



Molecular systematics of *Blepharida* beetles (Chrysomelidae: Alticinae) and relatives

Judith X. Becerra

Department of Entomology, University of Arizona, Tucson, AZ 85721, USA

Received 12 September 2002; revised 10 April 2003

Abstract

I investigated the phylogenetic relationships within the New World *Blepharida* and among related genera, using sequences of the Internal Transcriber Spacer 2 (ITS2) of nuclear ribosomal DNA and sequences of the COI and COII genes of the mitochondrial genome. Cladistic analyses were performed using parsimony, maximum likelihood, and Bayesian methods. These methods generated almost identical topologies using the combined data sets. The analyses suggest that *Blepharida rhois*, the type species, should be separated from the New World *Blepharida* and that the New World *Blepharida* might be congeneric with closely related *Notozona*. Also, according to this phylogeny, all of the New World *Blepharida* species that feed on *Bursera* (Burseraceae) form a single monophyletic clade, with the Afrotropical species forming its sister clade. The analyses also identified four main groups of species within the New World *Blepharida*.

© 2003 Published by Elsevier Science (USA).

1. Introduction

The genus *Blepharida* currently includes 73 described species, of which roughly one half are from tropical Africa, and the other half are distributed in the New World tropics (Furth, 1998). One species of outlying distribution, *Blepharida rhois*, is widespread in North America.

Throughout its evolution *Blepharida* has maintained a close relationship with its hosts. It is only known to feed on members of the Anacardiaceae and Burseraceae. These two groups of plants are very closely related, probably sister families (Terrazas and Chase, 1996). Reports of *Blepharida* hosts in Africa are scarce, but they all identify the Anacardiaceae genera *Rhus* and *Schinus* as well as the Burseraceae genus *Commiphora* as the primary hosts (Furth, 1998; Furth and Young, 1988; Koch, 1958).

Most of the known New World *Blepharida* are endemic to Mexico and all but three feed on *Bursera*, the sister genus of *Commiphora* (Becerra, 1997; Becerra and Venable, 1999b; Becerra et al., 2001; Furth, 1998).

Bursera also diversified in Mexico, where more than 100 species are found, and 70% are endemic. This group of plants is abundant in the Mexican tropical dry forests, especially on the Pacific slopes and in the Balsas River depression. They are also conspicuous constituents of desertscrub and thornscrub of the Mexican central and northern deserts (Becerra, 2003; Becerra and Venable, 1999a). *Blepharida* has been found in all these habitats as well and its geographic diversity matches *Bursera*'s geographic diversity. The interaction between *Blepharida* and their hosts is ancient, probably dating to before the separation of Africa and South America. Using a calibrated molecular clock of *Bursera* and their relatives and another clock for the *Blepharida* and related genera, Becerra (in preparation) deduced that the association of these two lineages is more than 100 Mya old. This makes it the oldest documented interaction between a herbivore and its hosts. The phylogenetic relationships of *Blepharida*, as well as their host plants have been central for testing coevolutionary ideas (Becerra, 1997; Hillis, 1997; Mitchell-Olds and Bergelson, 2000).

The complete life cycle and ecology have been intensively studied for several *Blepharida* species (Becerra, 1994; Becerra, 1993; Becerra et al., 2001; Evans et al., 2000; Frost, 1973; Furth and Young, 1988). Their life

E-mail address: becerra@ag.arizona.edu.

cycle starts at the beginning of the summer season when adult females lay clusters of eggs on their hosts' stems. After larvae feed for about 20 days, they drop from the hosts and burrow into the soil. After prepupal and pupal periods in a sealed cell adults emerge at the end of spring, usually coincident with the onset of the rainy season.

Many Mexican *Blepharida* are highly monophagous and feed on a single *Bursera* species, although a few are relatively more polyphagous, feeding on several species of *Bursera* (Evans et al., 2000). Many *Bursera* species exhibit spectacular anti-herbivore defensive mechanisms that are matched by remarkable counter-defensive strategies in *Blepharida* (Becerra, 1994; Becerra and Venable, 1990). The plants produce resin consisting of terpenes stored under pressure in a reticulating network of canals in the cortex of the stems and throughout the leaves. When a leaf is damaged there is an abundant release of resins, often as a squirt that may travel for over two meters. Besides being repellent and toxic, these resins solidify when exposed to air and may completely entrap insects. *Blepharida* species have evolved the ability to neutralize *Bursera*'s squirt gun defense by cutting the leaf veins before feeding on the leaves. There is variation among *Bursera* species in the amounts of resins released and larvae of *Blepharida* species that feed on highly squirting *Bursera* species tend to have larger teeth. These species also tend to spend more of their time cutting their hosts' leaf veins, which exposes them to predators, but they have also developed the ability use their hosts' chemistry for predator defense (Evans et al., 2000).

