical ant *P. 'inversa'* (Hymenoptera: Formicidae). In P. Rosenkranz and C. Garrido, eds., *Soziale Insekten, IUSSI Tagung Hohenheim*. Graz, Hohenheim, Würzburg.

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11. Curriculum vitae

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Studium	1990-1997	Studium der Biologie an der TU-Darmstadt
	1997	Abschluss: Diplom Diplomarbeit: „Charakterisierung von Arten und Koloniestruktur in der Ameisengattung *Tetramorium*, mit Hilfe der Isoenzym-Elektrophorese“
Promotion	1998-2001	Wissenschaftliche Angestellte (DFG) am zoologischen Institut für Verhaltensphysiologie und Soziobiologie der Universität Würzburg in einem Gemeinschaftsprojekt von Prof. Dr. Hölldobler (Würzburg) und Prof. Dr. Heinze (Regensburg). Dissertation „Co-operation and conflict in the ponerine ant genus *Pachycondyla*“