Chap. 8: Phylogenetic relationships and evolution of host plant use in the tortoise beetle community

8.1. Introduction

Plant feeding arose early in beetle history. Herbivorous species doubled beetle diversity by the mid-Jurassic and surpassed the nonherbivorous taxa by the beginning of the Tertiary; this interval coincided with the rise of the angiosperms (Farrell 1998). In Farells´ phylogeny of phytophagous beetles (subfamily-level), the total increase in beetle diversity seems to be directly attributable to a series of adaptive radiations onto the angiosperms (Farrell 1998) which gives support to the idea of a parallel evolution of phytophagous insects and their host plants. Recent molecular phylogenies on single genera of Chrysomelidae, however, do not always support a parallel evolution on this lower taxonomical level (e.g. Kelley and Farell (1998) and Garin et al (1999)). Thus, strict congruence of pylogenies still seems to be rare (Mitter et al., 1991).

A trait in which all phylogenetic studies of Chrysomelidae-genera agree in, is the conservatism of host associations (Futuyma & McCafferty 1990, Mitter et al. 1991, Kelley & Farell 1998, Köpf et al. 1998). Shifts in host plant use, therefore, tend to occur more frequently within a plant family than between different plant families. Closely related species of the genus *Phratora*, for example, generally feed on related species of plants (Köpf et al 1998). The evolution of new associations seems to be genetically constrained (Mitter et al. 1991, Keese 1998).

A central question in insect-plant interactions is the evolution of host plant specialization (Ehrlich and Raven 1964, Bernays & Chapman 1994, Kelley & Farrell 1998). Causes of specialization have been discussed controversially, to explain the evolution of a trait which at least at the first glance restricts the possibilities of an insect to gain food (overcome of plant secondary chemistry-hypothesis (Ehrlich & Raven 1964), freedom from natural enemies-hypothesis (Price et al. 1980, Bernays & Graham 1988); jack of all trades, master of none hypothesis (Simms & Rausher 1989, Robinson et al. 1996)). A question related to this subject is, whether host plant specialization is a derived or a primitive trait in herbivore phylogenies. Molecular phylogenies of different genera of the Chrysomelidae (and close relatives) and their host plant associations have become available only recently and show different results. Whereas the genera *Phratora* (Köpf et al. 1998)) and *Dendroctonus* (Scolytidae)(Kelley & Farrell 1998) show trends of increasing specialization, the phylogenies of *Ophraella* (Futuyma & McCafferty 1990) and *Oreina* (Dobler et

al. 1996) show little evidence that specialists tend to be more derived. In the genus *Chrysolina* (Garin et al. 1999) one clade even shifts from a specialized to a generalized feeding habit.

In this study I reconstruct the phylogeny of a group of Cassidinae by molecular data. The beetle group consists of three genera, which coexist sympatricly on the same host plant system but have developed different degrees of specialization for host plant species and microhabitats. I compare the molecular phylogeny of this group to current proposals of Cassidinae systematics (Borowiec 1994)(hypothesis 1: Fig. 8.1) and ecological data (this study, chap.2 and chap.5)(hypothesis 2: Fig 8.2). No former literature is available on the host associations of the beetle species investigated. Systematic work has been done by Spaeth (review see Borowiec 1994) and recently a revision of the whole afrotropical Cassidinae has been published by Borowiec (1994). Questions investigated in this chapter were: (1) Is the molecular phylogenetic tree of the beetle species in concordance with the systematic categorization based on morphological criteria (hypothesis 1)? (2) Or is the molecular tree in better agreement with ecological similarities between the species (e.g. host plant use, similar microhabitats)(hypotheses 2: species using the same host plants should be more closely related)? (3) Are specialized feeding patterns primitive or derived within the tortoise beetle community investigated?

Fig. 8.1: Hypothetical phylogenetic tree No. 1 based on morphological characters (hypothesis 1). Beetles investigated belong to the subfamily Cassidinae (Chrysomelidae).

