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Evolution of host utilization patterns in the seed beetle genus *Mimosestes* Bridwell (Coleoptera: Chrysomelidae: Bruchinae)

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ABSTRACT

The evolutionary history of diet breadth expansion and intergeneric host shifts in the seed beetle genus *Mimosestes* were reconstructed to investigate the process of host range expansion in phytophagous insects. The evolutionary correlation between diet breadth and variation in oviposition behavior of *Mimosestes* was also examined to estimate the process of generalist evolution within the genus. Ancestral state reconstruction based on a molecular phylogeny inferred from three mitochondrial markers (16S rRNA, 12S rRNA, and COI) and one nuclear marker (EF-1α) revealed that host utilization patterns were shaped by repeated colonizations to novel or pre-adapted host plants. Neither plant genus and species group level host conservatism nor an evolutionary tendency toward specialization was found in the genus, contrary to the expectations of plant–insect co-evolutionary theory. In addition, statistical analyses revealed that diet breadth was significantly correlated with oviposition behavior, suggesting that behavioral factors such as the oviposition preferences of female seed beetles affect the expansion of diet breadth in generalists.

1. Introduction

The extreme species diversity of phytophagous insects has given rise to one of the most attractive research fields in evolutionary biology (Farrell, 1998; Lopez-Vaamonde et al., 2003; Novotny et al., 2006). Ehrlich and Raven (1964) argued that reciprocal evolution between plant secondary metabolic compounds and the physiological adaptations of insects to those compounds is a main cause of the present high diversity of phytophagous insects. This is one of the adaptive radiation processes by which an insect lineage colonizes novel plants that are phylogenetically unrelated to the ancestral hosts (herein referred to as major host shift). The insect then diversifies, resulting in ecological specialization within these "novel adaptive zones" (Ehrlich and Raven, 1964; Schluter, 2000; Morse and Farrell, 2005). Thus, understanding the process of major host shifts is important for explaining the first step in diversification among phytophagous insects. However, the host expansion process in phytophagous insects remains controversial (Winkler and Mitter, 2008).

Most phytophagous insects use only one or a few related plants as hosts (i.e., specialist species); however, some utilize distantly related plants (i.e., generalist species). One conventional idea of ecological specialization suggests that specialist phytophagous insects are limited in their ability to utilize novel host plants by trade-offs and are thus more prone to extinction than are generalists (Joshi and Thompson, 1995; Kelley and Farrell, 1998; Schluter, 2000; Funk et al., 2002; Stireman, 2005). Therefore, many researchers have suggested that the evolution of diet breadth among phytophagous insects tends to proceed from generalists to specialists (Futuyma and Moreno, 1988; Kelley and Farrell, 1998). However, recent studies based on molecular phylogeny have demonstrated that no obvious general trends toward specialization exist for many phytophagous insects (Janz and Nylin, 1998; Janz et al., 2001; Nosil, 2002; Nosil and Mooers, 2005). Furthermore, such studies have shown the patterns that generalists have arisen from specialized ancestors (Morse and Farrell, 2005; Yotoko et al., 2005; Weingartner et al., 2006; Cho et al., 2008). These data imply that even though the identification of factors causing generalist evolution in phytophagous insects is still controversial (Morse and Farrell, 2005; Yotoko et al., 2005), diet breadth cannot be completely explained by ecological specialization processes such as chemical co-evolution between insects and plants (Ehrlich and Raven, 1964; Futuyma and Moreno, 1988).

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If generalists have evolved from specialists via the addition of novel plants to their host repertoire, one can hypothesize that the major host shift may be related to the evolution of generalist phytophagous insects. Indeed, several recent studies have shown that major host shifts and generalization have occurred simultaneously. For example, Winkler and Mitter (2008) reported that oligophagous insects in clades including at least one polyphagous species more frequently change their host plant family than do species in clades consisting of only oligophagous species. Additionally, Janz et al. (2001), Janz and Nylin (2008), and Weingartner et al. (2006) found that most changes in host utilization appeared to occur during the "expanded phase" of diet breadths in the *Polygonia* butterfly and argued that most major host shifts in the genus have occurred alongside generalization. However, few studies have focused on the relationship between major host shifts and generalization.

Here, we examined generalist evolution and major host shifts in the seed beetle genus *Mimosestes* (Coleoptera: Chrysomelidae: Bruchinae: Acanthoscelidini), whose component species exhibit considerable interspecific variation in diet breadth and host utilization.

1.1. Research target: The genus Mimosestes Bridwell, 1946

The genus *Mimosestes* consists of 17 known species from the United States to the Amazonian basin (Kingsolver and Johnson, 1978; Hopkins, 1983, 1984; Johnson, 1983b; Kingsolver, 1985). All species feed on the seeds of the two subfamilies of Leguminosae: the Mimosoideae (*Acacia*, *Enterolobium*, *Parkia*, and *Prosopis*) and the Caesalpinioideae (*Bauhinia*, *Caesalpinia*, and *Parkinsonia*; Kingsolver and Johnson, 1978; Hopkins, 1983, 1984; Johnson, 1983b; Kingsolver, 1985; Romero Nápoles et al., 2009).

Ten species of *Mimosestes* utilize only the *Acacia* subgenus *Acacia*, and four species are associated exclusively with non-*Acacia* host plants. Three species of *Mimosestes* utilize both *Acacia* and non-*Acacia* plants: *M. insularis* utilizes *Acacia* and *Prosopis*, and *M. amicus* and *M. mimosae* feed on both Mimosoideae and Caesalpinioideae (Table 1).

Host plant utilization patterns of *Mimosestes* exhibit two remarkable features. First, most host plants of the three multi-genera-utilizing species are also used by the specialists (Table 1). Second, differences in host utilization exist among multi-genera-utilizing species. For instance, *M. mimosae* does not use plants in common with hosts of *M. amicus*, except for *Parkinsonia aculeata*, even though these two species have the broadest diet breadths within the genus. These host utilization patterns suggest that multi-genera-utilizing species may represent an intermediate step between *Acacia* specialists and non-*Acacia* specialists and that multiple host expansion events have occurred in the genus. Thus, *Mimosestes* is an appropriate model genus for studying major host shifts and generalization.

As a first step in the comprehensive study of diet breadth evolution within *Mimosestes*, we also focused on the effects of variation in oviposition behavior on diet breadth within the genus. We hypothesized that diet breadth in *Mimosestes* is affected by differences in oviposition behavior. All known *Mimosestes* species, except *M. chrysocosmus*, oviposit on the surface of the seed pods of their host plants (Kingsolver, 1985; Johnson, 1987). Previous studies (Johnson, 1983b, 1987; Traveset, 1990, 1991; Siemens et al., 1992) and our field observations (T. Kato and M. Shimada,

Table 1
Species groups and previously published host plant utilizations of *Mimosestes* spp.

Mimosestes species group / *Mimosestes* species	Host plant group: Species group in *Acacia* subgen. *Acacia*	Other Mimosoideae	Caesalpinioideae
Enterolobii group			
Mimosestes enterolobii Kingsolver and Johnson, 1978		*Enterolobium* (1 sp.)	
Chrysocosmus group			
Mimosestes chrysocosmus Kingsolver, 1985		*Parkia* (5 spp.)	
Humeralis group			
Mimosestes humeralis (Gyllenhal, 1873)	Farnesiana (1sp.)*, Macracantha (2 spp.)		
Mimosestes janzeni Kingsolver and Johnson, 1978	Macracantha (1 sp.), Rigidula (1 sp.)*		
Mimosae group			
Mimosestes acaciestes Kingsolver and Johnson, 1978	Constricta (2 spp.), Rigidula (2 spp.)		
Mimosestes amicus (Horn, 1873)	Constricta (1 sp.), Farnesiana(2 spp.)*, Macracantha (2 spp.)*	*Prosopis* (4 spp.)	*Parkinsonia* (5 spp.)
Mimosestes anomalus Kingsolver and Johnson, 1978	Ant-acacia (2 spp.)*, Macracantha (2 spp.)		
Mimosestes cinerifer (Fähraeus, 1839)	Ant-acacia (1 sp.), Macracantha (1 sp.)*		
Mimosestes insularis Kingsolver and Johnson, 1978	Farnesiana (2 spp.), Macracantha (1 sp.)	*Prosopis* (2 spp.)	
Mimosestes mimosae (Fabricius, 1781)	Ant-acacia (4 spp.), Farnesiana (2 spp.), Macracantha (3 spp.), Rigidula (1 sp.)*	*Prosopis* (1 sp.)	*Bauhinia* (1sp.)*, *Caesalpinia* (2 spp.), *Parkinsonia* (3 spp.)
Mimosestes nubigens (Motschulsky, 1874)	Ant-acacia (3 spp.)* Farnesiana (3 spp.), Macracantha (1 sp)*	*Prosopis* (2 spp.)*	
Mimosestes viduatus (Sharp, 1885)	Ant-acacia (6 spp.), Macracantha (1 sp.)*		
Obscuriceps group			
Mimosestes brevicornis (Sharp, 1885)	Farnesiana (2 spp.), Ant-acacia (1 sp.)		
Mimosestes obscuriceps (Sharp, 1885)	Ant-acacia (2 spp.)		
Protractus group			
Mimosestes protractus (Horn, 1873)		*Prosopis* (2 spp.)	
Ulkei group			
Mimosestes playazul Johnson, 1983	Ant-acacia (1 sp.)		
Mimosestes ulkei (Horn, 1873)			*Parkinsonia* (2 spp.)

All data are collected from published literature (Kingsolver and Johnson, 1978; Johnson, 1979, 1983a, 1998; Kingsolver, 1985; Johnson and Siemens, 1996; Romero Nápoles et al., 2009).
Host plants listed by Zacher (1952) were also removed, following Kingsolver and Johnson's (1978) argument.
*Only one emergence record in the literatures.

unpublished data) point to considerable variation in the preferred seed pod status for oviposition in *Mimosestes*, with two possible scenarios: (A) females only oviposit on immature seed pods on the tree and (B) females oviposit on both immature and mature dry seed pods. Morse and Farrell (2005) reported that the evolution of generalists in the seed beetle genus *Stator* was significantly concentrated within clades in which females lay their eggs on the seed surface in ripe, opened seed pods. They argued that changes in oviposition behavior played an important role in the expansion of diet breadth within *Stator*. However, whether this correlation is a general evolutionary trend among seed beetles remains unknown.

