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Testing the trend towards specialization in herbivore–host plant associations using a molecular phylogeny of *Tomoplagia* (Diptera: Tephritidae)

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Abstract

Herbivorous insects are abundant and diverse and insect–host plant associations tend to be specialized and evolutionarily conserved. Some authors suggested that generalist insect lineages tend to become specialists, with host specialization leading to an evolutionary dead-end for the parasite species. In this paper, we have examined this tendency using a phylogenetic tree of *Tomoplagia* (Diptera: Tephritidae), a parasite of asteracean plants. We have tested the trend towards specialization in different hierarchical degrees of host specialization. The topology of the tree, the inference of ancestral hosts, and the lack of directional evolution indicated that specialization does not correspond to a phylogenetic dead-end. Although most *Tomoplagia* species are restricted to a single host genus, specialization does not seem to limit further host range evolution. This work emphasizes the advantages of the use of different levels of specialization and the inclusion of occasional hosts to establish a more detailed scenario for the evolution of this kind of ecological association.

Keywords: Insect–plant interactions; Host specialization; Tephritidae; Asteraceae; Molecular phylogeny; Host shifts; Cytochrome oxidase subunit 2; Ribosomal 16S

1. Introduction

Herbivorous insects are extraordinarily diverse in tropical and temperate biomes, with roughly a quarter of all eukaryotes being insects that feed on plants (Bernays, 1998). In addition to their diversity, insect–plant associations are specialized (Bernays and Graham, 1988; Fry, 1996; Funk et al., 2002; Jaenike, 1990; Lopez-Vaamonde et al., 2003) and show a high degree of phylogenetic conservation, i.e., related insect lineages tend to feed on related plants (Benson et al., 1975; Janz and Nylin, 1998; Wahlberg, 2001).

Several theories have been developed to explain these patterns. The classic coevolutionary theory suggests that insect–plant interactions arose through successive evolutionary innovations in plant defenses and in their circumvention by insects, thus producing alternating episodes of plant and insect radiation (Ehrlich and Raven, 1964). Such an evolutionary pattern would be expected to result in close similarity in the sequence of speciation events in the plants and insects (Thompson, 1994). These similarities, however, have been rarely observed and, consequently, have been assumed to play

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a minor role in the evolutionary associations between herbivores and their host-plants (Benson et al., 1975; Farrell and Mitter, 1993; Funk et al., 1995a,b; Janz et al., 2001; but see Farrell and Mitter, 1998; Percy et al., 2004).

On the other hand, herbivore lineages may switch their affinities to other plant groups after diversification of the host-plants, with no induction of any adaptive response in these plants (Jermy, 1984, 1993). Indeed, based on observations of host-shifts over short periods of time, many authors have proposed that, on an evolutionary scale, host affiliations of herbivorous insects may not be a consistent feature (Bernays and Graham, 1988; Rausher, 2001; Wasserman and Futuyma, 1981; but see also Feder et al., 2003). Nevertheless, if host-shifts are common, then one must explain the predominance of specialists over generalists, as well as the phylogenetic conservatism on an evolutionary scale.

Another classic hypothesis developed to explain these observations had suggested that specialization is a derived character (Brues, 1920). More specifically, any long-term association with a particular host may eventually result in the loss of the genetic variation associated with the ability to use alternate hosts, due to genetic drift or to the lack of selective pressure to maintain these alleles. This situation should culminate in an evolutionary dead-end for the parasite species. In this case, we should expect that the phylogenetic reconstruction of the parasites would show a trend towards specialization (Cope, 1896—*apud* Mayr, 1997); Futuyma and Moreno, 1988; Kelley and Farrell, 1998; Mayr, 1997; but see also Amadon, 1943; Janz et al., 2001; Termonia et al., 2001).

To properly test the tendency towards specialization in herbivorous insects using phylogenetic analysis, it is convenient to select a model in which insects and their hosts have the following characteristics: (1) the insect species should be closely related, (2) insect and plant groups should be speciose and of no economic value, (3) host ranges should be known accurately for all insect species, and (4) the insect species should show different degrees of host specialization. Unfortunately, most studies on this subject have neglected some or all of these characteristics (Dobler et al., 1996; Funk et al., 1995a; Nosil, 2002).

Therefore, in this paper, we studied the *Tomoplagia*–Asteraceae association, which shows all of the above characteristics, making it an exceptional model for studying insect–plant systems. For instance, *Tomoplagia* Coquillett is one of the most speciose and best studied genus of Neotropical tephritids (Prado and Lewinsohn, 1994; Prado et al., 2002), with 59 known species (Aczél, 1955; Prado et al., 2004). Their hosts belong to Asteraceae, the largest family of plants, with 23,000 species distributed in 1535 genera which, in turn, are subdivided into 17 tribes worldwide (Bremer, 1994). The host-records of *Tomoplagia* in Brazil reflect 17 years of an extensive inventory of endophagous insects among Asteracea (Lewinsohn, 1987, 1991; Prado et al., 2002) and are exceptionally complete and unequivocal. Finally, *Tomoplagia* can parasitize asteracean plants with different degrees of specialization (Headrick and Goeden, 1998), which means that some species are genus-, subtribe- or tribe-specialists, while others (generalists) parasitize various tribes among Asteraceae (Table 1).

We thus reconstructed the phylogeny of *Tomoplagia* based on two mitochondrial genes, *Cox*II and 16*S*, and tested the phylogenetic consistency of detailed host affiliations of *Tomoplagia*. To account for alternative explanations of the different patterns, we carefully analyzed different degrees and hierarchies of host specialization.

2. Materials and methods

2.1. The host-parasite system

The genus *Tomoplagia* belongs to the subfamily Tephritinae (Korneyev, 1999), the one with the most specialized insect–plant interactions among Tephritidae. *Tomoplagia* females, as well as the females of other endophagous insects, lay their eggs on the flower heads of the host, where the larvae develop. This characteristic is crucial to the unambiguity of the host-records of these flies, since the flower heads are sampled, transported, and maintained in laboratory until the emergence of the adults, providing accurate information about main and occasional hosts of each species. Furthermore, most of asteracean plants have no economical value, another important factor to determine their ecological habits and geographic distribution in more detail.

Since the meaning of the term "specialist" is not well defined in the literature (Kelley and Farrell, 1998), the different levels of specialization found in *Tomoplagia* are very important for testing evolutionary processes related to patterns of specialization. For instance, some authors consider as specialist a species which parasitizes a single host species, while others regard as specialist a species which is able to parasitize plants of an entire family (Nosil, 2002, and references therein).

We thus sampled 19 species of *Tomoplagia* from the Cerrado (Brazilian savannas) and Campos Rupestres (Highland grasslands) environments in the Brazilian states of Goiás (GO), Minas Gerais (MG), São Paulo (SP), and Santa Catarina (SC). The sampling sites for the individuals analyzed are shown in Fig. 1.