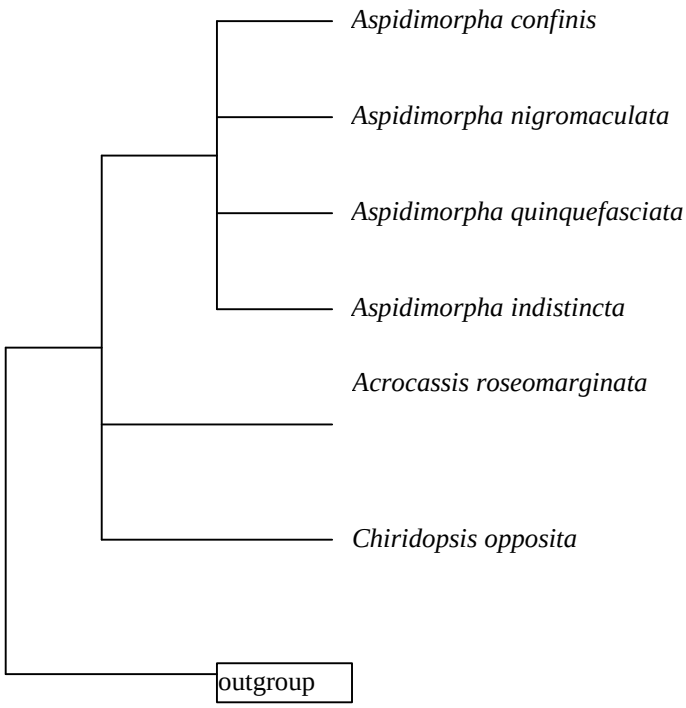
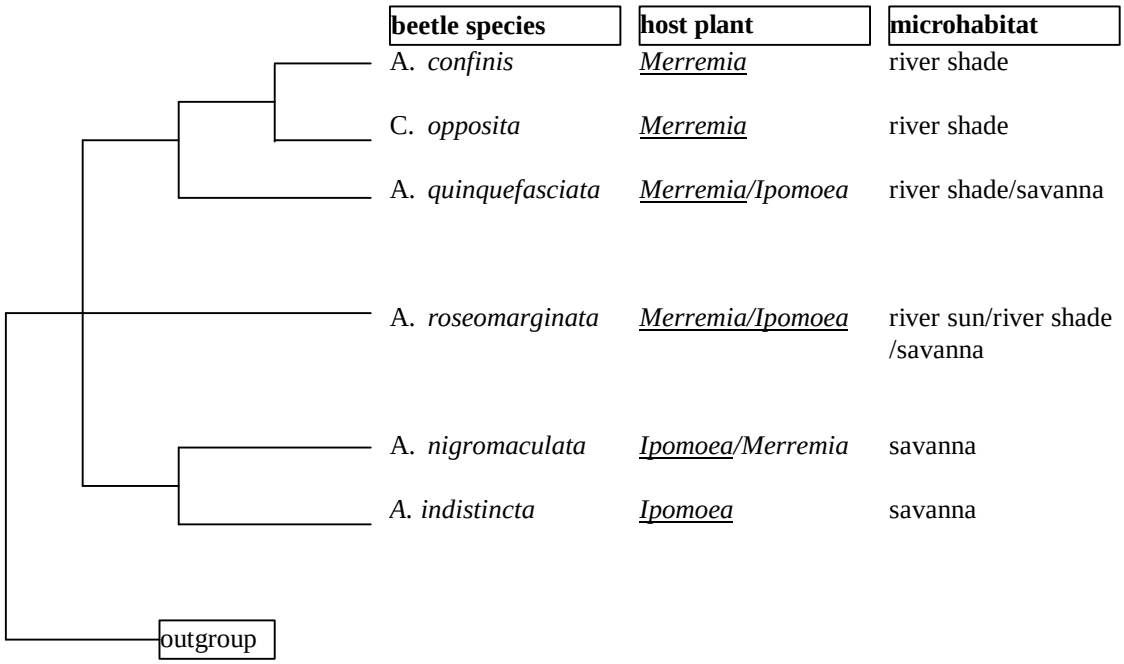


Fig. 8.2: Cladogram No. 2 based on ecological criteria (hypothesis 2).



8.2. Materials and methods

8.2.1. Beetle sampling

The six beetle species used for the phylogeny, are all members of the subfamily Cassidinae; the four outgroup species, whose sequences were used for the tree building, belong to the subfamily Chrysomelinae (Köpf et al. 1998). All beetle individuals of the six species were collected in the same area in the Comoé-National Park, Ivory Coast (Lola-camp) in 1996-1998. Five of these species were bred in the laboratory in Wuerzburg and individuals were taken from these populations for analyses, when available. Therefore, DNA was extracted from material, which was either deep frozen of species kept in the climatic chamber (*Aspidimorpha confinis, Aspidimorpha indisticta, Aspidimorpha quinquefasciata, Acrocassis roseomarginata, Aspidimorpha nigromaculata*), or preserved in alcohol (100%) in the field (*Chiridopsis opposita*).