To help determine the evolutionary history of generalists and host utilization in *Mimosestes*, we address the following questions concerning the phylogenetic patterns of host utilization within the genus: (1) Do host utilization patterns of *Mimosestes* follow those predicted by phytochemical co-evolutionary theory (i.e., phylogenetically conserved host utilization and an evolutionary trend from generalist to specialist)? (2) Is the occurrence of major host shifts related to generalization? (3) Is diet breadth statistically correlated with oviposition behavior, even when phylogeny is taken into account?

2. Materials and methods

2.1. Taxon sampling

During field sampling from Arizona to southern Mexico, we collected 25 samples, including 12 known species and one unidentified species (*Mimosestes* sp.1) of *Mimosestes* and three outgroup species (see Appendix A). We were unable to collect one species of *Mimosestes* endemic to Panama (*M. enterolobii*) and three very rare species (*M. chrysocosmus*, *M. playazul*, and *M. brevicornis*; *M. chrysocosmus* and *M. playazul* are only known as type series). In addition, *M. protractus* was not collected despite substantial sampling effort within its distribution range. This species is not very rare but is considered uncommon (Johnson, 1983c). Seed pods of host plants were collected from both trees and the ground beneath trees. All seed pods were placed in plastic jars and kept in the laboratory (27 °C, relative humidity = 40%) until adult emergence. A single adult of each species was preserved in 99.5% ethanol or 99.5% acetone for DNA extraction, and all other individuals were dried and pinned. T. Kato and J. Romero-Nápoles identified species according to the taxonomic key of Kingsolver and Johnson (1978). Voucher specimens were deposited in the Insect Museum, Natural Resources Inventory Center, National Institute for Agro-Environmental Sciences, Japan.

T. Kato identified the host plants, and voucher specimens of host plants were deposited in the Herbarium of the Institute de Ecologia, A. C. (XAL), Mexico, and the Herbarium of the University of Tokyo (TI), Japan.

For five *Mimosestes* species collected from multiple localities and/or host plant species (see Appendix A), we included multiple individuals from these localities and host plants in the analyses to account for geographic and inter-host variation within the species. Despite these sampling efforts, the geographic ranges of three widespread species (*M. insularis*, *M. mimosae*, and *M. nubigens*) were not completely covered.

Three Acanthoscelidini seed beetles, *Acanthoscelides oblongoguttatus* (Fähreus, 1839), *Merobruchus insolitus* (Sharp, 1885), and *Stator limbatus* (Horn, 1873), were chosen as outgroups based on both morphological and phylogenetic data. Johnson (1983a) pointed out the morphological similarity between *Mimosestes* and *A. oblongoguttatus*. However, our preliminary molecular phylogenetic analysis of the tribe Acanthoscelidini (T. Kato, unpublished data) suggested that *Mimosestes* was more closely related to the genera *Merobruchus* and *Stator* than to *A. oblongoguttatus*.

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). The thorax or hind leg of the specimen was separated from the entire body and incubated in 180 μl PBS buffer with 20 μl proteinase K at 55 °C for 72 h. After incubation, total genomic DNA was extracted following the manufacturer's instructions.

A fragment of approximately 1650 bp consisting of the mitochondrial 16S ribosomal RNA, tRNA-VAL, and 12S ribosomal RNA (16–12S); a 1563-bp fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene corresponded to positions 1–1563 of *Crioceris duodecimpunctata* (GenBank Accession Number AF467886); and a 544-bp fragment of the nuclear elongation factor 1 alpha subunit (EF-1α) gene corresponded to positions 213–756 of *Polypedilum vanderplanki* (GenBank Accession Number AB490338) were amplified using polymerase chain reaction (PCR). Two mitochondrial gene fragments (16–12S and COI) were amplified following the methods of Kato et al. (2006) using primers MtrA1adv 5′-AAACTAGGATTAGATACCCT-3′ (modified from Fukatsu et al., 2001) and MrrK1 5′-CATAATAAGATTCTAAATC-3′ (Kato et al., 2006) for16S-12S and COI-F2 5′-AACCTT CAAAGTTAAAAATAG-3′ and COI-R1 5′-TCCATTGCACTATTCTGCC-3′ (Yoshitake et al., 2008) for COI. The EF-1α fragment was amplified following the methods of Morse and Farrell (2005) using primers S149 5′-GARAARGARGCNCARGARATGGG-3′ and A692 5′-GGTGGGAGGATGGCATCAAGAG-3′ (Normark et al., 1999; Morse and Farrell, 2005). The sequencing primers for two mitochondrial genes are listed in Appendix B. PCR products were purified using Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA, USA) and served as template DNA for cycle sequencing reactions with CEQ Quick Start Mix (Beckman Coulter, Fullerton, CA, USA) following the manufacturer's instructions. The cycle sequencing products were purified using ethanol precipitation and were electrophoresed using the CEQ8000 Genetic Analysis System (Beckman Coulter). DNA sequences obtained in both directions were assembled and edited using ATGC 4.0 (Genetyx, Tokyo, Japan). When heterozygosities were detected in the EF-1α fragment, they were cloned using the pGEM-T Vector System (Promega, Madison, WI, USA) following the manufacturer's instructions.

All DNA sequences determined herein were deposited in GenBank under Accession Numbers AB499920–AB499969 and AB539669–AB539698 (see Appendix A).

2.3. Phylogenetic analyses

DNA sequences were aligned using ClustalX 1.83 (Thompson et al., 1997), and final alignments were adjusted manually using Bioedit 5.0.9 (Hall, 2007). Because all heterozygous sites of the EF-1α sequence were not shared among samples, and because the phylogenetic positions of heterozygous alleles from an individual were usually sister to each other in a preliminary analysis (data not shown), we randomly chose one of the two EF-1α sequences for each sample with a heterozygous genotype and treated it as representative of that sample. Incongruence length difference (ILD) tests (Farris et al., 1994) based on 1000 replicates of heuristic search algorithms (Swofford et al., 1996) were performed to examine conflicts among the loci. We performed ILD tests for all pairs of loci (pairwise ILD test) and discarded tRNA-VAL, which showed significant incongruence with other loci in the data set. Phylogenetic analyses were performed by applying both maximum parsimony (MP) and Bayesian inference. The MP analysis was conducted using PAUP* 4.0b10 (Swofford, 2002) by heuristic search with 1000 random stepwise addition replicates, tree bisection–reconnection branch swapping, and saving multiple trees (MulTrees). All gaps in the data set were treated as missing data, and ambiguous aligned sites were excluded from the analysis.

Support for the tree topology was evaluated using a bootstrap analysis (Felsenstein, 1985) with 10,000 replications using heuristic algorithms with tree bisection–reconnection branch swapping and simple addition sequences. For Bayesian inference, all sequenced regions (16S–12S ribosomal RNA, COI, and EF-1α) and codon positions (first, second, and third positions) of two protein coding genes were treated as partitioned data, as statistical analysis showed significant heterogeneity in base frequencies among sequenced regions ($\chi^2 = 10142.89$, df = 21, $P < 0.0001$) and codon positions ($\chi^2 = 3650.02$, df = 6, $P < 0.0001$) across taxa. We estimated the nucleotide substitution model for each partition using MrModeltest 2.2 (Nylander, 2004) under Akaike's information criteria (AIC). Bayesian inference was conducted using MrBayes 3.12 (Ronquist and Huelsenbeck, 2003). Two independent Metropolis-coupled Markov Chain Monte Carlo (MCMC) chains were run with a temperature of 0.2. Trees were sampled every 1000 generations for a total of 5,000,000 MCMC generations, and the first 1250 trees (125,000 generations) were discarded as burn-in. The convergence of chains and the appropriateness of burn-in values were evaluated by checking the average standard deviation of the split frequencies and comparing the graph of the likelihood of two independent MCMC samples using TRACER 1.41 (Rambaut and Drummond, 2007). Posterior probabilities of nodes were estimated based on 50% majority rule consensus of the 7500 post-burn-in trees.

When a clade in our estimated phylogeny was inconsistent with the taxonomic groupings of Kingsolver and Johnson (1978), we tested for monophyly of the clade using the "Constraint" command of MrBayes with the log-Bayes factor test (Kass and Raftery, 1995). We calculated the harmonic mean of the likelihood values for trees constrained to reflect previous taxonomic groupings. We then compared the harmonic mean of the likelihood values of the original and "constrained" trees. The test statistic was calculated as 2(log[harmonic mean (likelihood value for unconstrained trees)] − log[harmonic mean (likelihood value for constrained trees)]). For the log-Bayes factor test, we treated values greater than 10 as decisive evidence, 6–10 as strong evidence, 2–6 as positive evidence and 0–2 as weak evidence of support for unconstrained tree, based on Kass and Raftery (1995).

2.4. Ecological data collection

2.4.1. Determination of host plants

The host plant species from which *Mimosestes* samples emerged were treated as directly observed host plants. In addition to these direct emergence records, we used adult emergence (i.e., larval rearing) records from previously published literature (Kingsolver and Johnson, 1978; Johnson, 1979, 1983b; Siemens et al., 1992; Nilsson and Johnson, 1993; Johnson and Siemens, 1996; Romero Nápoles et al., 2009) and the database BRUCOL (Romero Nápoles and Johnson, 2002; Romero and Johnson, 2004), which includes rearing information about seed beetles, to infer the hosts for each sampled species of *Mimosestes*. Female *Mimosestes* lay their eggs on the seed pod, and hatched larvae spend their entire developing stage in a single seed pod. This behavior allowed us to both collect accurate information about hosts of each species by rearing seed pods in the laboratory and to extract highly reliable host information from the literature.

The records extracted from the literature and the database inevitably include occasional host use of *Mimosestes* species. Indeed, 22 of 75 recorded host plants have been reported only once in the literature and can potentially serve as an occasional host. These occasional host utilizations cause overestimation of the host range for *Mimosestes* species. Thus, we adopted two strategies for determining the host(s) of each species: the narrowest host estimation strategy and the broadest host estimation strategy. In the narrowest strategy, we excluded all plant records that were reported only once. In the broadest strategy, all rearing records in both the literature and the database were included in the analyses. Comparing the narrowest and broadest host range is the most conservative method for host estimation and may allow us to infer a detailed evolutionary history of host utilization of *Mimosestes*, as suggested by several authors (Yotoko et al., 2005; Weingartner et al., 2006; Janz and Nylin, 2008).