1.1. Systematics of *Blepharida*

Blepharida belongs in the Alticinae, a highly diversified Chrysomelidae subfamily that encompasses more than 10,000 species in over 550 genera. Members of this subfamily are characterized by having greatly enlarged metafemora and by their ability to jump, sometimes over 100 times their length. This has led to them being given the common name of flea beetles. The relationships within Alticinae are still unknown, and no formal tribal arrangements have been proposed yet. Based on a morphological examination of the methaphoral spring of 91 genera of Alticinae found in the Neotropical region, Furth (1989) separated *Blepharida* along with *Acrocynum*, *Euplectroscelis*, *Crimissa*, *Elithia*, *Iphitroides*, and *Notozona* from other genera studied. The relationships and boundaries among these genera are not clear although morphological evidence suggests that *Notozona*, *Acrocynum*, and *Euplectroscelis* are phylogenetically closer to *Blepharida* than the other genera (Furth, 1992; Wicox, 1975). *Blepharida* is also thought to be very close to *Podontia*, an Asian group, and several *Blepharida* species were first considered to be *Podontia*

(Furth, 1982; Heikertinger and Csiki, 1940; Takizawa, 1978; Weise, 1897).

What constitutes *Blepharida* has been a source of continuous disagreement. In their *Coleopterorum Catalogus*, Heikertinger and Csiki (1940) considered *Blepharida* to include three subgenera: *Blepharida* from tropical Africa and the New World, *Blepharidella*, from East and South Africa, and *Calotheca*, from East and South Africa. Bechyné (1968) elevated these subgenera to generic status and separated Afrotropical *Blepharida* from New World *Blepharida*. In his arrangement, the Afrotropical *Blepharida* species constituted a new genus, *Blepharidina*. He also divided New World *Blepharida* into *Blepharida* and *Notozona*. He proposed that *Blepharida* should consist of only three species (*B. rhois*, *B. irrorata*, and *B. haitiensis*), while all other New World *Blepharida* species should be congeneric with *Notozona*. Seeno and Wilcox (1982) dismissed Bechyné's arrangement and followed Heikertinger and Csiki's three-subgenera treatment, except that they considered *Blepharidina* to be a valid genus. Scherer (1983) in turn, considered *Blepharida* to include *B. rhois* and many of the Afrotropical species, while recognizing that the Neotropical *Blepharida* species were too difficult to separate from *Notozona*. More recently Furth (1992) lowered *Blepharidina* from genus to being a subgenus of *Blepharida*. He also transferred *Blepharida haitiensis* to *Acrocynum*, and argued that *Notozona* is a valid genus that is distinct from *Blepharida*.

The systematics of the New World *Blepharida* are not well known either. Until 1998 only 19 of the current 38 species had been described. Also, finding morphological synapomorphies with a strong phylogenetic signal to divide species within this group has been difficult. In 1997, a phylogeny of 23 *Blepharida* species and varieties was reconstructed under the assumptions of maximum parsimony using the nucleotide variation of the second Internal Transcribed Spacer region (ITS2) of 18S–26S of nuclear ribosomal DNA. This phylogenetic analysis was part of a study directed at understanding the factors that promote host-shifts in herbivorous insects using *Bursera* and *Blepharida* as the study system. For that phylogeny, samples of all *Blepharida* species known to feed on Mexican *Bursera* species were collected from field sites in Mexico. Many of the *Blepharida* species found were new and therefore undescribed. Also, that phylogeny included beetles that were known only from larvae and could not be identified to species.

That study documented the major role that plant chemical similarity has played in *Blepharida*'s host use evolution. Yet, because many of the beetle specimens used were not described or identified, the phylogeny was not as useful for advancing the systematics of the genus. While this problem was partially solved when many of these species were described (Becerra and Venable, 1999a,b; Furth, 1998), more new species have been

found in Mexico during recent studies on *Bursera* herbivory (Evans et al., 2000).