8.2.2. DNA extraction, PCR and nucleotide sequencing

I analyzed one adult per beetle species. Only head and thorax of each individual were used for DNA extraction. DNA was extracted by use of a standard phenol/chloroform procedure as described in Garnery et al. (1991). Purified DNA was stored in H_2O dest. at -20°C. Aliquots of these DNA samples were then used in polymerase chain reactions (PCR). Four primers (Simon et al. 1994, Köpf et al 1998) were used to amplify either one (P2: *A. confinis*; P4*: A. roseomarginata, A. nigromaculata*) or two double stranded DNA fragments which overlap in 134 basepairs (P2 and P4: *A. indistincta, A. quinquefasciata, Ch. opposita*) (Tab. 8.1). The sequences examined are part of the mitochondrial COI gene which codes for an enzyme of the oxygen-chain. The COI gene was used in several studies examining the molecular phylogeny of a Chrysomelidae genus (Köpf et al 1998).

Tab. 8.1: Four primers used for the partial amplification and sequencing of the COI gene (described in Simon et al 1994 and Köpf et al 1998).

Primer	Sequence
P2: C1-J-1718	5' GGAGGATTTGGAAATTGATTAGTTCC 3'
P2: C1-N-2329	5' ACTGTAAATATATGATGAGCTCA 3'
P4: C1-J-2195	5' TTGATTTTTGGTCATCCAGAAGT 3'
P4: TL2-N-3014	5' TCCAATGCACTAATCTGCCATATTA 3'

PCRs were carried out in total reaction volumes of 50 µl, containing reaction buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each primer, 0.2 mM dNTP, and 2.5 U of Taq Polymerase (Promega). The thermocycling profile (1) consisted of 30 cycles with 1 min at 92°C, 1 min at 37°C, and 1 min at 72°C, the thermocycling profile (2) of 40 cycles with 30 sec. at 94°C, 30 sec. at 35°C, and 1 min at 72°C. PCRs were performed either on an MJ Research model PTC-100 temperature cycler or on a Perkin-Elmer GeneAmp PCR System 2400.

PCR fragments were purified with a JETquick PCR purification kit (Genomed) to remove unincorporated primers and dNTPs (single nucleotids). Primers were used to sequence both strands of the PCR fragment to identify all sites unambiguously. Sequencing was done by the dideoxy method (Sanger et al. 1977) using AmpliTaq DNA Polymerase FS and fluorescent labelled dNTP's. Sequencing reactions were set up according to the supplier's recommendations (Perkin-Elmer 1995) and purified using ethanol precipitation at room temperature. Automated DNA sequencing was performed on an ABI PRISM™310 Genetic Analyzer.

8.2.3. Sequence alignment

The Navigator PPC software (Applied Biosystems) was used for proofreading and sequence alignment. In the first step the two single strands of each fragment were aligned and compared for errors. Of the three species, where all four primers had amplified, the two fragments were combined and their overlapping region controlled for conformity. There was a very good concordance in the overlapping region of each species. In a second step the COI sequences of these 3 species were aligned manually with each other and with four outgroup species (Köpf et al. 1998, see below). In the third step those seven species were aligned either with *A. confinis* which had amplified only with primer pair P2 or with *A. roseomarginata* and *A. nigromaculata* which

had amplified at P4. For each primer-pair the sequences of all species which had amplified for that pair, were cut to the length of the shortest fragment in the respective group. Those three data groups were used for tree building.

8.2.4. Tree-Building methods

Different methods for phylogeny reconstruction make very different assumptions about the underlying evolutionary process. We used the maximum parsimony, the neighbor joining and the maximum likelihood method to reconstruct phylogenetic relationships (PAUP 4.0 Beta1).

8.2.5. Outgroups and rooting

Four outgroup taxa (Chrysomelinae: Chrysomelidae) were included as additional taxa in the tree. These were *Chrysomela vigintipunctata* (Scop.), *Gastrophysa viridula* (Deg.), *Phratora vitellinae* (L.) and *Phratora aklaviki* (Carr.). Nucleotid sequences of these species were analyzed by Köpf et al (1998).