Synonyms of the host plants included in BRUCOL and the published literature were checked using the World Database of Legumes of the International Legume Database and Information Service (Roskov et al., 2005, http://www.ildis.org/LegumeWeb/).

2.4.2. Estimation of oviposition type

We categorized oviposition behavior into two types: (A) ovipositing on only immature seed pods on the tree, and (B) ovipositing on both immature and mature seed pods. For each sampled *Mimosestes* species, we estimated the preferred stages for oviposition based on field observations (T. Kato, unpublished data) and data from the literature (Johnson, 1983c; Traveset, 1990, 1991).

2.5. Comparative analyses

2.5.1. Construction of phylogeny for comparative analyses

Allcomparative analyses were performed on a simplified phylogenetic tree to avoid bias caused by inclusion of multiple specimens from a single species in the phylogeny. We randomly selected an individual for each multi-sampled *Mimosestes* species and treated it as representative of the species (see Appendix A). All outgroup species were included in the phylogeny for comparative analyses. The simplified phylogeny was constructed using MrBayes under the same conditions described above. To account for phylogenetic uncertainty, all 7500 post-burn-in trees were used for the Bayesian comparative analyses described below.

2.5.2. Character settings

We used the phylogenetic diversity (PD) index (Faith, 1992, 1996) as a gauge of diet breadth for each *Mimosestes* species. The PD index allows the direct comparison of the diet breadth of *Mimosestes* species that utilize multiple *Acacia* species with species that utilize both *Acacia* species and non-*Acacia* host plants under the assumption that the phylogenetic distances serve as a proxy of trait relatedness among host plants (Symons and Beccaloni, 1999; Beccaloni and Symons, 2000; Morse and Farrell, 2005). Due to the lack of molecular data for some host plant species, we used the method of Morse and Farrell (2005), which calculates the PD index from a supertree of host plants. The supertree was constructed based on previously published phylogenies of Leguminosae and host plant genera: *Acacia*, *Prosopis*, *Parkinsonia*, and Caesalpinia (Miller and Bayer, 2003; Heil et al., 2004; Lavin et al., 2005; Catalano et al., 2008; Bruneau et al., 2008). When host plants were not included in the above analyses, they were placed in a clade that included related species (or species groups in the case of *Acacia*) as an unresolved polytomy, based on the taxonomic literature of host plants (Seigler and Ebinger, 1995; Lee et al., 1989; Clarke et al., 1989, 1990; Ebinger et al., 2000; Haston et al., 2005; Rico-Arce, 2007). Branch lengths of the supertree were calculated using two alternative methods: Grafen's method and the minimal extension (ME) method using CACTUS 1.13 (Schwilk and Ackerly, 2001), following Morse and Farrell (2005). The PD index for each *Mimosestes* species was then calculated by summing the branch lengths of all host plants in the supertree.

All ecological data except diet breadth were treated as discrete characters. Host plants were categorized into three groups according to host plant genera (Acacia, Prosopis, and Parkinsonia). This host categorization was based on the assumption that phylogenetic structure is a proxy of the biological entity of host

plants (e.g., seed compounds and/or seed pod structure). The existence of *Mimosestes* species that specialize on a particular host plant genus (Acacia, Prosopis, or Parkinsonia) supports this assumption. We removed Caesalpinia and Bauhinia from host plant categories for comparative analyses because these two host plants are utilized by only M. mimosae and it was apparent that these host utilizations evolved only once. Additionally, placing M. mimosae in a unique category was not practical, given the purpose of the present study.

We also conducted comparative analyses for *Acacia* species groups to evaluate the degree of phylogenetic conservatism at the species group level. *Acacia* subgenus *Acacia* in the New World consists of seven informal species groups based on both morphological and molecular data (Lee et al., 1989; Clarke et al., 1989, 1990, 2009; Seigler and Ebinger, 1995; Ebinger et al., 2000; Miller and Bayer, 2003; Heil et al., 2004). These species groups are quite diverse in seed pod structure (Janzen, 1969b; Johnson, 1987) and myrmecophilous habit (Janzen, 1966; Seigler and Ebinger, 1995), although both taxonomic and molecular studies have demonstrated the close relatedness of the species groups. If host plant utilization is constrained at the *Acacia* species group level, we can assume that these character differences may play a role in the determination of host plants in *Mimosestes*.

2.6. Estimation of the phylogenetic conservation of ecological traits

A permutation tail probability (PTP) test (Faith and Cranston, 1991) using PAUP* was conducted to estimate the phylogenetic conservation of discrete ecological traits (host utilization and oviposition behavior). A single ecological character state was randomly reshuffled 10,000 times among the ingroup species without changing the tree topology. The number of steps required to explain each randomly permuted character was compared to the number of steps needed to explain the observed character distribution. If the observed number of steps was significantly fewer than the number of steps for randomized characters, the null hypothesis that the character is not phylogenetically conserved was rejected.

PTP tests tend to overestimate phylogenetic conservatism (Slowski and Crother, 1998; Harshman, 2001). Thus, we also tested phylogenetic conservatism using a likelihood framework. Using the "Constraint" option of MrBayes, we calculated the harmonic mean of likelihood values of trees under the constraint that species utilizing the same host or sharing the same oviposition behavior were placed as monophyletic. We then compared the harmonic mean of likelihood values of original and "constrained" trees using the log-Bayes factor test.

We tested the phylogenetic conservatism of diet breadth as a continuous character using the BayesContinuous module of BayesTraits 1.0 (Pagel and Meade, 2006) with a generalized least square (GLS) model. We estimated maximum likelihood values of the scaling parameter, lambda (λ, Pagel, 1997; Pagel and Meade, 2006), which describes whether a given phylogenetic tree correctly predicts the pattern of covariance among species. We calculated harmonic means of λ-values for all combinations of host estimation strategies and branch length estimation methods using an MCMC approach. The "Ratedev" parameter was adjusted to achieve an acceptance rate of 20–40%. Rate coefficients and ancestral character states were sampled every 10,000 generations to avoid autocorrelation. The MCMC chain was run for 5,000,000 iterations with 1,250,000 burn-in periods. We conducted log-Bayes factor tests to evaluate whether likelihood values under estimated λ were significantly different from likelihood values under $\lambda = 1.0$. A λ-value near 1.0 indicates a strong phylogenetic signal, whereas a λ-value near 0.0 indicates that the trait is evolving among species independently of the phylogeny (Pagel, 1997; Pagel and Meade, 2006).

2.7. Detection of evolutionary direction for specialist

We assessed the evolutionary trend towards specialization in host utilization of *Mimosestes* using a GLS model in BayesContinuous. We compared two models of evolution incorporated in BayesContinuous: a model that included one directional (from generalist to specialist) change parameter (directional random-walk model) and a model without a directional change parameter (non-directional model). We calculated the logarithm of the harmonic mean of likelihoods for both models using MCMC under the same conditions as those used for the analysis for conservatism; harmonic means were then compared using the log-Bayes factor test. If the directional random-walk model described patterns of diet breadth significantly better than the non-directional random-walk model did, we treated the results as support for an evolutionary trend towards specialization.

2.8. Ancestral state reconstruction

Ancestral states of host utilization were reconstructed under a maximum likelihood framework with Bayesian inference using BayesMultistate module in BayesTraits. We categorized host utilization into four types (*Acacia* specialist; *Parkinsonia* specialist; *Acacia* and *Prosopis*; *Acacia*, *Prosopis*, and *Parkinsonia*) based on the actual host utilization patterns of sampled *Mimosestes* species.

We conducted preliminary maximum likelihood analysis using BayesMultistates to estimate the best fit model of character transition rate parameters and the midpoint of parameter distribution (see BayesTraits manual). We compared model sets that included the most complex evolutionary model (i.e., all parameters could move independently of one another) and simpler evolutionary models for each of 7500 trees by using AIC statistics, which were calculated from the results of ML analyses and the numbers of parameters. Simpler models were obtained by equalizing the forward and backward rates of transition between characters, based on the recommendation of Schluter et al. (1997). Bayesian ancestral state reconstruction using MCMC was conducted with the obtained evolutionary model and midpoint value of the prior parameter. The conditions of the MCMC analyses (5,000,000 iterations, 1,250,000 burn-in, 20–40% of acceptance rate) were the same as those used in the analysis for conservatism. We reconstructed the ancestral states at a particular node using the "most recent common ancestor approach" (command "AddMRCA" in BayesTraits). The likelihoods of alternative characters for each internal node were calculated using the "Fossil" command in BayesMultistate and compared using the log-Bayes factor test. The states of all outgroups were treated as missing data. We also reconstructed the ancestral states for each subgroup of *Acacia* to investigate whether species group level host utilization originated from a single colonization event.

2.9. Correlation tests between diet breadth and oviposition behavior

Tests of correlated evolution between diet breadth and oviposition behavior were performed with the BayesContinuous module using the MCMC option with 7500 post-burn-in trees to account for phylogenetic uncertainty. Given a pair of diet breadth and oviposition behavior characters, the logarithm of the harmonic mean of likelihood values was calculated under two evolutionary models: a model postulating that the two characters evolved independently (independent model) and a model assuming that the two characters evolved in a correlated manner (dependent model; Pagel and Meade, 2006). The MCMC analyses were conducted using the same conditions as in the other comparative analyses. The harmonic means of the independent and dependent models were compared using the log-Bayes factor test.

3. Results

3.1. Phylogenetic analysis

The strict consensus tree of the MP trees is presented in Fig. 1. A total of 3768 nucleotides for 25 samples from 16 species were determined and aligned. The pairwise ILD test indicated that tRNA-VAL (69 bp) significantly differed from other genes. Therefore, we removed tRNA-Val from the data set and used 3699 bp for the molecular phylogenetic analyses. We combined the 16SrRNA, 12SrRNA, COI, and EF-1α data sets, because the pairwise ILD test indicated no significant conflict among these genes.

The data set contained 1054 variable characters, of which 741 were parsimoniously informative. The MP analysis of the combined data set resulted in the six most parsimonious trees. Topological differences among all MP trees only depict differences in the arrangement of specimens within the species *M. amicus* and *M. mimosae*.