2.2. DNA preparation, amplification, and sequencing

Insects were identified soon after their emergence and were immediately stored in liquid N_2 until DNA extraction. Wings and terminalia of each individual were preserved for a taxonomic reevaluation, if necessary. Total

Table 1
Host affiliation of *Tomoplagia* species analyzed showing the main and occasional hosts and the degree of specialization of each species, represented by the total host range

Species	Range of main hosts	Occasional hosts	Total host range
T. argentiniensis	*Cyrtocymura*[a]		Genus
T. pseudopenicillata			
T. minuta	*Vernonanthura*[a]		Genus
T. fiebrigi			
T. rudolphi (gall maker)			
T. grandis	*Lessingianthus*		Genus
T. brasiliensis			
T. voluta	*Lychnophora*[b]		Genus
T. trivittata	*Gochnatia*[c]		Genus
T. sp3	*Eremanthus*[b]		Genus
T. sp2	*Eremanthus*[b]	*Lychnophora*[b]	Subtribe
T. rupestris	*Lychnophora*[b]	*Eremanthus*[b]	Subtribe
T. costalimai	*Trixis*[d]	*Jungia*[d]	Subtribe
T. achromoptera	*Lessingianthus*[a]	*Proteopsis*[b]	Tribe
T. sp1	*Chrysolaena*[a], *Echinochoryne*[a], *Lepidaploa*[a], *Lessingianthus*[a],	*Vernonanthura*[a] and subtribe Lychnophorinae[b]	Tribe
T. tripunctata	*Lessingianthus*[a]	*Trixis*[d]	Tribes
T. incompleta	*Chrysolaena*[a], *Echinochoryne*[a], *Lepidaploa*[a], *Lessingianthus*[a]	Many spp from Subtribes Vernoniinae and Lychnophorinae, and from tribes Eupatorieae and Mutisieae	Tribes
T. bicolor	*Lychnophora*[b], *Eremanthus*[b], *Minasia*[b], *Piptolepis*[b]	*Chresta*[a], *Moquinia*[e]	Tribes
T. reimoseri	*Vernonanthura*[a]	*Cyrtocymura*[a], *Minasia*[b], *Baccharis*[f]	Tribes

[a] Vernonieae: Vernoniinae.
[b] Vernoniinae: Lychnophorinae.
[c] Mutisieae: Mutisiinae.
[d] Mutisieae: Nassauviinae.
[e] Moniquinieae.
[f] Astereae.

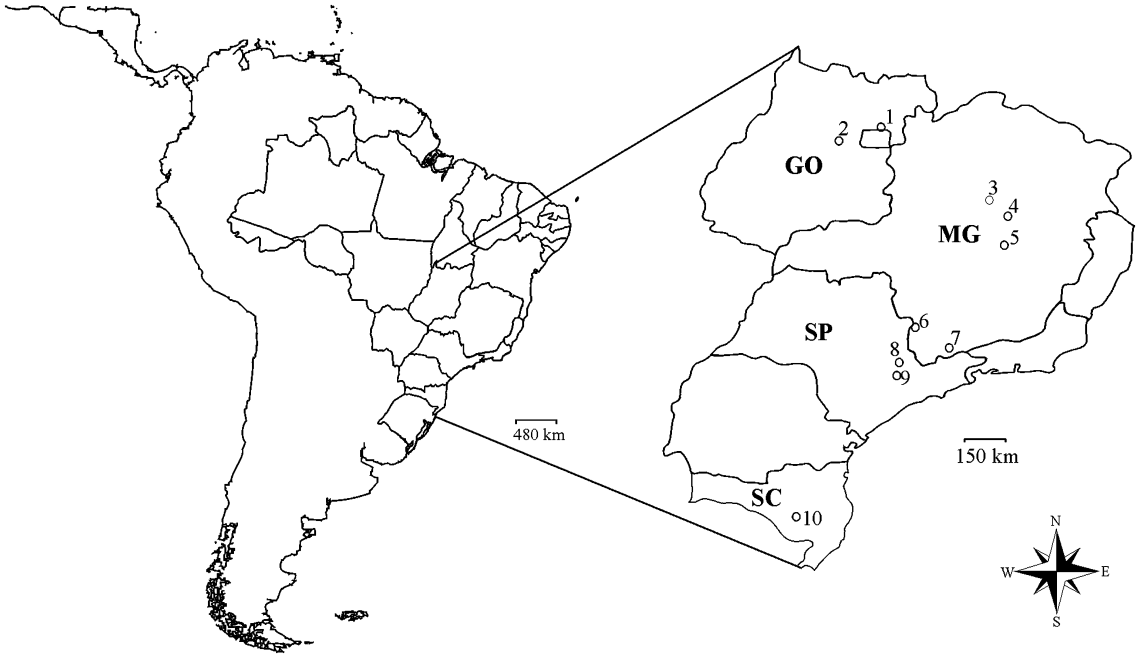

Fig. 1. Map showing collection sites for *Tomoplagia* species. The exact position of each site is available in GenBank through the accession number of each species (see Fig. 2). The site labels are: 1: Unaí, GO; 2: Pirenópolis, GO; 3: Joaquim Felício, MG; 4: Diamantina, MG; 5: Santana do Riacho, MG; 6: Poços de Caldas, MG; 7: Itajubá, MG; 8: Campinas, SP; 9: Cabreúva, SP; and 10: Lages, SC.

DNA was isolated from each adult using a modified phenol–chloroform protocol (Azeredo-Espin et al., 1991).

A 683 bp fragment of the *Cox*II gene and a 642 bp fragment of the mitochondrial ribosomal 16*S* were amplified by the standard PCR technique. These sequences correspond, respectively, to positions 3083–3766 (*Cox*II) and 12,756–13,398 (16*S*) in the *Drosophila yakuba* mitochondrial sequence (Clary and Wolstenholme, 1985). Typical amplification conditions included a 2 min denaturation at 95 °C, followed by 40 cycles of 94 °C for 30 s, 40–52 °C for 30–50 s, and 72 °C for 1 min with a final extension at 72 °C for 4 min. PCR products were cleaned out of non-specific fragments, primers and enzymes using the Concert rapid gel extraction system (Gibco-BRL), and recovered in 30 μl of sterilized water.

The PCR product of each specimen was directly sequenced using dye terminator chemistry in an ABI377A DNA sequencer (Applied Biosystems). PCR primers were also used in the sequence protocol: TL2-J-3034 (AAT ATG GCA GAT TAG TGC A) and TK-N-3785 (GTT TAA GAG ACC AGT ACT TG), for *Cox*II; and N1-J-12585 (GGT CCC TTA CGA ATT TGA ATA TAT CCT), and LR-N-13398 (GCG CTG TTT AAC AAA AAC AT) for 16*S*, as described by Simon et al. (1994). Sequence chromatograms were evaluated and edited in the Consed program (Gordon et al., 1998).

Out groups were chosen based on availability of *Cox*II and 16*S* sequences and their taxonomic position relative to *Tomoplagia*. Both gene sequences were obtained from the *Ceratitis capitata* (Spanos et al., 2000) and *Drosophila yakuba* (Clary et al., 1992) mitochondrial genome sequences, accessible in GenBank (http://www.ncbi.nlm.nih.gov), through Accession Nos. NC-000857 and NC-001322, respectively.

2.3. Molecular phylogenetic analysis

*Cox*II and 16*S* sequences for *Tomoplagia* and the outgroup species were initially aligned using CLUSTAL W (http://www.ebi.ac.uk/clustalw) and the alignment was corrected manually using the Se-Al program (Rambaut, 2002). For *Cox*II sequences, the fragment sequenced ranged from 482 to 651 bp, representing a total of 274 variable sites out of 651 aligned positions. For 16*S* sequences, 592 positions were aligned (508–592 bp sequenced), with 227 variable sites.