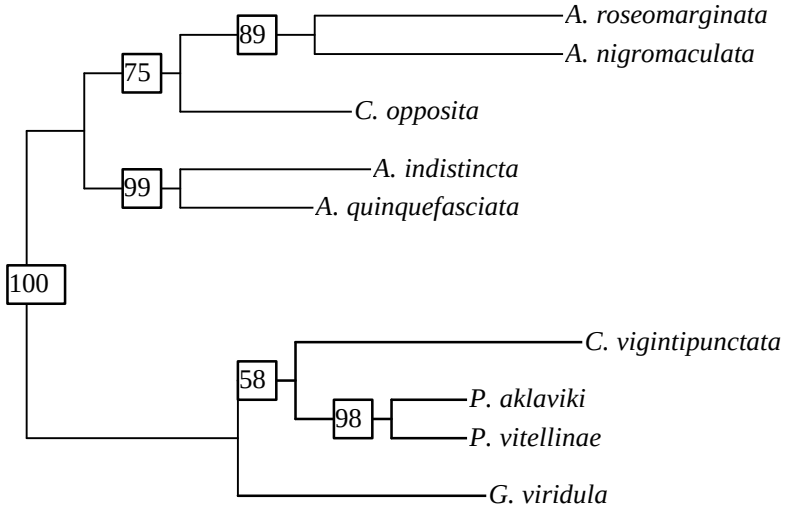
8.3. Results

8.3.1. The combined molecular phylogenetic tree

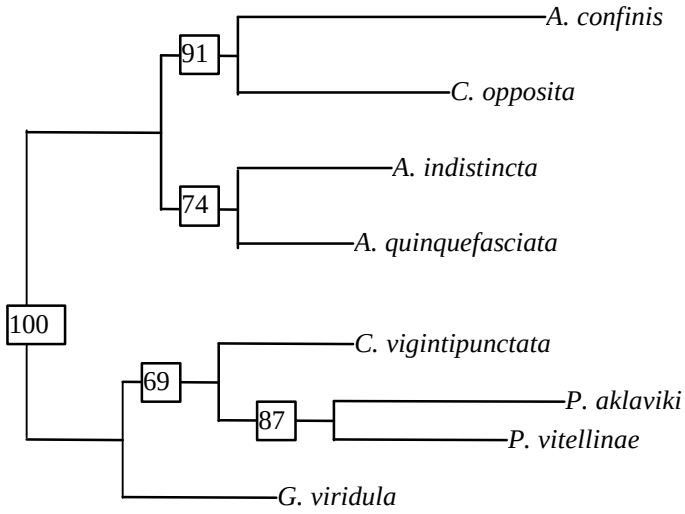
Not all 6 beetle species, investigated, could be amplified at the same region of the gene. Four species could be amplified at a first (”P2”, Fig. 8.3) and five at a second sequence (”P4”, Fig. 8.3) within the mitochondrial COI-gene. These sequences and a short overlapping sequence (”short”, Fig. 8.3) of 134 base pairs, where all beetle species had amplified, were used for a combined phylogenetic tree (Fig. 8.4). This third tree clarified the position of *A. confinis* in the final tree. For each tree, all three methods (maximum likelyhood, maximum parsimony and neighbor joining) agreed in the tree they proposed. Bootstrap values for both trees (”short” not included) were reliable (Fig. 8.3). Bootstrap proportions supporting single nodes are given in the figures; the branch lengths are proportional to the number of mutations.

Fig. 8.3: Molecular trees built on the basis of three sequences (P4, P2, short) of the mitochondrial COI-gene. Bootstrap values >50% suggest a high validity of the respective relation of a species group. Length of tree branches relates to the number of mutations.