For Bayesian inference, three substitution models were selected for analyzed partitions using AIC (GTR+I+G model: 16SrRNA, 12SrRNA, and the first and third positions of COI; GTR+I model: second position of COI and first position of EF-1α; F81 model: second position of EF-1α; and SYM+I model: third position of EF-1α). For ingroup taxa, the species level topology of a 50% majority rule consensus tree of 7500 trees obtained from Bayesian inference was identical to the species level topology of the MP trees (Fig. 2). The only difference between the two topologies was the relationship among the three outgroup species.

Mimosestes obscuriceps was placed as the most basal ingroup taxon in the tree. Ingroup taxa formed two major clades (clades I and II; see Figs. 1 and 2). Clade I (with 91.6% bootstrap support and 1.00 Bayesian posterior probability) comprised *M. acaciestes*,

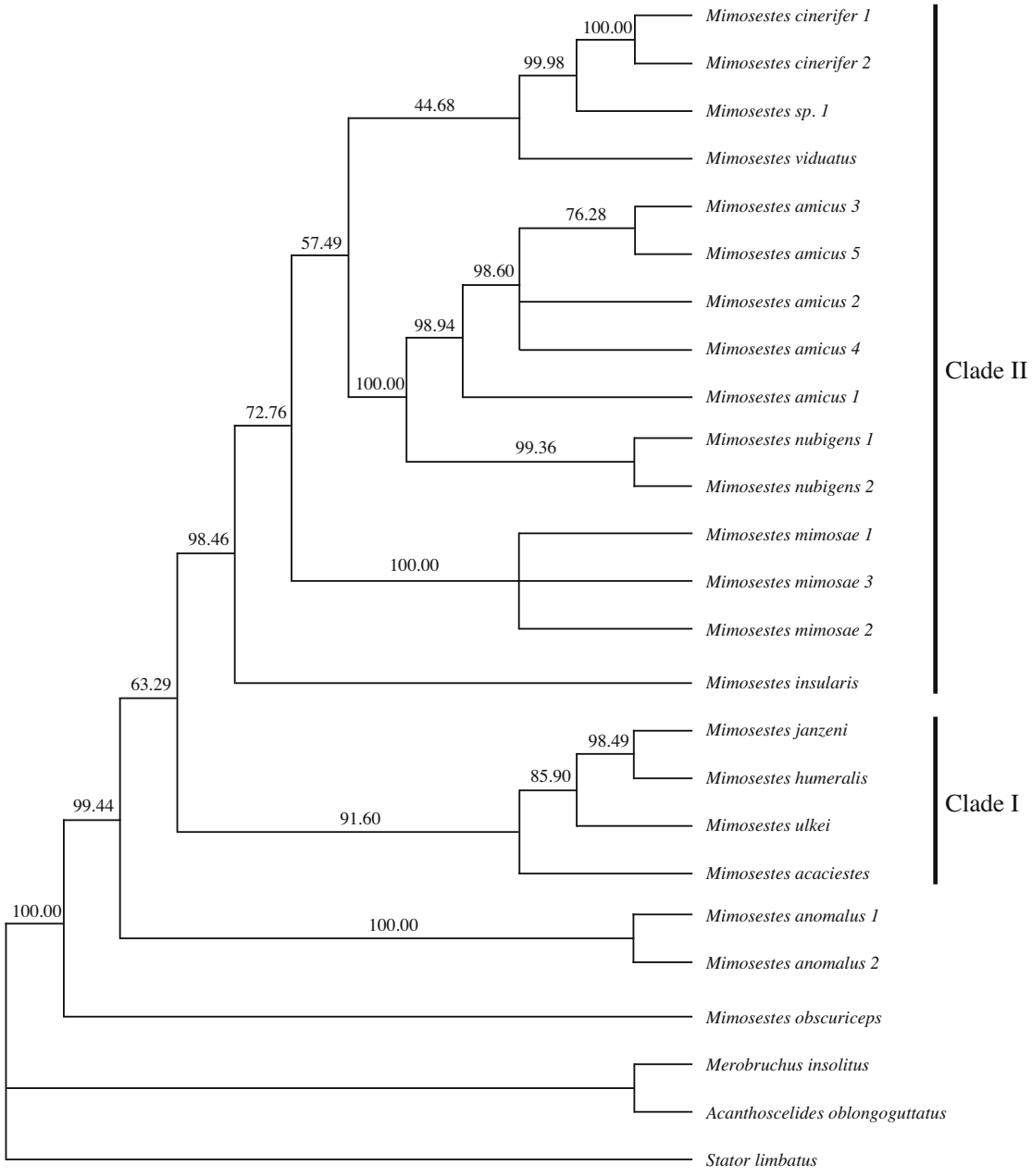

Fig. 1. Strict consensus of six most parsimonious trees for *Mimosestes* (tree length = 2773 steps, Consistency index = 0.528, Retention index = 0.583) based on the combined mitochondrial COI, 16–12S and nuclear EF-1α sequences. Bootstrap values based on 10,000 replications are noted above the corresponding nodes.

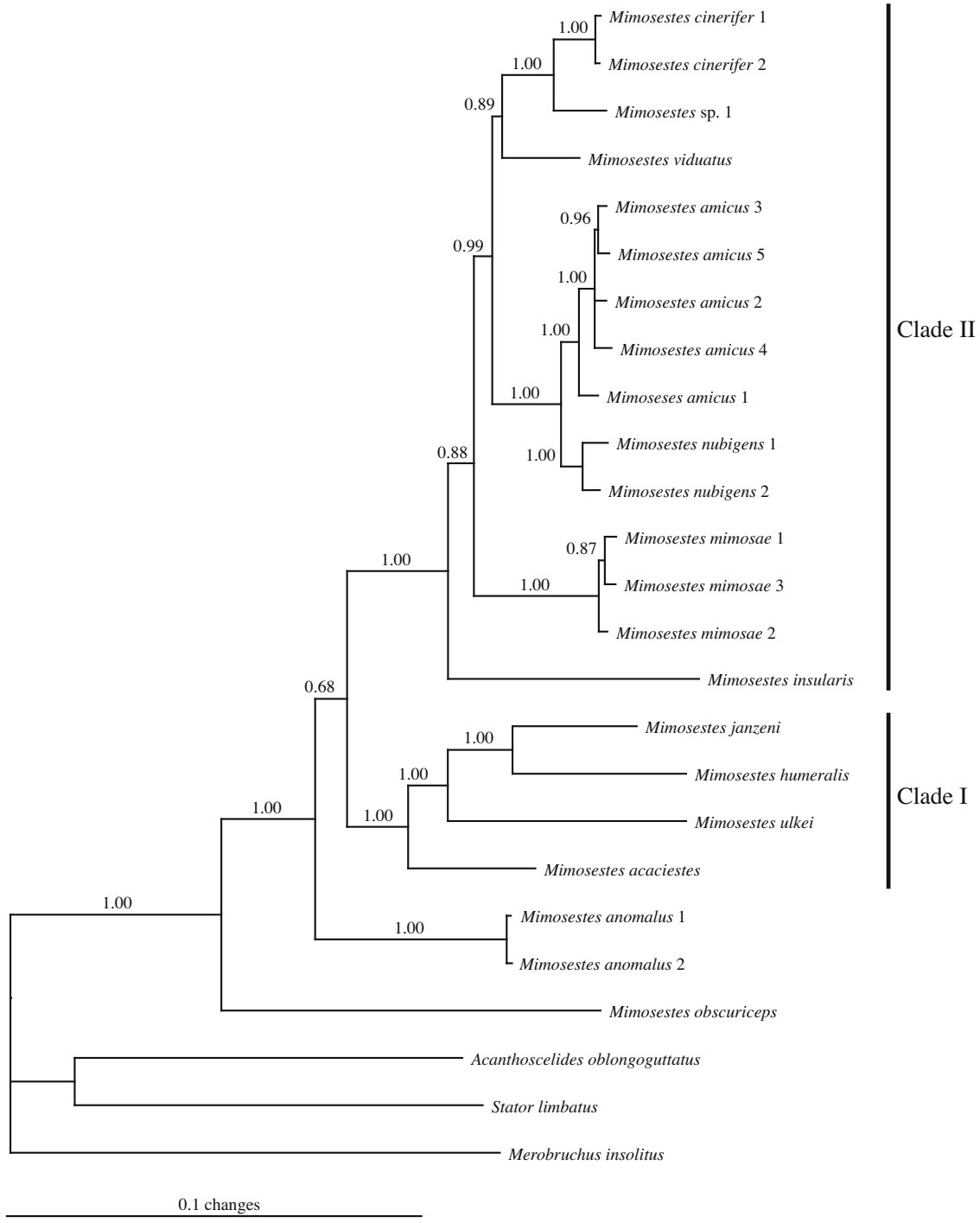

Fig. 2. 50% consensus of 3750 trees of Bayesian phylogram of the *Mimosestes*. Bayesian posterior probability of each node are noted above the corresponding nodes.

M. ulkei, *M. humeralis*, and *M. janzeni*. Clade II (with 98.46% bootstrap support and 1.00 Bayesian posterior probability) consisted of all remaining species except *M. obscuriceps* and *M. anomalus*. Within clade II, *M. insularis* was placed as the outermost branch. *Mimosestes* sp.1 collected from *Acacia collinsii*, formed a subclade in clade II with two other Ant-acacia feeding species, *M. cinerifer* and *M. viduatus*. *Mimosestes anomalus* was placed as a sister of clade I + II, although it had low bootstrap support (63.29%) and Bayesian posterior probability (0.68). Even though Kingsolver and Johnson (1978) placed *M. acaciestes* and *M. anomalus* within the Mimosae group (Table 1), these species were not included in the clade containing other species of the Mimosae group (Figs. 1 and 2). The placement of these two species in our phylogeny was strongly supported by the results of the log-Bayes factor test (logarithm of harmonic mean of likelihood values: unconstrained trees = −17212.98; trees constrained to include all species in the Mimosae group into one clade = −17228.35, log-Bayes factor = 30.74 > 10), a finding that contradicts the taxonomic grouping of Kingsolver and Johnson (1978).