The maximum likelihood (ML) (Cavalli-Sforza and Edwards, 1967; Felsenstein, 1981) phylogenetic tree was inferred using the concatenated *Cox*II and 16*S* sequences and the analysis was done with the PAUP* program (Swofford, 1998). In ML phylogenies, a careful selection of the evolutionary model is necessary to ensure the reliability of the tree. Therefore, we used the ModelTest 3.06 program (Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998) to establish the model of DNA evolution that best fit our data. The model of choice was the general time-reversible (GTR) (Rodriguez et al., 1990), considering 31% of invariable sites and a value of gamma shape parameter = 0.7167. This model will be referred to in the text as the concatenated model. To find the maximum likelihood tree, the heuristic search algorithm used an initial neighbor-joining (NJ) tree (Saitou and Nei, 1987) built with default options. Then, it used a TBR (tree-bissection-reconnection) heuristic algorithm to search for the ML tree under the maximum likelihood framework. This algorithm is known to generate the most accurate results by computer simulations (Takahashi and Nei, 2000). To estimate the robustness of each internal branch of our ML tree, we used the non-parametric bootstrap test (PB) (Efron et al., 1996; Felsenstein, 1985), whose values were inferred through a fast-heuristic search, with 1000 replications. This analysis was also done with PAUP* program (Swofford, 1998).

Bootstrap values tend to be conservative (Efron et al., 1996; Sanderson and Shaffer, 2002; Sitnikova et al., 1995), thus, we decided to also perform a Bayesian analysis (Yang and Ranalla, 1997) using the Mr. Bayes 3.0 software (Huelsenbech and Ronquist, 2001). The Bayesian procedure allows the use of distinct substitution models for different parts of the sequence (Yang and Ranalla, 1997). In this case, a model, hereafter named the mixed model, that combines the one that best fits the 16*S* sequences (Felsenstein, 1981, with a gamma shape parameter = 0.2875) with the model chosen for the *Cox*II sequences (GTR, with a gamma shape parameter = 0.3740) was used. One million generations of Monte Carlo Markov chains with a sampling frequency of 100 in a Bayesian approach were run to generate a majority rule consensus tree. The frequencies of the observed bipartitions (i.e., posterior probabilities) that were higher than 50% were also included in the internal branches of our ML tree (Fig. 2).

2.4. Host affiliation analyses

After building the phylogenetic tree, we used the MultiState (Pagel, 1994, 1999b) program to estimate ancestral states of *Tomoplagia* in terms of tribe and subtribe of main hosts, and in terms of host specialization level as well (considering both main and occasional hosts—see Table 1). To do that, the current states were mapped on exterior branches of the tree for each estimative. The host-record of *Tomoplagia* was divided in three different levels of specialization and, for each level, a different test was performed. For instance, the first test considered species which were restricted to a single host genus as specialists and included all remaining species as generalists. The second analysis defined species confined to a single host subtribe as specialists, whereas the third analysis considered those limited to a single host tribe as specialists.

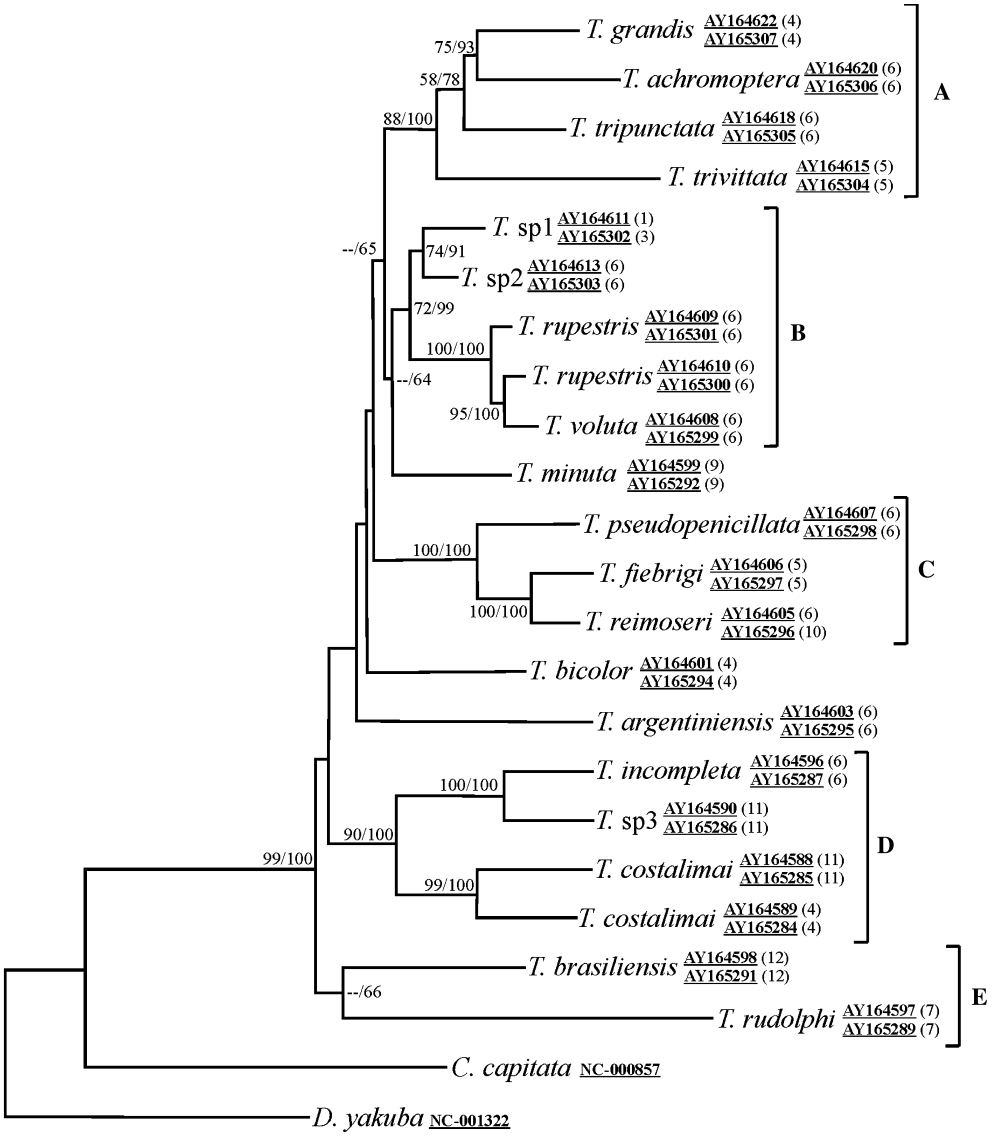

Fig. 2. Maximum likelihood tree constructed using concatenated *Cox*II and 16*S* sequences of 19 *Tomoplagia* species. The bootstrap values (PB—10,000 replications) and the bi-partitions values found in the majority rule consensus tree of the Bayesian analysis (BA—1,000,000 replications) are shown besides each interior branch, respectively. Each *Tomoplagia* specimen is identified by its access number in NCBI (*Cox*II/16*S*) and each sequence is identified by a number that corresponds to a collection site in Fig. 1.