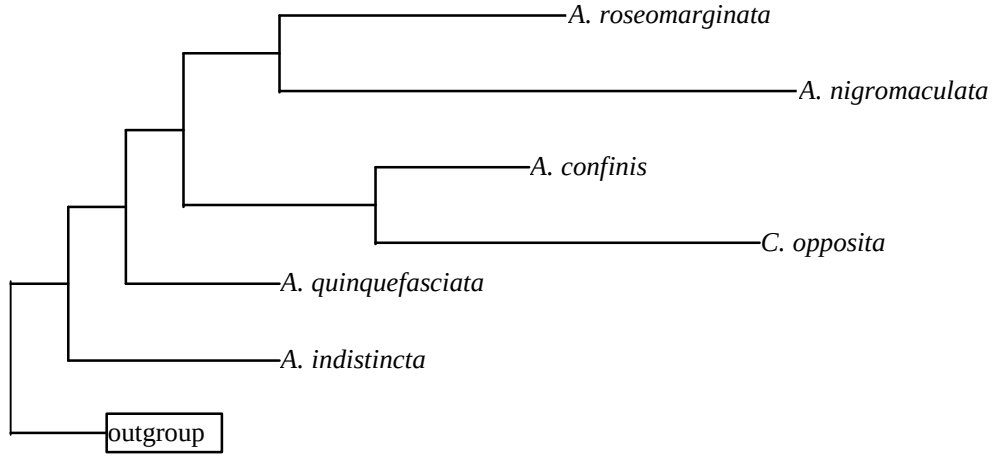
P4:



P2:



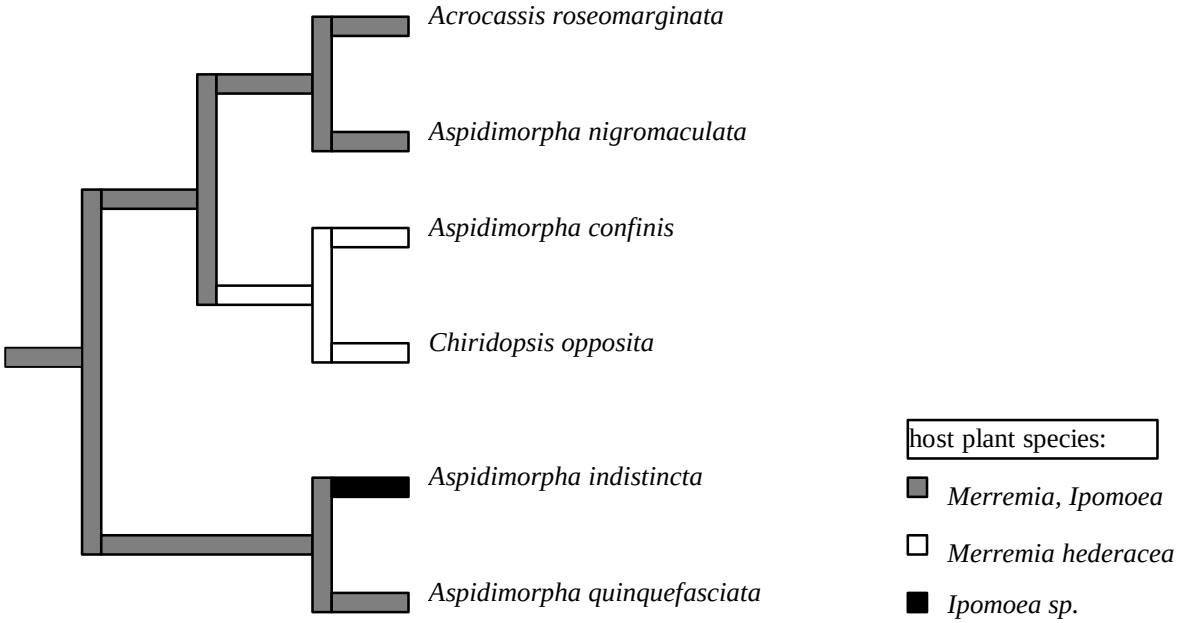
short:



8.3.2. Comparison of the molecular phylogenetic tree with the systematic (morphological) tree

My first hypothesis was, that the molecular tree (Fig. 8.4) should agree with the systematic tree of the beetles, that distinguished three different genera based on morphological criteria (Fig. 8.1). The three genera *Aspidimorpha, Acrocassis* and *Chiridopsis* were not strictly separated from each other in the molecular phylogenetic tree. Some species of the genus *Aspidimorpha* were more closely related to the two other genera than to their congeners. The cladogram therefore suggests that the large genus *Aspidimorpha* is polyphyletic or that the two other genera have no justification. *Acrocassis roseomarginata* was most closely related to *Aspidimorpha nigromaculata* and *Chiridopsis opposita* to *Aspidimorpha confinis*. *Aspidimorpha indistincta* and *Aspidimorpha quinquefasciata* were grouped close to each other, but more distant to the other species within the genus *Aspidimorpha*. The two subfamilies Cassidinae and Chrysomelinae (outgroup of the molecular tree), however, were clearly separated in the molecular tree.