3.2. Ecological traits

All ecological data are shown in Table 2. The host range of *M. nubigens* differed considerably at the genus level between the

Table 2
Summary of estimated oviposition behavior and diet breadth estimated as phylogenetic diversity (PD) index of 13 studied *Mimosestes* species. Oviposition behavior: (A) oviposit only on immature seedpods; (B) oviposit on both immature and dry mature seedpods. PD index is calculated based on two host estimation strategies (narrowest and broadest host estimation) and two branch length estimation methods: Grafen's (Grafen) and minimal extension (ME) methods.

Species name	Oviposition behavior	PD diet breadth Narrowest estimation		PD diet breadth Broadest estimation	
		ME	Grafen	ME	Grafen
M. acaciestes	A	1.38	1.15	1.50	1.24
M. amicus	B	4.00	3.62	5.38	4.85
M. anomalus	B	0.13	0.06	0.75	0.85
M. cinerifer	A	0.25	0.41	0.63	0.79
M. humeralis	A	0.13	0.06	0.88	0.82
M. insularis	B	2.13	2.15	2.13	2.15
M. janzeni	A	0.25	0.12	0.25	0.12
M. mimosae	B	4.88	4.79	5.88	5.97
M. nubigens	B	0.38	0.18	3.50	3.59
M. obscuriceps	A	0.25	0.41	0.25	0.41
M. ulkei	A	0.50	0.18	0.50	0.18
M. viduatus	A	0.38	0.62	1.13	1.62
Mimosestes sp.1	A	0.13	0.21	0.13	0.21

two estimation methods. Under the narrowest host estimation, only three species of *Acacia* were estimated as the hosts of *M. nubigens*. However, under the broadest host estimation strategy, *Prosopis* and *Parkinsonia* were included as host plants of *M. nubigens*. This difference of estimated host range led to considerable differences in PD indices for *M. nubigens* depending on the strategy used (Table 2). At the *Acacia* species group level, the estimated hosts of eight of 12 *Mimosestes* species differed between the two estimation strategies. In the narrowest host estimation strategy, these eight species utilized only one *Acacia* species group, whereas they utilized two or more species groups in the broadest host estimation strategy (Table 1).

Most species of *Mimosestes* exhibited oviposition behavior type A, ovipositing exclusively on immature seed pods. Several species of *Acacia* have seed pods that dehisced soon after they had matured and dried (Janzen, 1969b). Thus, *Mimosestes* species that exclusively utilize such *Acacia* species were treated as type A because all of these species oviposited on the surface of seed pods and were unlikely to lay their eggs on dehisced seed pods. *Mimosestes* sp.1 was only obtained from immature seed pods of *Acacia collinsii* and was therefore provisionally regarded as type A. Our data revealed that five of the 13 examined species oviposited on both fleshy and dry seed pods. These species were categorized as exhibiting oviposition behavior B (Table 2).

3.3. Comparative analyses

3.3.1. Phylogenetic conservatism of ecological traits

The results of the PTP tests and log-Bayes factor tests for phylogenetic conservatism are summarized in Table 3. The PTP test showed that non-*Acacia* host utilization was not significantly correlated with the *Mimosestes* phylogeny in both host estimation strategies. The PTP test also indicated that no significant correlation existed between host utilization of *Mimosestes* and their phylogeny at the *Acacia* species group level, even if occasional or unusual host utilization was taken into consideration. The log-Bayes factor tests showed that, for all host categories, the unconstrained model provided a significantly better fit than did the model hypothesizing phylogenetic conservatism of host utilization (Table 3).

Our results indicated that diet breadth was not correlated with *Mimosestes* phylogeny (Table 4). The λ parameters estimated by the GLS model fell between 0.0 and 1.0 for all combinations of host and PD index estimation methods. The results of the log-Bayes factor test revealed that a model in which λ was allowed to take its maximum likelihood value provided a significantly better fit than the model in which λ was set to 1.0 (Table 4).

Oviposition behavior was the only ecological trait significantly correlated with phylogeny in the PTP test (Table 3). However, the

Table 3
Results of analyses of phylogenetic conservatism for host plant utilizations and oviposition behavior. Significant result (α-value < 0.05, indicated as an asterisk) of permutation tail probability (PTP) test indicates that character is phylogenetically conserved. log-Bayes factors are calculated by 2(log[harmonic mean (likelihood value for unconstrained model: −16366.65)] − log[harmonic mean (likelihood value for constrained model in which *Mimosestes* species utilizing the same host plant group constrained to be monophyletic)]). Positive value of the log-Bayes factor indicates that the unconstrained model has a better fit than the model estimating phylogenetic conservatism (Kass and Raftery, 1995). Interpretations of obtained log-Bayes factor (0–2, weak evidence; 2–6, positive evidence; 6–10, strong evidence; >10, very strong evidence) are based on discussion of Kass and Raftery (1995). Phylogenetic conservatisms of *Acacia* genus level host utilization and Constricta species group utilization were reconstructed only for the narrowest host estimation because host utilization pattern of these categories were identical in both host estimation methods. Phylogenetic conservatism of the Rigidula species group utilization was tested only for the broadest host estimation because only one *Mimosestes* species (*M. acaciestes*) was estimated to utilize this *Acacia* species group in the narrowest host estimation.

	Narrowest estimation			Broadest estimation		
	PTP test	Mean likelihood of constraint model	log-Bayes factor	PTP test	Mean likelihood of constraint model	log-Bayes factor
Host plant group						
Acacia	1.000	−16,402.55	71.8	–	–	–
Constricta	1.000	−16,535.48	337.66	–	–	–
Macracantha	0.490	−16,532.67	332.04	0.852	−16,603.13	472.96
Ant-acacia	0.130	−16,432.26	131.22	1.000	−16,538.82	344.34
Farnesiana	1.000	−16,442.28	151.26	0.524	−16,594.57	455.84
Rigidula	–	–	–	0.319	−16,586.50	439.7
Prosopis	1.000	−16,445.63	157.96	0.324	−16,373.49	13.68
Parkinsonia	1.000	−16,558.13	382.96	0.347	−16,477.66	222.01
Oviposition behavior	0.040*	−16,462.58	191.86	–	–	–

Table 4
Results of analyses of phylogenetic conservatism of diet breadth based on two host estimation strategies (narrowest and broadest strategies) and two branch length estimation methods (minimal extension and Grafen's methods). log-Bayes factors are calculated by 2(log[harmonic mean (likelihood value for unconstrained model)] − log[harmonic mean (likelihood value for constrained model in which diet breadth evolved with direction)]). Positive value of the log-Bayes factor indicates that the unconstrained model has a better fit than the model estimating evolutionary direction. Interpretations of obtained log-Bayes factor (0–2, weak evidence; 2–6, positive evidence; 6–10, strong evidence; >10, very strong evidence) are based on discussion of Kass and Raftery (1995).

Host estimation and branch length inferring method	log-harmonic mean with ML λ (unconstrained)	log-harmonic mean with $\lambda = 1.0$ (conservative)	log-Bayes factor
Narrowest estimation			
ME branch length	−27.79	−29.55	3.52
Grafen's branch length	−26.73	−29.04	4.62
Broadest estimation			
ME branch length	−27.46	−29.11	3.30
Grafen's branch length	−27.29	−28.00	1.43

log-Bayes factor test showed that the unconstrained model was more favored than the model hypothesizing phylogenetic conservatism of oviposition behavior (Table 3). This difference may be due to the liberal nature of the PTP test as argued by several authors (Slowski and Crother, 1998; Harshman, 2001).

3.3.2. Evolutionary direction from generalist to specialist

The harmonic mean of log likelihoods and the results of the log-Bayes factor test for directional and non-directional evolutionary models are summarized in Table 5. For all combinations of host and branch length estimation methods, the non-directional model exhibited a higher mean of log likelihoods than the directional model, with weak or positive support (0.69–2.97 log-Bayes factor). These results suggest that no or only weak evolutionary trends exist toward host specialization in *Mimosestes*.

3.3.3. Correlation between diet breadth and other ecological traits

log-Bayes factors and parameter estimates for PD indices and oviposition behavior are presented in Table 6. Although the PD indices took on different values according to host range estimation strategies, oviposition behavior was strongly correlated with diet breadth for all combinations of host estimation strategies and branch length estimation methods (6.03–8.97 log-Bayes factor).

3.3.4. Ancestral state reconstruction

The results of the ancestral state reconstruction of host utilization at the genus level are shown in Fig. 3. For both host estimation strategies (narrowest and broadest), one parameter model (all rate parameters were set as equal) was selected as the best fit model by comparing AIC statistics for 7500 trees.

In both host estimation strategies, the ancestral condition for host utilization in *Mimosestes* was reconstructed as an *Acacia* specialist. The log-Bayes factor test indicated that the posterior probability of an *Acacia* specialist at nodes 1–6, 9, 11, and 12 significantly exceeded the posterior probability of other states in both strategies (Fig. 3). The ancestral host of clade I (nodes 3 and 4) was reconstructed as an *Acacia* specialist using both host estimation strategies (Fig. 3). This implies that the *Parkinsonia* specialist in clade II (*M. ulkei*) originated directly from the *Acacia* specialist in the clade and that *Parkinsonia* utilization evolved at least twice (one in clade I and the other in clade II) in the *Mimosestes*. A significant support for the presence of *Acacia* specialist over other host utilization states at node 9 suggests that multi-genera-utilizing species evolved at least twice in clade II.

The two host estimation strategies resulted in some differences between state reconstructions. Under the narrowest host estimation strategy, host utilization of two basal nodes of clade II (nodes 7 and 8) were reconstructed as *Acacia* specialists (Fig. 3). In contrast, under the broadest host estimation strategy, the posterior probability of an *Acacia* specialist in these nodes did not significantly exceed the posterior probabilities of alternative states. In addition, the posterior probability of *Acacia*, *Prosopis*, and *Parkinsonia* utilization at node 10 significantly exceeded the posterior probability of an *Acacia* specialist under the broadest estimation, whereas the posterior probability of these two states at the nodes under the narrowest estimation did not significantly differ.

At the *Acacia* species group level, ancestral state reconstruction under the narrowest host estimation revealed that utilization of four species groups (Ant-acacia, Farnesiana, Macracantha, and Constricta) evolved twice or more times in the *Mimosestes*, though reconstruction under the broadest host estimation did not show significant support for multiple-evolutionary event of three host plant utilizations (Appendix C).