For each analysis, once current states were mapped on the external branches of the tree, putative ancestral states were estimated. In this procedure, the branch lengths are taken into account and ancestral states are calculated by a continuous-time Markov model, in a maximum likelihood framework (Pagel, 1994; Schluter et al., 1997). Maximum likelihood solutions are those which maximize the probability of observing the data from a particular model of the process under investigation. To select the most appropriate model for the ancestral host association, two nested models which describe the evolution of host association changes at each taxonomic level were tested. Concerning the analysis performed at the level of the tribes, for example, the first model assumed that the transition rate from Mutisieae to Vernonieae and vice versa were constrained to be equal, whereas the second model assumed that these two transition rates were free to vary. The same procedure was done regarding subtribe level associations and the levels of specialization. The LR test was used to verify whether the two models considered in each estimative were significantly different. Since they are nested models, the LR statistic is asymptotically distributed as χ^2 (Pagel, 1999b) and may be tested with a single degree of freedom. If the difference were non-significant, the simpler model would lead to a significant improvement in the fit of the model to the data, and it would be chosen.

After evolutionary models were selected, the likelihood of each alternative ancestral state was inferred. The support for one ancestral state over others, at a given

node, was also tested by means of an LR test. In this case, however, since alternative models were not nested, the test would be considered significant if the difference between log-likelihoods was greater than 2 (Pagel, 1999a, 2000).

Besides providing the ancestral states inference, the LR test (with LR statistic asymptotically distributed as χ^2) also allows the test of whether there is a trend towards specialization in *Tomoplagia*. This inference is simply given by the test if the rate towards specialization is different from the rate backwards, which was performed when we compared the model in which these rates were constrained to be equal to the model in which they were free to vary, as previously explained.

3. Results

3.1. Phylogenetic analysis

The ML tree constructed with the concatenated sequences of *Cox*II and 16*S* of 19 species of *Tomoplagia* is shown in Fig. 2. The values alongside ancestral nodes correspond to bootstrap values and the majority rule consensus values of the Bayesian analysis. The phylogenetic tree (Fig. 2) shows that *Tomoplagia* diversity may be divided into five groups of species, named from A to E. Although most of the groups are remarkably consistent, showing high support, their phylogenetic relationships are weakly resolved.

Group A tightly encompassed *T. grandis* Prado et al. (AY164622, AY165307), *T. achromoptera* Prado et al. (AY164620, AY165306), *T. tripunctata* Hendel (AY164618, AY165305), and *T. trivittata* (Lutz and Lima) (AY164615, AY165304) (PB = 88%, BA = 100%). *T. trivittata* is the sister species of the remaining taxa and appears to have switched to parasitizing the genus *Gochnatia* Kunth (tribe Mutisieae), while the remaining species (*T. tripunctata*, *T. achromoptera*, and *T. grandis*) parasitized the Vernoniinae genus *Lessingianthus* (Less.) H. Rob. as their main host.

Connected with Group A, is Group B, with *T. minuta* Hering (AY164599, AY165292) as a sister species. Cluster B splits into two subgroups, the first of which includes two *T. rupestris* Prado et al. (AY164609, AY165301; AY164610, and AY165300) individuals with an individual of *T. voluta* Prado et al. (AY164608, AY165299) (PB = 100%, BA = 100%). The second subgroup contains two individuals which failed to match the diagnosis of any described species (PB = 74%, BA = 91%). These individuals probably belong to a pair of very closely related species that present reticulate wing patterns. They are currently under genetic and morphometric studies and these new species will be described elsewhere (Aluana G. Abreu, Paulo I. Prado, Allen L. Norrbom, and Vera N. Solferini unpublished data). Thus, in the present study, they will be simply referred to as *Tomoplagia* sp1 (AY164611, AY165302) and *T.* sp2 (AY164613, AY165303).

All species in Cluster B are restricted to the subtribe Lychnophorinae, more specifically, to the genera *Eremanthus* Less and *Lychnophora* Mart., whereas *T. minuta* is restricted to the Vernoniinae genus *Vernonanthura* H. Rob. Remarkably, the division of Group B agrees well with the main host associations of these four species, i.e., *T. rupestris* and *T. voluta* parasitize mainly the genus *Lychnophora* whereas *T.* sp1 and *T.* sp2 feed on *Eremanthus* most of the time. Nevertheless, for occasional hosts, the association is not straightforward, suggesting that major host-shifts are less labile than occasional host-shifts, which may be too frequent to be phylogenetically consistent.

Cluster C appears as a sister-group of A and B (plus *T. minuta*). It consists of a well-supported group (PB = 100%, BA = 100%) which includes *T. pseudopenicillata* Aczél (AY164607, AY165298), *T. fiebrigi* Hendel (AY164606, AY165297), and *T. reimoseri* Hendel (AY164605, AY165296). In terms of host affiliations, *T. pseudopenicillata* and *T. fiebrigi* are both restricted to a genus of hosts (*Cyrtocymura* H. Rob. and *Vernonanthura*, respectively), while *T. reimoseri* is a host generalist, which that can parasitize plants of different tribes (see Table 1).

Tomoplagia bicolor Mart. (AY164601, AY165294) and *T. argentiniensis* Aczél (AY164603, AY165295) appear as separated sister groups of A, B, and C. It is important to remark that *T. bicolor* is a host generalist and uses as its main hosts plants of the subtribe Lychnophoriinae, while *T. argentiniensis* is a genus specialist that parasitizes only plants of the genus *Cyrtocymura*.

As a sister group of all mentioned species is cluster D, which contains *T. incompleta* (Williston) (AY164596, AY165287), *T.* sp3 (AY164590, AY165286), and two individuals of *T. costalimai* Aczél (AY164588, AY165285; AY164589, AY165284), (PB = 90%, BA = 100%). The species with the most basal branching in this group was *T. costalimai*, which parasitizes species of *Trixis* P. Browne, from the tribe Mutisieae. The remaining species are clustered in significant values (PB = 100%, BA = 100%) and are all generalists in the tribe Vernonieae.

Finally, Cluster E appears as a sister group of all remained species and encompasses *T. brasiliensis* Prado et al. (AY164598, AY165291) and *T. rudolphi* (Lutz and Lima) (AY164597, AY165289) with a non-significant support (PB < 50%, BA = 66%). Each of these species is restricted to a single but different Vernoniinae genus, such as *Lessingianthus* and *Vernonanthura*, respectively.

3.2. Levels of specialization

The question of whether specialization is an evolutionary trend in herbivorous insect lineages (Kelley and Farrell, 1998; Nosil, 2002) must be approached using a

cross-scale analysis. As stated before, the reconstruction of ancestral characters was performed under the simplest model that fit our data.

Regarding the inference of the ancestral hosts, our phylogenetic tree unmistakably indicated that the putative ancestral of the genus *Tomoplagia* parasitized plants of the tribe Vernonieae (Fig. 3—the ancestral nodes whose state could be inferred through statistical support were highlighted with an asterisk). In this sense, both shifts to plants of the tribe Mutisieae occurred independently during the evolution of this genus. Unfortunately, it was not possible to directly assert ancestral host preferences in *Tomoplagia* at a subtribe level (Fig. 3) but, through our tribe analysis, we have concluded that the ancestral *Tomoplagia* species must feed on hosts from subtribe Vernoniinae or Lychnophorinae (subtribes of Vernonieae).