Fig. 8.4: Combined molecular tree of the tortoise beetle community associated with the *Merremia-*, *Ipomoea*-complex in Ivory Coast. Ancient host plant relations were reconstructed according to the least number of host plant switches necessary. Grey: generalist species feeding on *Merremia* and *Ipomoea*; white: species feeding only on *Merremia hederacea*; black: species feeding exclusively on *Ipomoea sp.*



8.3.3. Comparison of the molecular phylogenetic tree with the ecological cladogram

To test the second hypothesis, the molecular tree (Fig. 8.4) was compared with the ecological cladogram of the beetle community (Fig. 8.2), based on data discussed in other chapters (chap.2, chap. 5). In this hypothesis, beetle species which used the same main host plant species and/or live in the same habitat were predicted to be more closely related with each other than with species which used different host plant species/genera or lived in different habitats.

This two trees, the molecular and the ecological tree, were more similar than the molecular and the morphological tree. There was a good agreement in the close molecular phylogenetic relation of *Aspidimorpha confinis* and *Chiridopsis opposita,* in spite of their systematic position in different genera. Both occurred only in river side shade habitats and on the host plant *Merremia hederacea*. The other two "species-pairs" of the molecular tree also had at least one host plant genus and habitat in common, but one partner in both cases was less specialized than the other and habitat preferences differed (Fig. 8.4). (*Acrocassis roseomarginata* and *Aspidimorpha nigromaculata* both could occur in the savanna on *Ipomoea* and at the river side on *Merremia,* but *A. nigromaculata* was nearly exclusively found in the savanna. *Aspidimorpha indistincta* and *Aspidimorpha quinquefasciata* both occurred in the savanna on *Ipomoea,* but *A.quinquefasciata* was most abundant at the river side on *Merremia*.

8.3.4. Evolution of host plant use within the beetle community

Evolution of host plant affiliations of the beetle species, could be, at least in part, reconstructed with the phylogenetic tree. The present host plant use of the species was known from field observations (chap.2). The host plant use of the common ancestor(s) was reconstructed by using the least number of host plant switches to the present situation, possible. In the combined tree (Fig. 8.4), two host plant switches were necessary, when a use of both host plant genera, *Merremia* and *Ipomoea,* by the ancestor was assumed. In another reconstruction, where *Merremia hederacea* was the only host of a common ancestor, three host plant switches up to the present state would have been necessary.

In the first example of a generalistic common ancestor, generalists would have been in a primitive and specialists, like *C. opposita*, *A. confinis* and *A. indistincta*, in a derived position in the phylogenetic tree of the beetle community investigated. *Acrocassis roseomarginata* was by far the most generalistic species among the six beetle species compared here. It had the largest niche

breadth in terms of microhabitat use (chap. 5) and was the most abundant species on both host plant genera (chap. 3). Within the molecular tree it could have, in contrast to related species, maintained a broad niche during evolution.

8.4. Discussion

8.4.1. Molecular and morphological-systematic phylogenetic tree

The molecular phylogenetic tree was only partly consistent with the morphological-systematic tree. In both trees the outgroup (Uf. Chrysomelinae) was clearly separated from the Cassidinae-group and the species *Aspidimorpha indistincta* and *Aspidimorpha quinquefasciata* were very closely related. The two trees differed, however, in the close relationship of *Aspidimorpha nigromaculata* with *Acrocassis roseomarginata* and of *Aspidimorpha confinis* with *Chiridopsis opposita* in the molecular tree, in both cases species-pairs of different genera.

Borowiec has set up a key to the genera (in the first volume of his monograph of the afrotropical Cassidinae (Coleoptera: Chrysomelidae) (Borowiec 1994)). Cassidinae-genera are distinguished by body size, body shape, shape of pronotum and elytra and punctation, length of antennal and tarsal segments. The genus *Aspidimorpha* is one of the largest genera within the afrotropical Cassidinae. Many species are described as extremely variable in color and structure and the genus is said to be one of the most difficult in taxonomical practice (Borowiec, 1994). This could be a hint which supports a polyphyletic origin of the genus *Aspidimorpha,* as the molecular results of sequences of the COI-gene suggest.