We were unable to estimate the ancestral oviposition behavior of the genus from our data because the ancestral state of the genus was reconstructed as equivocal (data not shown).

Table 5
Results of the log-Bayes factor test for directional evolution of diet breadth based on two host estimation strategies (narrowest and broadest strategies) and two branch length estimation methods (minimal extension and Grafen's methods). log-Bayes factors are calculated by 2(log[harmonic mean (better fit model)] − log[harmonic mean (worse fit model)]). For all combinations of host estimation strategies and branch length estimation methods, the unconstrained model had a better fit than the directional evolution models. Interpretations of obtained log-Bayes factor (0–2, weak evidence; 2–6, positive evidence; 6–10, strong evidence; >10, very strong evidence for better fit model) are based on the discussion of Kass and Raftery (1995).

Host estimation and branch length inferring method	log-harmonic mean with direction	log-harmonic mean without direction	log-Bayes factor
Narrowest estimation			
ME branch length	−30.25	−29.11	2.29
Grafen's branch length	−29.2	−28.86	0.69
Broadest estimation			
ME branch length	−30.47	−28.98	2.97
Grafen's branch length	−28.57	−27.63	1.88

Table 6
Harmonic mean of likelihood scores and results of the log-Bays factor test for trait correlation between oviposition behavior on two host estimation strategies and two branch length estimation methods. log-Bayes factors are calculated by 2(log[harmonic mean (better fit model)] − log[harmonic mean (worse fit model)]). For all combinations of host estimation strategies and branch length estimation methods, the correlation model had a better fit than the uncorrelation models. Interpretations of obtained log-Bayes factor (0–2, weak evidence; 2–6, positive evidence; 6–10, strong evidence; >10, very strong evidence for better fit model) are based on the discussion of Kass and Raftery (1995).

Host estimation and branch length inferring method	log-harmonic mean independent model	log-harmonic mean correlation model	log-Bayes factor
Narrowest estimation			
ME branch length	−45.37	−41.53	7.68
Grafen's branch length	−43.36	−40.34	6.03
Broadest estimation			
ME branch length	−42.19	−38.37	7.65
Grafen's branch length	−40.92	−36.44	8.97

4. Discussion

4.1. Relationships among Mimosestes species

In general, our reconstructed phylogeny was consistent with the species groupings of Kingsolver and Johnson (1978) (Table

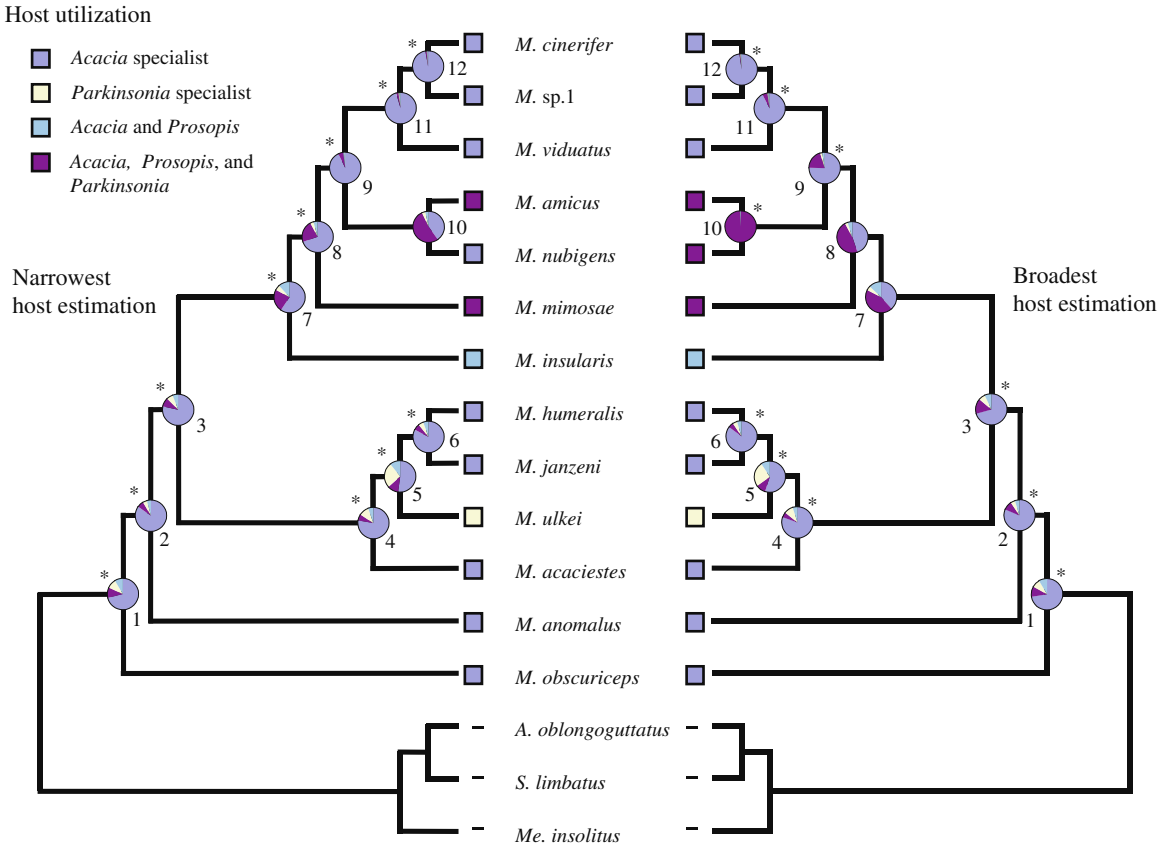

Fig. 3. Ancestral character reconstruction for the evolution of genus level host utilization in *Mimosestes* based on 7500 post burn-in Bayesian trees. Left phylogram reconstruction based on narrowest host estimation strategy; right phylogram reconstruction based on broadest host estimation strategy. Pie graphs represent posterior probabilities of the four host utilization states at each node. Asterisks indicate that the state with the highest posterior probability significantly exceeded the state with the second highest posterior probability in the log-Bayes factor test (as indicated by a log-Bayes factor >2) at the node. Outgroups were coded as missing data.

1). However, our data provide new insights into the relationships among *Mimosestes* species. For example, the positions of *M. acaciestes* and *M. anomalus* within the genus differed significantly from previous species groups proposed by Kingsolver and Johnson (1978). Kingsolver and Johnson (1978) recognized several morphological differences between these two species and the remaining species within the Mimosae group. *Mimosestes acaciestes* was placed into clade I in our phylogeny. Johnson (1987) reported that all species placed into clade I in our phylogeny share the trait that first-stage larvae crawl onto the seed pod surface before they enter the seeds, whereas the larvae of the remaining species enter the seed pod directly through the bottom of the egg. We suggest that this behavior is a synapomorphic character and provides reliable support for the validity of clade I in our phylogeny.

Mimosestes anomalus represents a sub-basal branch in the phylogeny (Figs. 1 and 2). Kingsolver and Johnson (1978) referred to morphological similarities between *M. anomalus* and the Obscuriceps group, which held the outermost branch in our phylogeny. We suggest that these similarities may represent plesiomorphic characters.

4.2. Evolution of host utilization in Mimosestes

The present analyses revealed that non-*Acacia* genera utilization and *Acacia* species group level host utilization are not phylogenetically conserved and that diet breadth does not exhibit an evolutionary trend toward specialization in *Mimosestes*, even though evolutionary conservatism and trends for specialization are possible outcomes of strict co-evolution between phytophagous insects and their host plants (Moran, 1988). These results are consistent with recent studies demonstrating that host utilization patterns in phytophagous insects are not solely explained by physiological specialization (i.e., subsequent loss of the ability to utilize alternative hosts) of the insects (Janz and Nylin, 1998; Janz et al., 2001; Nosil, 2002; Nosil and Mooers, 2005; Yotoko et al., 2005; Weingartner et al., 2006). We emphasize that this lack of conservatism depended on the level at which host associations were coded (Kelley and Farrell, 1998; Yotoko et al., 2005). For example, all *Mimosestes* species specialize on only one host family (Leguminosae), and Kergoat et al. (2007) demonstrated a statistically significant bias in the preference for *Acacia* in *Mimosestes*.

The ancestral state reconstruction showed that host expansion to *Prosopis* and *Parkinsonia* occurred multiple times (twice and three times, respectively) in *Mimosestes*, at least for the sampled species. Two of the three inferred evolutionary events of *Parkinsonia* utilization and all inferred evolutionary events of *Prosopis* utilization appear to have led to the evolution of multi-genera-utilizing species (Fig. 3). These results support the speculations of several recent studies that shifts to newly incorporated host plants and the evolution of generalists are related phenomena (Janz et al., 2001; Weingartner et al., 2006; reviewed in Janz and Nylin, 2008), and also suggest that gaining the ability to utilize new host plants does not require the loss of ancestral *Acacia* utilization abilities. The estimated host expansion events and the

host shift from an *Acacia* specialist to a *Parkinsonia* specialist (*M. ulkei*) in clade I suggest that specialization may not be an evolutionary dead end in *Mimosestes*. We emphasized that our estimation of the evolutionary history of *Prosopis* utilization may be changed if *M. protractus* (a *Prosopis* specialist) is included in the analysis.

Mimosestes nubigens is the only species whose host range changed at the genus level across the two host estimation strategies (Tables 1 and 2). The ancestral host utilization of *M. nubigens* depended on the host estimation strategy (Fig. 3). We consider *M nubigens* to have derived from a generalist ancestor, despite the lack of significant support in their ancestral host utilization under the narrowest host estimation. Under laboratory conditions, *M. nubigens* can develop in seeds of *Prosopis juliflora* with high survival rates (T. Kato, unpublished data). This preliminary result suggests that *M. nubigens* can utilize *Prosopis*, similar to its sister species *M. amicus*, at least at the physiological level, thus potentially supporting our inference of their ancestral host utilization.

Our analyses indicated that the utilization patterns at the *Acacia* species group level were not phylogenetically conserved using either host estimation strategy, suggesting that differences in morphological and ecological characters among *Acacia* species groups may not be a limiting factor of host utilization in *Mimosestes*.