Table 2 shows the differences between the rates towards and backwards specialization for the three levels considered (specialists in a genus, in a subtribe or in a tribe). Using the model in which the rates from specialization towards generalization and from generalization towards specialization were free to vary, the rates towards specialization were higher than the ones towards generalization in all cases (1.1 times for genus specialists, 2.0 times for subtribe specialists, and 3.7 times for tribe specialists). However, when we compared this model with that in which these rates were constrained to be equal, for the three levels of specialization separately, the LR tests showed that the differences among both models were not significant. This was true for all three specialization levels (Table 2) and it clearly indicates that

Table 2
Results of maximum likelihood analyses estimating the evolutionary transition rate toward specialization considering three levels of specialization (genus-, subtribe-, and tribe-specialists)

Level of specialization	E/G	−2 Log LR	*P*
Genus	1.11	0.00183	0.96
Subtribe	2.04	0.0578	0.81
Tribe	3.71	0.2115	0.64

E/G stands for is the rate toward specialization divided by the rate toward generalization when the rates toward specialization or generalization were free to vary. Since the model where transition rates toward specialization and toward generalization were free to vary and a model where these two rates were forced to be equal are nested, the LR test (−2 Log LR) was used to verify whether the difference between models was significant through by a χ^2 test with a single degree of freedom. A significant result ($P < 0.05$) implies direcional evolution toward specialization.

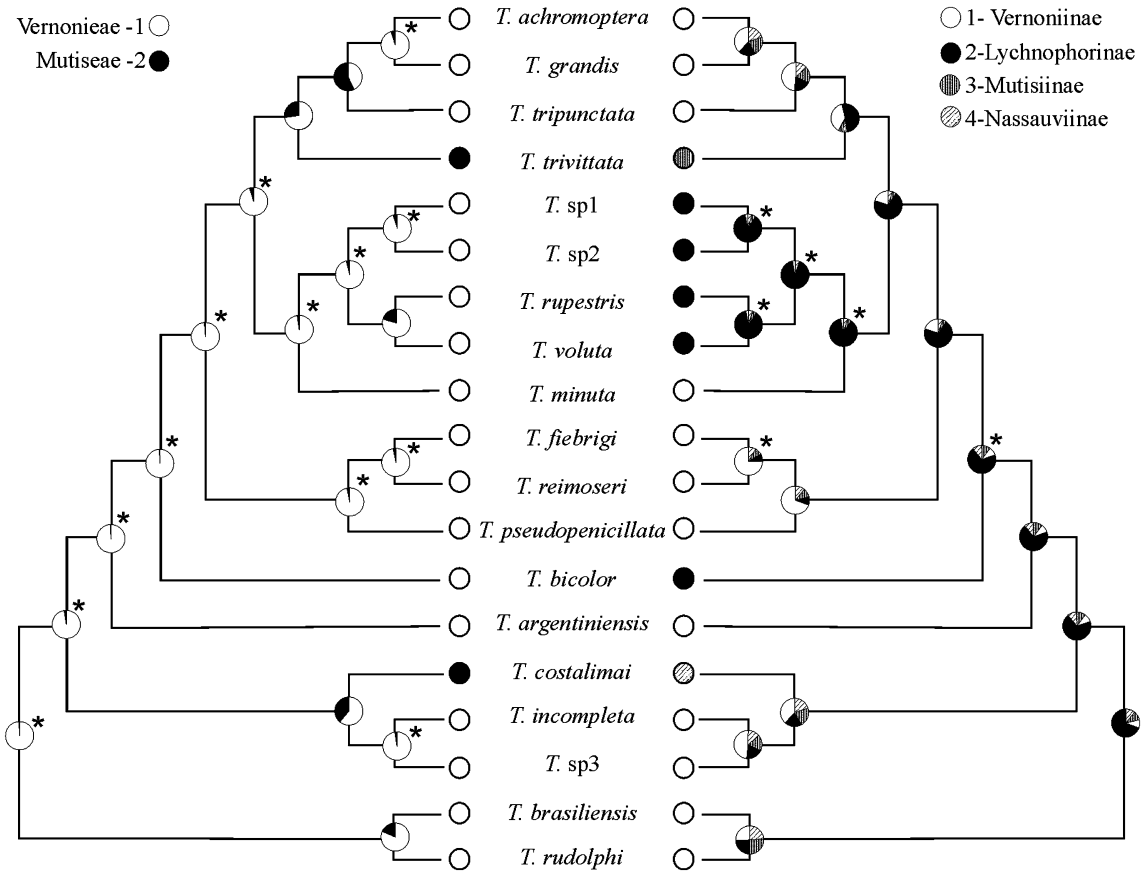

Fig. 3. Maximum likelihood ancestor states regarding main host tribe (left) and subtribe (right) assignments. Pie charts indicate the relative support for different hosts. Whenever one of the alternative ancestors was significantly more probable than the remaining ones in a given node, it was highlighted with the symbol (*).

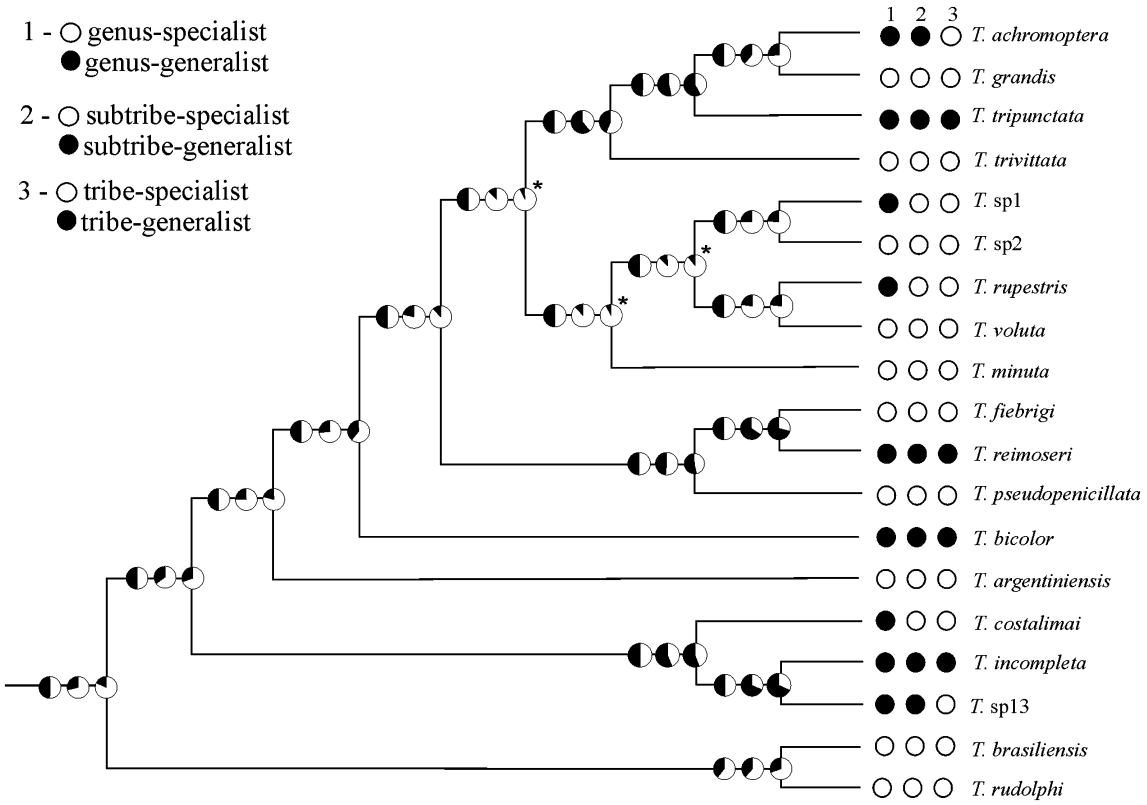

Fig. 4. Maximum likelihood ancestor states regarding levels of specialization. From left to right, each ancestral node is described by three pie graphs which correspond to the probability that a given ancestral is 1, a specialist of a genus or a generalist at this level; 2, a specialist of a subtribe or a generalist at this level or 3, a specialist of a tribe or a generalist at this level. Whenever one of the alternative ancestors was significantly more probable than the other in a given pie, it was highlighted with the symbol (*).

the trend towards specialization indicated above is artifactual. Fig. 4 indicates the inference of the specialization status of the ancestral nodes in the tree and reinforces that there is no phylogenetic signal for the levels of specialization.