Here, I present a phylogenetic analysis of the New World *Blepharida* that uses nucleotide data of the ITS2 region, as well as the COI/COII region of the mitochondrial genome to infer the relationships within 31 *Blepharida* species and varieties. This study also includes members of 13 other species from related genera, including African *Blepharida* (*Blepharidina*), to gain insight into New World *Blepharida*'s relationships with other groups.

2. Materials and methods

2.1. Taxa examined

Thirty-one species and varieties of New World *Blepharida* were analyzed in this study. I also included five species of African *Blepharida* (*Blepharidina*) and seven species of the allied genera *Notozona*, *Eupectroscelis*, *Podontia*, and *Diamphidia* to help elucidate their relationships with respect to the New World *Blepharida*. A Chrysomelinae species, *Timarcha tenebricosa* was used as outgroup.

All *Blepharida* species included in this study have been described, except five (four known from adults and one only from larvae) that were discovered recently. The unidentified larvae appear to correspond to a monophagous species and its host (*Bursera chaemapodicta*) is not attacked by other *Blepharida* species. Thus, there is a high probability that if *Blepharida* adults are found on this hosts in the future, the sequenced larvae will be of the same species as the adults.

I collected most analyzed taxa in the field (Table 1). Leg tissue of South African *Blepharida vittata*, *B. nigromaculata*, and *B. undetermined* sp. 2, was provided by Catherine Duckett, larvae of *B. rhois* were donated by Frederick Vencl, and *Podontia affinis*' genomic DNA was provided by Brian Farrell. Sequences for *T. tenebricosa* were obtained from NCBI (AJ279769 and AJ236560; Gomez-Zurita et al., 2000a; Gomez-Zurita et al., 2000b). David Furth from the Smithsonian Institution identified the New World *Blepharida*, *Notozona*, *Eupectroscelis* and *Podontia* specimens included in this study. African *Blepharida* species were identified by B. Groobelaar from the National Collection of Insects, South Africa.

2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was isolated from whole bodies or from body parts using a Dneasy Tissue Kit (Qiagen) according to the instructions of the manufacturer. The ITS2 and COI/COII regions were amplified using the polymerase chain reaction (PCR). Typical PCR conditions were an initial denaturation at 94 °C for 2 min,

followed by denaturation at 94 °C for 1 min, annealing at 48–55 °C, depending on the species, and extension at 72 °C for 1 min (ITS2) or 2 min (COI/COII). These amplifications were performed in 50- μ L reaction volumes including 5.0 μ L of 10 \times PCR buffer, 5.0 μ L of 200 μ mol/L dNTPs in an equimolar ratio, 4.0 μ L of 25 mmol/L MgCl₂ solution, 1.0 μ L of 10 pmol of each primer and 2.5 U of Platinum *Taq* polymerase (Life Technologies). ITS2 reactions included the primer ITS3 (GCATCGATGAAGAACGCAGC; Baldwin, 1992) and the primer ITSJ that I designed for these insects (GCTCTGAGGTCGATAAACG). Amplifications of the COI/COII region were done with primers Jerry (mtD-08: CAACATTTATTTTGGATTTTTGG) and Barbara (mtD-18: CCACAAATTTCTGAACATTGACCA; Simon et al., 1994). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced in both directions. Sequencing reactions used the same primers as for amplification and were conducted on an ABI automated sequencer at the DNA sequencing facility of the Biotechnology Division of the University of Arizona.

2.3. Sequence alignment and phylogenetic analyses

Sequences were assembled using the program Sequencher 4.1 for Macintosh (Genes Codes Corporation). Alignments of the ITS2 and COI/COII sets of sequences were performed with ClustalW 1.6 (Thomson et al., 1996). Correction of small misalignments was done with MacClade 4 (Maddison and Maddison, 2000). Two fractions in the ITS2 sequences encompassing 16 and 25 base pairs were difficult to align and were trimmed from the data.

Cladistic analyses were performed using the ITS2 and COI/COII aligned matrices using PAUP 4.08 (Altevec; Swofford, 2000) on three G4 Power Macintosh computers. Because agreement among phylogenies estimated using more than one method can be an index of the reliability of those reconstructions (Kim, 1993), phylogenies were inferred using parsimony, maximum likelihood, and Bayesian methods. Gaps were coded as missing data.