We suggest that host utilization of *Mimosestes* can change dynamically within a restricted set of plants, as argued by recent studies of several phytophagous insects (Weingartner et al., 2006; Yotoko et al., 2005; Janz and Nylin, 2008). Although subsequent physiological studies are needed, in general, the evolutionary pattern of host utilization in *Mimosestes* presented here may be better explained by similarities in secondary compounds among host plants, as put forth by Ehrlich and Raven (1964).

4.3. Evolutionary correlation between diet breadth and oviposition behavior

Diet breadth was significantly correlated with oviposition behavior (Table 6). This result is consistent with a recent study of the seed beetle genus *Stator* (Morse and Farrell, 2005) and support Morse and Farrell's argument that oviposition behavior sets up an ecological axis on host plant utilization in seed beetles.

We hypothesize three causal mechanisms for the positive correlation between oviposition behavior and diet breadth in *Mimosestes*. First, changes in oviposition behavior may reduce the phenological constraint of host utilization in generalists. Seed beetles that only oviposit on immature seed pods restrict their oviposition season because the life cycle of the species should synchronize with the phenology of the host plant. This phenological constraint may cause and/or maintain factors that strictly limit host utilization (Bernays and Chapman, 1994). Conversely, a species that can infest dry and mature seed pods maintains a longer oviposition period, which may increase opportunities to use plants that exhibit similar chemical compounds in different fruiting seasons.

Second, oviposition on dry seed pods may have released generalists from the constraints put forth by the specific defense mechanisms of immature seed pods. Indeed, immature seed pods of several *Lathyrus* (pea) species exhibit a defense mechanism induced by the bruchid egg (Annis and Okeeffe, 1984; Doss et al., 2000, see Kergoat et al., 2006). Tuda et al. (2006) also suggested that the secondary metabolites in immature seeds are more deleterious than are those in dry mature seeds. Siemens et al. (1992) showed that the mortality rate of generalist *M. amicus* larvae at the stage of penetrating the dry seed pod accounted for very small fractions of overall mortality rate. This may support the second hypothesis, even though there are no studies comparing *Mimosestes* larvae mortality rates between immature and dry seed pods.

Third, the changes in oviposition cue accompanying changes in oviposition behavior may decrease host specificity. Jaenike (1990) suggested that chemical oviposition cues determining a female's ability to recognize the preferred host plant play an important role in determining the diet breadth of phytophagous insects. Changing host recognition cues may occur in concert with changes in oviposition behaviors, which may increase the chance of oviposition mistakes by females and cause further expansion of diet breadth in generalist species. In addition, differences in host searching efficiency (e.g., maturing seed pods are easier for female beetles to find than dry matured seed pods) between oviposition behaviors might also affect diet breadth of seed beetles. These causal hypotheses explain the case of *Mimosestes* and can also provide a general explanation for the diet breadth evolution of the seed beetle, including *Stator* (see Morse and Farrell, 2005).

In addition to these causal mechanisms, community-level factors, such as local host abundance (Jaenike, 1990; Yotoko et al., 2005), differences in susceptibility to parasitoids among oviposition behaviors and differences in the intensity of interspecific competition may cause correlations between diet breadth and oviposition behavior, as discussed by Morse and Farrell (2005). These community-level factors should be examined in further studies.

The present study provides novel insights into the evolution of diet breadth in phytophagous insects. The lack of phylogenetic conservatism and the evolutionary tendency toward diet breadth specialization in *Mimosestes* imply that specialist and generalist traits may have been molded by environmental factors, such as local host abundance (Bernays and Chapman, 1994; Yotoko et al., 2005), interspecific competition (Janzen, 1973; Denno et al., 1995; Kaplan and Denno, 2007), and predator avoidance (Crespi and Sandoval, 2000; Vencl et al., 2005; Nosil and Crespi, 2006) as well as by phylogenetic constraint factors, such as a plant secondary compounds and the physiological adaptation of insects to these chemicals (Ehrlich and Raven, 1964; Janzen, 1969a; Becerra, 1997). Further comparative analyses of changes in ecological traits, including oviposition behavior between generalists and specialists, as well as chemical compound analyses should further elucidate the evolution of generalist phytophagous insects.

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Appendix A. Samples examined in the present study

Taxon name	Locality	Longitude	Latitude	Date	Collector	Host plant	GenBank Accession Nos.		
							16-12S	COI	EF-1α
Mimosestes acaciestes	Saguaro National park, Arizona, U.S.A	N 32.2036	W 110.7256	05/IX/ 2007	T. Kato and H. Yoshitake	*Acacia constricta*	AB499920	AB499945	AB539669
Mimosestes amicus 1*	San Juan Guegoyache, Oaxaca, Mexico	N 16.6694	W 96.2684	15/II/ 2005	T. Kato, M. Shimada and A. Bonet	*Parkinsonia* sp.	AB499921	AB499946	AB539670
Mimosestes amicus 2	Between Tehuantepec and Juchitan, Oaxaca, Mexico	N 16.3949	W 95.1041	17/II/ 2005	T. Kato, M. Shimada and A. Bonet	*Parkinsonia* sp.	AB499922	AB499947	AB539671
Mimosestes amicus 3	Rest area near Eloy on HWY10, Arizona, U.S.A	N 33.0424	W 111.7857	07/IX/ 2007	T. Kato and H. Yoshitake	*Parkinsonia florida*	AB499923	AB499948	AB539672
Mimosestes amicus 4	Rest area near Arizona city on HWY10, Arizona, U.S.A	N 32.7726	W 111.6196	07/IX/ 2007	T. Kato and H. Yoshitake	*Prosopis velutina*	AB499924	AB499949	AB539673
Mimosestes amicus 5	Pearl harbor West Loch Park, Oahu, Hawaii, U.S.A	N 21.3664	W 158.0193	11/IX/ 2007	T. Kato and K. Teramoto	*Prosopis pallida*	AB499925	AB499950	AB539674, AB539675**
Mimosestes anomalus 1*	Estanzuela, Veracruz, Mexico	N 19.4535	W 96.8632	02/III/ 2003	T. Kato, M. Shimada and A. Bonet	*Acacia pennatula*	AB499926	AB499951	AB539676
Mimosestes anomalus 2	El cedral, Veracruz, Mexico	N 18.3684	W 96.4817	06/VI/ 2007	T. Kato and A. Bonet	*Acacia pennatula*	AB499927	AB499952	AB539677
Mimosestes cinerifer 1*	Apazapan, Veracruz, Mexico	N 19.3281	W 96.7153	02/III/ 2003	T. Kato, M. Shimada and A. Bonet	*Acacia sphaerocephala*	AB499928	AB499953	AB539678
Mimosestes cinerifer 2	Temascal, Oaxaca, Mexico	N 18.2736	W 96.3922	06/VI/ 2007	T. Kato and A. Bonet	*Acacia cornigera*	AB499929	AB499954	AB539679
Mimosestes humeralis	Estanzuela, Veracruz, Mexico	N 19.4535	W 96.8632	02/III/ 2003	T. Kato, M. Shimada and A. Bonet	*Acacia pennatula*	AB499930	AB499955	AB539680, AB539681**
Mimosestes insularis	Pearl harbor West Loch Park, Oahu, Hawaii, U.S.A	N 21.3664	W 158.0193	11/IX/ 2007	T. Kato and K. Teramoto	*Prosopis pallida*	AB499931	AB499956	AB539682
Mimosestes janzeni	Ocotepec, Oaxaca, Mexico	N 16.8274	W 96.3545	15/II/ 2005	T. Kato, M. Shimada and A. Bonet	*Acacia cochliacantha*	AB499932	AB499957	AB539683
Mimosestes mimosae 1	Actopan, Veracruz, Mexico	N 19.4497	W 96.5818	04/III/ 2003	T. Kato, M. Shimada and A. Bonet	*Acacia macracantha*	AB499933	AB499958	AB539684
Mimosestes mimosae 2*	Rio Los Perros, Oaxaca, Mexico	N 16.6265	W 95.2413	17/II/ 2005	T. Kato, M. Shimada and A. Bonet	*Acacia cochliacantha*	AB499934	AB499959	AB539685
Mimosestes mimosae 3	Between Tehuantepec and Oaxaca, Oaxaca, Mexico	N 16.3445	W 95.3263	17/II/ 2005	T. Kato, M. Shimada and A. Bonet	*Caesalpinia coriaria*	AB499935	AB499960	AB539686, AB539687**
Mimosestes nubigens 1*	Rio Santa Maria, Queretaro, Mexico	N 21.397	W 99.5817	09/III/ 2004	T. Kato, M. Shimada and A. Bonet	*Acacia farnesiana*	AB499936	AB499961	AB539688, AB539689**
Mimosestes nubigens 2	Near Tequixtepec, Oaxaca, Mexico	N 17.7937	W 97.3575	08/III/ 2006	T. Kato, M. Shimada and A. Bonet	*Acacia schaffneri*	AB499937	AB499962	AB539690
Mimosestes obscuriceps	Boca del Monte, Veracruz, Mexico	N 19.4384	W 96.6088	07/XII/ 2006	T. Kato C. Morales and A. Bonet	*Acacia sphaerocephala*	AB499938	AB499963	AB539691
Mimosestes ulkei	Saguaro National park, Arizona, U.S.A	N 32.2051	W 110.7238	06/IX/ 2007	T. Kato and H. Yoshitake	*Parkinsonia florida*	AB499939	AB499964	AB539692, AB539693**

(*continued on next page*)

Appendix A (*continued*)

Taxon name	Locality	Longitude	Latitude	Date	Collector	Host plant	GenBank Accession Nos.		
							16-12S	COI	EF-1α
Mimosestes viduatus	Cuauhtémoc, Oaxaca, Mexico	N 17.0995	W 94.9254	10/VI/2007	T. Kato and A. Bonet	*Acacia chiapensis*	AB499940	AB499965	AB539694
Mimosestes sp. 1	Route Playa Azul-Arteaga, Michoacan, Mexico	N 18.2181	W 102.2513	15/I/2007	A. Bonet	*Acacia collinsii*	AB499941	AB499966	AB539695
Acanthoscelides oblongoguttatus	Apazapan, Veracruz, Mexico	N 19.3281	W 96.7153	02/III/2003	T. Kato, M. Shimada and A. Bonet	*Acacia sphaerocephala*	AB499942	AB499967	AB539696
Merobruchus insolitus	Rinconada, Veracruz, Mexico	N 19.3514	W 96.5439	03/III/2003	T. Kato, M. Shimada and A. Bonet	*Lysiloma* sp.	AB499943	AB499968	AB539697
Stator limbatus	Jalcomulco, Veracruz, Mexico	N 19.3471	W 96.7731	02/III/2003	T. Kato, M. Shimada and A. Bonet	*Acacia* aff. *villosa*	AB499944	AB499969	AB539698

* Treated as a representative of the species in the comparative analyses.
** Chosen as a representative of heterozygous alleles from an individual.