4. Discussion

Herbivorous insects are extremely diverse and abundant, and most of them present a high degree of specialization in relationships with their hosts (Bernays and Graham, 1988; Fry, 1996; Jaenike, 1990). In this paper, we have used the *Tomoplagia*–Asteraceae model to test the theory that states that this pattern has arisen from a trend towards specialization (Brues, 1920). As previously mentioned, our tree shows poor resolution in the deeper nodes connecting major groups of *Tomoplagia*. Nevertheless, this aspect affects neither the major conclusions of ancestral host inference nor the analysis regarding the specialization levels.

The analyses performed in this study have provided convincing evidence to support the conclusion that specialization does not correspond to a phylogenetic dead-end in *Tomoplagia*, which differs from the previously suggested theory to explain herbivorous insects associations (Cope, 1896—*apud* Mayr, 1997); Crespi and Sandoval, 2000; Futuyma and Moreno, 1988; Kelley and Farrell, 1998).

The first evidence against the tendency towards specialization is based on the inference of the ancestral hosts, regarding their main tribe and subtribe of hosts in our phylogenetic tree. Following the dead-end hypothesis, it was expected that shifts to a different tribe, such as Mutisieae, should occur only once during the phylogeny of *Tomoplagia* with a subsequent loss of the ability to parasitize Vernonieae. Nevertheless, Fig. 3 shows that this kind of change occurred twice, with a subsequent return to Vernonieae.

Regarding the subtribe level, the shifts were even more frequent, which was demonstrated by the fact that few ancestral nodes could be clearly assigned through statistical support. These results are evidence of the lack of evolutionary correspondence in this host-parasite association, which grants some insights on key aspects of the evolution of this trait. The most conspicuous of these are the unexpectedly low evolutionary constraints for these shifts, as far as the host range considered in our analysis is concerned.

This dearth of constraints can be better understood if we focus on species that parasitize plants of the subtribe Lychnophorinae. This is due to the fact that *Tomoplagia*

spp. presents different levels of association with these plants, ranging from an occasional use to strict specialization (see Table 1). Some species, such as *T. achromoptera*, *T. incompleta*, and *T.* sp1, use flower heads of Lychnophorinae as occasional hosts (Table 1), while *T. bicolor*, *T. rupestris*, *T. voluta*, *T.* sp2, and *T.* sp3 parasitize them as their main hosts.

It is also important to notice that Lychnophorinae plants, except for those of the genus *Eremanthus* (MacLeish, 1987), are restricted to high altitude habitats, where they are exceptionally speciose and abundant (Coile and Jones, 1981). As previously mentioned, the subtribe assignment of most ancestral nodes of our tree was unclear. Nevertheless, in five of these nodes, the most probable hosts belong to the subtribe Lychnophorinae (Fig. 3). Supposing that members of this subtribe were the ancestral host of *Tomoplagia* spp., it would follow that host-shifts to other subtribes may have taken place in environments in which these original hosts were unavailable. Conversely, if the ancestral hosts were plants of the subtribe Vernoniinae, some species of *Tomoplagia* may have switched host preferences to Lychnophorinae once they reached the high altitude habitats.

An important point, therefore, is that the host-shifts may be dictated by local plant availability. In fact, similar patterns were observed in two genera of beetles (Coleoptera: Crysomelidae), *Oreina* and *Timarcha*. In *Oreina*, no host fidelity was observed below plant family levels, and in high altitudes, in which many *Oreina* species live, host plant availability might actually be the decisive limiting factor (Dobler et al., 1996). For the genus *Timarcha*, this pattern is even more prominent, since some species of this genus use hosts belonging to different plant families, depending on their availability at high altitudes (Gómez-Zurita et al., 2000).

The second piece of evidence that there are no trends towards specialization arose from a directional evolution test in three different levels of specialization. Our results have shown that, although rates towards specialization were higher than rates towards generalization for the three levels considered, the differences between these rates were not statistically supported (see Table 2). Nosil (2002) performed this same analysis using 15 groups of phytophagous insects that presented within-group variation in degrees of specialization. In that analysis, he detected a general, yet weak, tendency for phytophagous insects to exhibit directional evolution towards an increased specialization. Nonetheless, he also found that, in 11 out of the 15 tested groups, the directional evolution could not be statistically confirmed. Based on these results, he concluded that this lack of evidence of directional evolution towards increased specialization is bound to support a dynamic view of evolution of the specialization trait in phytophagous insects.

Even though 10 out of the 19 species of *Tomoplagia* included in this work are restricted to a single host genus, our results have clearly indicated that this specialization does not limit further host range evolution. In this sense, how can the predominance of specialists over generalists in *Tomoplagia* be explained? This question can be partially answered by the speculation that *Tomoplagia* associations with their hosts are strongly influenced by ecological and geographical features such as the availability of hosts at a given place, as previously mentioned. In addition, some authors have speculated about other factors which may promote specialization, such as resource competition (MacArthur et al., 1964), resistance to predators (Bernays, 1989; Bernays and Graham, 1988; Dyer, 1995), systems of female search behavior (Janz and Nylin, 1997), local coevolution or genetic differentiation (Thompson, 1994).

Remarkably, the species considered as the most generalists in this genus (those able to feed on more than a tribe of plants) also present a wide geographical distribution, which means that they may be "forced" to find different hosts in different geographical areas. Indeed, our host-record of *Tomoplagia* species (Lewinsohn, 1991; Prado et al., 2002) shows that some *Tomoplagia* larvae are capable of developing on genera, subtribes (e.g., *T. achromoptera* and *T.* sp1) and even tribes (e.g., *T. tripunctata*, *T. reimoseri*, *T. incompleta*, and *T. bicolor*), differently from those assigned as their main hosts, which emphasizes the importance of including occasional hosts in such studies. This clearly shows that although *Tomoplagia* has been considered as a specialist genus, its specialization, at least for some species, does not imply a loss of the genetic ability to feed on alternative hosts. Similar conclusions have been reached for some phytophagous insects in experiments involving the transfer of insect larvae from their main host to alternative hosts, which showed that larvae frequently survived on alternative related hosts (e.g., Janz et al., 2001; Roininen and Tahvanainen, 1989; Smiley, 1978; but see Keese, 1998; Futuyma et al., 1993, 1994).