2.3.1. Parsimony analysis (MP)

Heuristic searches were conducted on the ITS2 and COI/COII matrices using 100 random taxon-addition replicates, tree bisection reconnection (TBR) branch swapping, and the MulTrees option in effect. To estimate the support of individual clades, I implemented bootstrap analysis that consisted of 200 bootstrap searches with 10 replicates of random addition, TBR branch swapping and MulTrees in effect. Since a partition homogeneity test (Farris et al., 1994) indicated that the ITS2 and COI/COII data sets were combinable (the null hypothesis of combinability cannot be rejected;

Table 1
Species of *Blepharida* and related taxa included in the analysis

Species location ^a	Collection site(s)	Host (^b)	Voucher
New World <i>Blepharida</i>			
<i>B. alternata</i> Jacoby	Chamela, Jal., Volcan Ceboruco, Nay., Mazatlan, Sin. Xochipala, Gro., Compostela, Nay., Izucar de Matamoros, Pue., Playa de Oro, Col.	<i>Bursera instabilis</i> (1,2,3,5), <i>B. attenuata</i> (1,2,3), <i>B. mirandae</i> (1,2,4), <i>B. submoniliformis</i> (1) <i>B. fragilis</i> (1,5), <i>B. penicillata</i> (1,2), <i>B. palmeri</i> (2), <i>B. chaemapodicta</i> (?), <i>B. bicolor</i> (2), <i>B. cuneata</i> (2), <i>B. excelsa</i> (1)	USNM-Becerra06/18/1993 USNM-Becerra06/30/1993 USNM-Becerra06/16/1993
<i>B. atripennis</i> Horn	La Paz, BCS	<i>Bursera odorata</i> (1,2), <i>B. ruticola</i> (2)	USNM-Becerra09/15/1995
<i>B. balyi</i> Bryant	Chilpancingo, Gro., Salto Valadez, Gro., Tejupilco, Mex. Placeres del Oro, Gro.	<i>Bursera copallifera</i> (1,3,4,5), <i>B. diversifolia</i> (1,5), <i>B. discolor</i> (?), <i>B. bipinnata</i> (1)	USNM-Becerra07/06/1993
<i>B. conspersa</i> (Horn)	La Paz, BCS	<i>Bursera filicifolia</i> (1,2), <i>B. epinnata</i> (1,2), <i>B. hindsiana</i> (1,2,4)	USNM-Becerra07/06/1993
<i>B. flavocostata</i> Jacoby	Temascaltepec, Mex., Tejupilco, Mex., Zapotitlan, Pue., Chilpancingo, Gro., Placeres del Oro, Gro.	<i>Bursera biflora</i> (1,2,5), <i>B. submoniliformis</i> (1,2,5), <i>B. bipinnata</i> (1,2), <i>B. bonetti</i> (2), <i>B. velutina</i> (2), <i>B. hintonii</i> (1,2), <i>B. asplenifolia</i> (1,2,4), <i>B. xochipalensis</i> (?), <i>B. sarukhanii</i> (2)	USNM-Becerra07/24/1993 USNM-Becerra07/03/1993 USNM-Becerra07/12/1995 USNM-Becerra06/03/1993
<i>B. flohri</i> Jacoby	Tejupilco, Mex.	<i>Bursera bipinnata</i> (1,2,5)	USNM-Becerra07/13/1993
<i>B. gabriellae</i> Furth	Infiernillo, Mich., Luvianos, Gro., Bejucos, Mex, Palmillas, Mex.	<i>Bursera trifoliolata</i> (1,2,5), <i>B. fagaroides</i> var. <i>purpusii</i> (1,2,5), <i>B. paradoxa</i> (1,2,3,5), <i>B. fagaroides</i> var. <i>fagaroides</i> (1,2,3,4,5), <i>B. discolor</i> (1,2)	USNM-Becerra06/18/1993 USNM-Becerra07/1992
<i>B. hinchahuevosi</i> Furth	Cuernavaca, Mor.	<i>Pseudosmodium perniciosum</i> (Anacardiaceae) (1)	UA-JXB 2000-048