8.4.2. Molecular and ecological phylogenetic tree and the coexistence of the beetle community

A comparison between the ecological and the molecular tree showed that similar ecological niches could be realized in closely related species but that in more cases niches have diverged. Two of the species-pairs of the shield beetle community, *Acrocassis roseomarginata-Aspidimorpha nigromaculata* and *Aspidimorpha indistincta-Aspidimorpha quinquefasciata*, showed a divergent development in habitat and host plant use (one species of the two was always more polyphagous than the other and species differed in their preferences for different habitats), one species-pair showed a non-divergent development (*Aspidimorpha confinis-Chiridopsis opposita*). Divergent ecological resource use would be expected in the case of a sympatric speciation with ecological

niche differentiation and character displacement due to competition (Hutchinson 1959, Leibold 1998) or specialization of one of the species. The mechanisms which have led to the evolution of specialization or formation of new species, can, however, not be inferred from the present data.

The hypothesis that *A. roseomarginata* represented an own phylogenetic lineage which separated early in evolution from the rest of the Cassidinae community, had to be rejected. The hypothesis was based on the restricted seasonal occurrence (beginning of the rainy season)(chap. 3) and the better tolerance of high temperatures (chap. 6) of *A. roseomarginata* in comparison to the other beetle species which occurred during the whole vegetation period and could tolerate only much lower temperatures (e.g. *Aspidimorpha confinis*). The molecular phylogenetic tree showed that *A. roseomarginata* was a relatively derived species which was closely related to *A. nigromaculata* within the community.

8.4.3. Evolution of host plant use

There are three possibilities for the evolution of host plant use in the beetle community investigated. The ancestor(s) could have been specialized on *Merremia,* on *Ipomoea,* or they could have been oligophagous on both genera. The solution with the least host plant switches would result in a more generalistic ancestor, feeding on *Merremia* and *Ipomoea* (two host plant switches/specializations necessary) and monophagy would be a derived character.

Most recent studies of molecular phylogenies of Chrysomelidae-genera agree in the pronounced conservatism in beetle-host plant associations on the plant family level (Futuyma & McCafferty 1990, Mitter et al. 1991, Thompson 1993, Brown et al 1994, Farell 1998, Kelley & Farell 1998, Köpf et al. 1998). The strong phylogenetic component in many insect-plant associations suggests that host shifts are strongly constrained by the ancient host plant use (Mitter et al. 1991). Species of the leaf beetle genus *Ophraella*, like other phytophagous insects most readily adapted to related plants (Futuyma et al. 1993, Futuyma et al. 1994, Futuyma et al. 1995). This was supported also by results of Keese (1998) who found that in two closely related leaf beetle species *O. notulata*, the species with the derived host association, retained a considerable ability to utilize the ancestral host plant, while *O. slobodkini*, the species with the ancestral host association, did not show a similar ability to utilize the derived host. In contrast to these studies Garin et al (1999) found in the leaf beetle genus *Chrysolina* a minimum of five host plant switches from the ancestral state at the family level and suggested the absence of parallel evolution of

beetles and their host plants. For the community of six afrotropical Cassidinae-species investigated in this study I found host plant associations only within one plant family (Convolvulaceae) which seemed to support the hypothesis of the phylogenetic constraints on host plant use. No host records of these beetle species are available in the literature.

The evolution of host specificity in phytophagous insects still is one of the central questions in insect-plant studies (Ehrlich & Raven 1968, Bernays & Graham 1988, Kelley & Farrell 1998). Beside the ecological mechanisms which have lead to specialization, it is still an unsolved problem whether specialization is a primitive or a derived trait. An analysis of a bark beetle community (*Dendroctonus*) suggested that specialists evolved from generalists for at least six times independently (Kelley & Farrell 1998). By contrast in other Chrysomelidae genera (*Ophraella* (Futuyma & McCafferty 1990), *Oreina* (Dobler et al. 1996), *Chrysolina* (Garin et al. 1999)) specialists did not tend to be more derived. In this study probably an oligophagous beetle species ("generalist") was the ancestor. Some of the present species, evolved, were monophagous specialists (*Aspidimorpha indistincta, Chiridopsis opposita*), others oligophagous with broader niches in terms of host plants and microhabitats (*Acrocassis roseomarginata*)(host plant range in the field). Thereafter specialists seemed to have evolved from generalists in this group studied here.

In conclusion, although in the field beetle species differed considerably and predictably in their host plant preferences (chap.2) and abundances in different habitats/microhabitats (chap.3), differences and number of species investigated were too small to make a definite statement about the direction of the evolution of specialization in this beetle community.