Certainly, the results obtained in this work have made some aspects of the evolution of *Tomoplagia* species clearer, such as the unexpected low constraints for host shifts and the putative relationships of these shifts with the geographic host availability. These results suggest that the approaches proposed here, concerning the use of different levels of specialization and the inclusion of occasional hosts are bound to permit the definition of a more detailed scenario to explain the evolution of one of the most intriguing patterns observed in insect–plant relationships: their extreme level of specialization. In fact, our results have shown that, if one considers the dead-end as a decrease of host options by an evolutionary trend, the high level of specialization found in *Tomoplagia* has been molded by environmental factors instead of evolutionary constraints; i.e., the specialization in this

genus, and probably in most of herbivorous insects, is an "option" rather than a "lack of options."

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References

Aczél, M., 1955. Fruit flies of the genus *Tomoplagia* Coquillet (Diptera: Tephritidae). U. S. Natl. Mus. Proc. 104, 321–411.

Amadon, D., 1943. Specialization and evolution. Am. Nat. 77, 133–141.

Azeredo-Espin, A.M.L., Schroder, R.F.W., Huettel, M.D., Sheppard, W.S., 1991. Mitochondrial–DNA variation in geographic populations of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera, Chrisomelidae). Experientia 47, 483–485.

Benson, W.W., Brown, K.S., Gilbert, L.A., 1975. Coevolution of plants and herbivores: passion flower butterflies. Evolution 29, 659–680.

Bernays, E.A., 1998. Evolution of feeding behaviour in insect herbivores: success seen as different ways to eat without being eaten. BioScience 48, 35–44.

Bernays, E.A., 1989. Host range in phytophagous insects: the potential role of generalist predators. Evol. Ecol. 3, 299–311.

Bernays, E.A., Graham, M., 1988. On the evolution of host specificity in phytophagous arthropods. Ecology 69, 886–892.

Bremer, K., 1994. Asteraceae: Cladistics and Classification. Timber Press, Portland, Oregon.

Brues, C.T., 1920. The selection of food-plants by insects, with special preference to lepidopterous larvae. Am. Nat. 54, 313–332.

Cavalli-Sforza, L.L., Edwards, A.W.F., 1967. Phylogenetic analysis: models and estimation procedures. Am. J. Hum. Genet. 19, 122–257.

Clary, D.O., Goddard, J.M., Martin, S.C., Fauron, C.M., Wolstenholme, D.R., 1992. *Drosophila* mitochondrial DNA: a novel gene order. Nucleic Acids Res. 10, 6619–6637.

Clary, D.O., Wolstenholme, D.R., 1985. The mitochondrial–DNA molecule of *Drosophila yakuba* nucleotide sequence, gene organization, and genetic-code. J. Mol. Evol. 22, 252–271.

Coile, N.C., Jones Jr., S.B., 1981. *Lychnophora* (Composite: Vernonieae), a genus endemic to the brazilian planalto. Brittonia 33 (4), 528–542.

Crespi, B.J., Sandoval, C.P., 2000. Phylogenetic evidence for the evolution of ecological specialization in *Timena* walking-sticks. J. Evol. Biol. 13, 249–262.

Dobler, S., Mardulyn, P., Pasteels, J.M., Rowell-Rahier, M., 1996. Host-plant switches and the evolution of chemical defense and life history in the leaf beetle genus *Oreina*. Evolution 50, 2373–2386.

Dyer, L.A., 1995. Tasty generalists and nasty specialists? Antipredator mechanisms in tropical Lepidopteran Larvae. Ecology 76, 1483–1496.

Efron, B., Harlloran, E., Holmes, S., 1996. Bootstrap confidence levels for phylogenetic trees. Proc. Natl. Acad. Sci. USA 93, 13429–13434.

Ehrlich, P.R., Raven, P.H., 1964. Butterflies and plants: a study in coevolution. Evolution 18, 586–608.

Farrell, B., Mitter, C., 1993. Phylogenetic determinants of insect/plant community diversity. In: Ricklefs, R.E., Schluter, D. (Eds.), Species Diversity in Ecological Communities, Historical and Geographical Perspectives. University of Chicago Press, Chicago, pp. 253–266.

Farrell, B., Mitter, C., 1998. The timing of insect/plant diversification: might Tetraopes (Coleoptera: Cerambycidae) and Asclepias (Asclepiadaceae) have co-evolved?. Biol. J. Linnean Soc. 63, 553–577.

Feder, J.L., Berlocher, S.H., Roethele, J.B., Dambroski, H., Smith, J.J., Perry, W.L., Gavrilovic, V., Filchac, K.E., Rull, J., Aluja, M., 2003. Allopatric genetic origins for sympatric host-plant shifts and race formation in *Rhagoletis*. Proc. Natl. Acad. Sci. USA 100, 10314–10319.

Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376.

Felsenstein, J., 1985. Confidence limits on phylogenies and approach using the bootstrap. Evolution 39, 783–791.

Fry, J.D., 1996. The evolution of host specialization: are trade-offs overrated?. Am. Nat. 148, S85–S107.

Funk, D.J., Futuyma, D.J., Ortí, G., Meyer, A., 1995a. Mitochondrial DNA sequences and multiple data sets: a phylogenetic study of phytophagous beetles: (Chrysomelidae: *Ophraella*). Mol. Biol. Evol. 12, 627–640.

Funk, D.J., Futuyma, D.J., Ortí, G., Meyer, A., 1995b. A history of host association and evolutionary diversification for *Ophraela* (Coleoptera: Chrysomelidae): new evidence from mitochondrial DNA. Evolution 49, 1008–1017.

Funk, D.J., Filchak, K.E., Feder, J.L., 2002. Herbivorous insects: model systems for the comparative study of speciation ecology. Genetica 116, 251–267.

Futuyma, D.J., Keese, M.C., Scheffer, S.J., 1993. Genetic constraints and the phylogeny of insect–plant associations: responses of *Ophraella communa* (Coleoptera: Chrysomelidae) to host plants of its congeners. Evolution 47, 888–905.

Futuyma, D.J., Moreno, G., 1988. The evolution of ecological specialization. Annu. Rev. Ecol. Syst. 19, 207–233.

Futuyma, D.J., Walsh, J.S., Morton, T., Funk, D.J., Keese, M.C., 1994. Genetic variation in a phylogenetic context: response of two specialized leaf beetles (Coleoptera: Chrysomelidae) to host plants of their congeners. J. Evol. Biol. 7, 127–146.

Gómez-Zurita, J., Juan, C., Petitpierre, E., 2000. The evolutionary history of the genus *Timarcha* (Coleoptera: Chrysomelidae) inferred from mitochondrial COII gene and partial 16*S* rRNA sequences. Mol. Phylogenet. Evol. 14, 304–317.

Gordon, D., Abajian, C., Green, P., 1998. Consed: a graphical tool for sequence finishing. Genome Res. 8, 195–202.

Headrick, D.H., Goeden, R.D., 1998. The biology of the nonfrugivorous tephritid fruit flies. Annu. Rev. Entomol. 43, 217–241.

Huelsenbeck, J., Crandall, K.A., 1997. Phylogeny estimations and hypothesis testing using maximum likelihood. Annu. Rev. Ecol. Syst. 28, 437–466.

Huelsenbech, J.P., Ronquist, F., 2001. Mr. Bayes: Bayesian inference of phylogenetic tree. Bioinformatics Applications Note 17, 754–755.