Appendix B. PCR primers and sequencing primers used in the present study

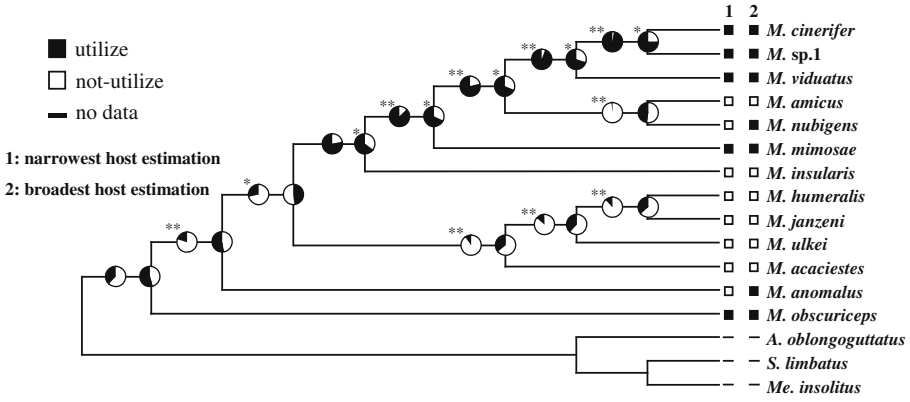
	Primer name	Sequence (5′–3′)
16–12S	MtrA1adv[a,c]	AAA CTA GGA TTA GAT ACC CT
	MtrK1[a,d]	CAT AAT AAG ATT CTA AAT C
	LR-N-13398[b,c]	CAC CTG TTT ATC AAA AAC AT
	MtriD1r[b,d]	TGG AAT AAG TCG TAA CAA AG
	MtriE1f[b,d]	AAA ATA CCG CGG CTT TAA
	MtriE1r[b,d]	TTA AAG CCG CGG TAT TTT
	MtriI1f[b,d]	CCC TGA TAC ACA AGG TAC
	MtriI1r[b,d]	GTA CCT TGT GTA TCA GGG
	MtriJ1f[b]	TCT ATA GGG TCT TCT CGT C
COI	COI-F2[a]	AAC CTT CAA AGT TAA AAA TAG
	COI-R1[a,e]	TCC ATT GCA CTA TTC TGC C
	C1J-1751Mim2[b,f]	TAG GRG CYC CWG AYA TAG C
	COI2-1[b,e]	CTT TAT CAA CAT TTA TTT TGA TTT TTT
	COI-iF1R2[b]	ACT ACN TAR TAW GTA TC
	COI-iF7[b]	GGT ATY GTY CAA TGA TT
	COI-iR2f[b]	ATA ATT TAT GCN ATA ATA GC
	HCO2198[b,g]	TAA ACT TCA GGG TGA CCA AAA AAT CA
	S1665[b]	CCA ATT AWW ATW GGT AT
	S1665re2[b]	CCN CCA ATT ATA ATR GG
EF-1α	S149[a,h]	GAR AAR GAR GCN CAR GAR ATG GG
	A692[a,h]	GGT GGG AGG ATG GCA TCA AGA G

[a] PCR primers.
[b] Sequencing primers.
[c] Modified from Fukatsu et al. (2001).
[d] Kato et al. (2006).
[e] Yoshitake et al. (2008).
[f] Modified from Simon et al. (1994).
[g] Folmer et al. (1994).
[h] Normark et al. (1999).

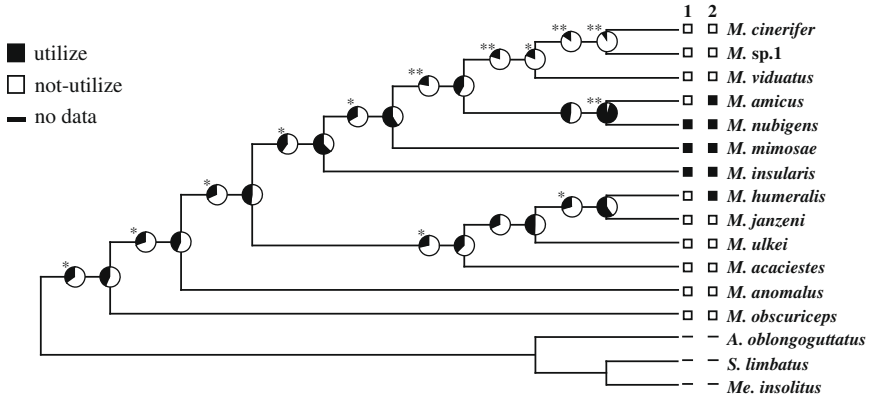
Appendix C

Bayesian ancestral state reconstruction of host utilization in *Mimosestes* for five *Acacia* species groups (Ant-acacia, Farnesiana, Macracantha, Constricta, and Rigidula) based on two host estimation methods (narrowest and broadest host estimation). 500,000 iterations of Markov Chain Monte Carlo were conducted under the following conditions: 20–40% acceptance rate and 125,000 iterations of burn-in periods. Evolutionary model and midpoint value of the prior parameter were estimated by preliminary maximum likelihood analyses. All outgroups were coded as missing data. Posterior probability of host utilization at each node is described by two pie graphs: the left pie graph describes posterior probability based on narrowest host estimation (all unusual host records are excluded from the data set); the right pie graph describes posterior probability based on broadest host estimation (all unusual host records are included in the data set). Asterisk(s) indicate that posterior probability of one state significantly exceeded probability of the other state as determined by the log-Bayes factor test (asterisk: 0–2, double asterisks:>2 log-Bayes factor, respectively). Ancestral host utilization for the Rigidula species group was reconstructed only for the broadest host estimation because only one species (*M. acaciestes*) was estimated to utilize this *Acacia* species group in the narrowest host estimation. Ancestral host utilization for the Constricta species group was reconstructed only for the narrowest host estimation because host utilization pattern of the Constricta species group is identical in both host estimation methods.

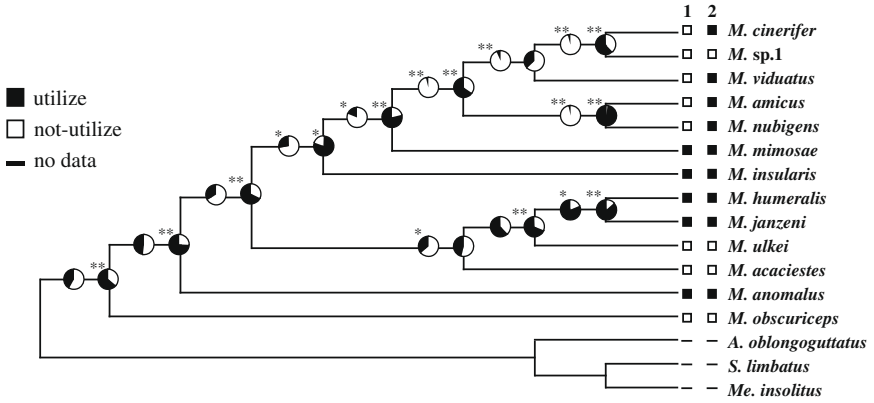
C.1. Ant-acacia species group



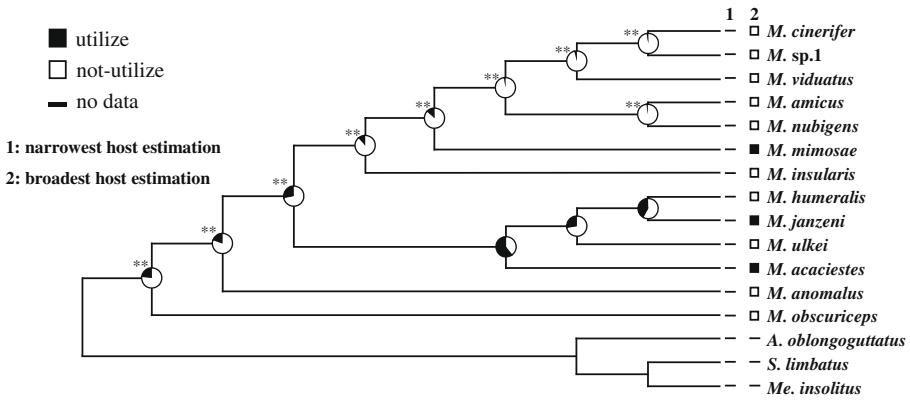
C.2. Farnesiana species group



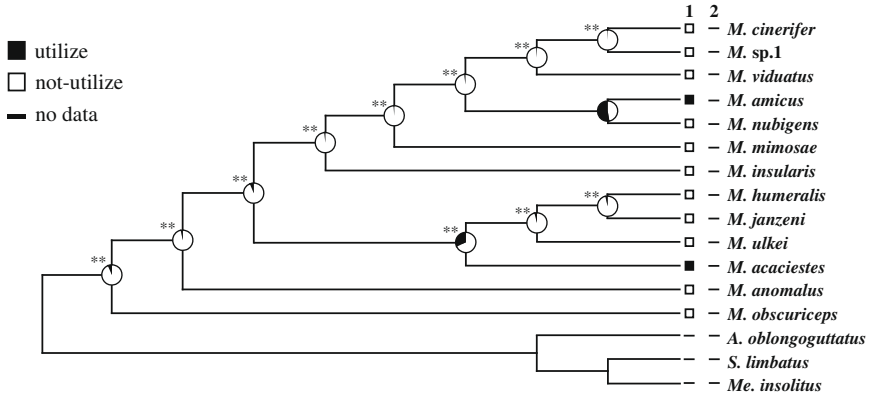
C.3. Macracantha species group



C.4. Rigidula species group



C.5. Constricta species group



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