Jaenike, J., 1990. Host specialization in phytophagous insects. Annu. Rev. Ecol. Syst. 21, 243–273.

Janz, N., Nyblom, K., Nylin, S., 2001. Evolutionary dynamics of host-plant specialization: a case study of the Tribe Nymphalini. Evolution 55, 783–796.

Janz, N., Nylin, S., 1997. The role of female search behavior in determining host plant range in plant feeding insects: a test of the information processing hypothesis. Proc. R. Soc. Lond. 264, 701–707.

Janz, N., Nylin, S., 1998. Butterflies and plants: a phylogenetic study. Evolution 52, 486–502.

Jermy, T., 1984. Evolution of insect/host plant relationships. Am. Nat. 124, 609–630.

Jermy, T., 1993. Evolution of insect–plant relationships—a devil's advocate approach. Entomol. Exp. Appl. 66, 3–12.

Keese, M.C., 1998. Performance of two monophagous leaf feeding beetles (Coleoptera: Chrysomelidae) on each other's host plant: do intrinsic factors determine host plant specialization?. J. Evol. Biol. 11, 403–419.

Kelley, S.T., Farrell, B., 1998. Is specialization a dead-end? The phylogeny of host use in *Dendroctonus* bark beetles (Scolytidae). Evolution 52, 1731–1743.

Korneyev, V., 1999. Phylogeny of the subfamily tephritinae: relationships of the tribes and subtribes. In: Martin, A., Norrbom, A.L. (Eds.), Fruit Flies (Tephritidae) Phylogeny and Evolution of Behavior. CRC press, Boca Raton, Florida, pp. 549–580.

Lewinsohn, T.M., 1987. Plantas Hospedeiras, Heterogeneidade Ambiental e Diversidade de Insetos Fitofagos. Publicações Aciesp 32.

Lewinsohn, T.M., 1991. Insects in flower heads of Asteraceae in southeast Brazil: a tropical case study on species richness. In: Price, P.W., Lewinsohn, T.M., Fernandes, G.W., Benson, W.W. (Eds.), Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. Wiley/Interscience, New York, pp. 525–560.

Lopez-Vaamonde, C., Godfray, C.J., Cook, J.M., 2003. Evolutionary dynamics of host-plant use in a genus of leaf-mining moths. Evolution 57, 1804–1821.

MacArthur, R.H., Lewins, R., 1964. Competition, habitat selection, and character displacement in a patchy environment. Proc. Natl. Acad. Sci. USA 51, 1207–1210.

MacLeish, N.F.F., 1987. Revision of *Eremanthus* (Compositae, Vernonieae). Ann. Mo. Bot. Gard. 74, 265–290.

Mayr, E., 1997. The emergence of evolutionary novelties. In: Evolution and the Diversity of Life. Harvard University Press, Cambridge, Massachusetts, pp. 88–113.

Nosil, P., 2002. Transition rates between specialization and generalization in phytophagous insects. Evolution 56, 1701–1706.

Pagel, M., 1994. Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. P. Roy Soc. Lond. B Bio. 255, 37–45.

Pagel, M., 1999a. Inferring the historical patterns of biological evolution. Nature 401, 877–884.

Pagel, M., 1999b. The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. Syst. Biol. 48, 612–622.

Pagel, M., 2000. Statistical analysis of comparative data. TREE 15, 418.

Percy, D.M., Page, R.D.M., Cronk, Q.C.B., 2004. Plant–insect interactions: double-dating associated insect and plant lineages reveals asynchronous radiations. Syst. Biol. 53, 120–127.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model DNA substitution. Bioinformatics 14, 817–818.

Prado, P.I., Lewinsohn, T.M., 1994. Genus *Tomoplagia* (Diptera: Tephritidae) in the Serra do Cipó, MG, Brazil: host ranges and notes of taxonomic interest. Revta. Bras. Entomol. 38, 669–680.

Prado, P.I., Lewinsohn, T.M., Almeida, A.M., Norrbom, A.L., Buys, B.D., Macedo, A.C.C., Lopes, M.B., 2002. The fauna of Tephritidae (Diptera) from capitula of Asteraceae in Brazil. Proc. Wash. Entomol. Soc. 104, 1006–1027.

Prado, P.I., Norrbom, A.L., Lewinsohn, T.M., 2004. New species of *Tomoplagia* Conquillett (Diptera: Tephritidae) from Capitula of Asteraceae in Brazil. 2004. Neotrop. Entomol. 33 (2), 189–211.

Rambaut, A., 2002. Sequence Alignment Editor, v. 2.0, Copyright Univ. Oxford. Available from: <http://evolve.zoo.ox.ac.uk/software/Se-Al/main.html>.

Rausher, M.D., 2001. Co-evolution and plant resistance to natural enemies. Nature 411, 857–864.

Rodriguez, F., Oliver, J.L., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitutions. J. Theo. Biol. 142, 485–501.

Roininen, H., Tahvanainen, J., 1989. Host selection and larval performance of two willow-feeding sawflies. Ecology 70, 129–136.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sanderson, M.J., Shaffer, H.B., 2002. Troubleshooting molecular phylogenetic analyses. Annu. Rev. Ecol. Syst. 33, 49–72.

Schluter, D., Price, T., Mooers, A., Ludwig, D., 1997. Likelihood of ancestor states in adaptive radiation. Evolution 51, 1699–1711.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.

Smiley, J., 1978. Plant chemistry and the evolution of host specificity: new evidences from *Heliconius* and *Passiflora*. Science 201, 745–747.

Sitnikova, T., Rhzetsky, A., Nei, M., 1995. Interior-branch and the bootstrap tests of phylogenetic trees. Mol. Biol. Evol. 12, 319–333.

Spanos, L., Koutroumbas, G., Kotsyfakis, M., Louis, C., 2000. The mitochondrial genome of the Mediterranean fruit fly, *Ceratitis capitata*. Insect Mol. Biol. 9, 139–144.

Swofford, D.L., 1998. PAUP*—A computer program for phylogenetic inference using Maximum Parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Takahashi, K., Nei, M., 2000. Efficiencies of fast algorithms of phylogenetic inference under the criteria of Maximum Parsimony, Minimum Evolution and Maximum Likelihood when a large number of sequences are used. Mol. Biol. Evol. 17, 1251–1258.

Termonia, A., Hsiao, T.H., Pasteels, J.M., Milinkovitch, M.C., 2001. Feeding specialization and host-derived chemical defense in Chrysomeline leaf beetles did not lead to an evolutionary dead end. Proc. Natl. Acad. Sci. USA 98, 3909–3914.

Thompson, J.N., 1994. The Coevolutionary Process. University of Chicago Press, Chicago.

Wahlberg, N., 2001. The phylogenetics and biochemistry of host-plant specialization in Melitaeine butterflies (Lepidoptera: Nymphalidae). Evolution 55, 522–537.

Wasserman, S.S., Futuyma, D.J., 1981. Evolution of host plant utilization in laboratory populations of the southern cowpea weevil, *Callosobruchus maculates* Fabricius (Coleoptera: Bruchidae). Evolution 35, 605–617.

Yang, Z., Ranalla, B., 1997. Bayesian phylogenetic inference using DNA sequences: a Markovchain Monte Carlo method. Mol. Biol. Evol. 14, 717–724.