<i>B. humeralis</i> Furth	Izucar de Matamoros, Pue.	<i>Bursera submoniliformis</i> (1,5)	USNM-Becerra06/18/1993
<i>B. judithae</i> Furth	Salto Valadez, Gro.	<i>Bursera ariensis</i> (1,5)	USNM-Becerra07/15/1992
<i>B. lineata</i> Furth	Placeres del Oro, Gro. Ziquiran, Mich., Acahuato, Mich.	<i>Bursera crenata</i> (1,2,3,4,5), <i>B. trimera</i> (1,3,5), <i>B. denticulate</i> (1,2,3), <i>B. kerberii</i> (1)	USNM-Becerra07/22/1993
<i>B. melanoptera</i> (Fall)	Infiernillo, Mich., Hermosillo, Son.	<i>Bursera infernidialis</i> (1,2), <i>B. laxiflora</i> (1)	USNM-Becerra07/18/1993
<i>B. multimaculata</i> Jacoby	Cuernavaca, Mor.	<i>Bursera fagaroides</i> var. <i>purpusii</i> (1,2,3,5).	USNM-Becerra07/10/1992
<i>B. pallida</i> Blake	Acahuato, Mich. Salto Valadez, Gro., Placeres del Oro, Gro.	<i>B. coyucensis</i> (2), <i>B. grandifolia</i> (1,2,3,4,5)	USNM-Becerra06/05/1996
<i>B. parallela</i> Furth	Zapotitlan, Pue., Tejupilco, Mex.	<i>Bursera schlechtendalii</i> (1,2,3,4,5), <i>B. discolor</i> (?)	USNM-Becerra06/16/1991
<i>B. rhois</i> (Foster)	New York	<i>Rhus glabra</i> (1,2) <i>Rhus</i> spp., <i>Schinus terebinthifolius</i> , <i>Cotinus abovatus</i> (6)	Priv. collection, F. Vencl
<i>B. schlechtendalii</i> Furth	Zapotitlan, Pue.	<i>Bursera schlechtendalii</i> (1,2,3,4,5), <i>B. aptera</i> (1)	USNM-Becerra08/18/1993
<i>B. sonorstriata</i> Furth	Hermosillo, Son.	<i>Bursera laxiflora</i> (1,2,5).	USNM-Becerra08/08/1993
<i>B. sparsa</i> (Clark)	Placeres del Oro, Gro., Acahuato, Mich.	<i>Bursera kerberii</i> (1,2,3,4,5), <i>B. submoniliformis</i> (?)	USNM-Becerra07/22/1992
<i>B. verdea</i> Furth	La Laja, Mor., Iguala, Gro., San Pablito, Pue.	<i>Bursera morelensis</i> (1,2,3,4,5), <i>B. lancifolia</i> (1,2,4), <i>B. rzedowski</i> (1,3,5)	USNM-Becerra06/30/1993
<i>B. xochipala</i> Furth	Xochipala, Gro.	<i>Bursera mirandae</i> (1)	USNM-Becerra06/04/1993
<i>B. unknown</i> sp. 1	Salto Valadez, Gro.	<i>Bursera glabrifolia</i> (1)	UA-JXB2000-0234
<i>B. unknown</i> sp. 2 (only larvae known)	Chilpancingo, Gro.	<i>Bursera chaemapodicta</i> (2)	UA-JXB2000-067L
<i>B. unknown</i> sp. 3	Chilpancingo, Gro.	<i>Bursera vejar-vazquezii</i> (1)	UA-JXB2000-0298
<i>B. unknown</i> sp. 4	Chilpancingo, Gro., Zapotitlan, Pue.	<i>Bursera longipes</i> (1,2), <i>B. biflora</i> (1)	UA-JXB2000-0354
<i>B. unknown</i> sp. 5	Xochipala, Gro.	<i>Bursera xochipalensis</i> (1,2,3,5)	UA-JXB2000-033
African <i>Blepharida</i> (<i>Blepharidina</i>)			
<i>B. nigromaculata</i> Jacoby	Bayala, South Africa	<i>Rhus</i> sp. (1)	NCI-CND2000-056
<i>B. vittata</i> Balyi	Santa Lucia, South Africa	<i>Rhus</i> sp. (1)	NCI-CND2000-078
<i>B. natalensis</i> Jacoby	Pilansburg, South Africa	<i>Rhus lancea</i> (1)	NCI-JXB2000-0136
<i>B. undetermined</i> sp. 1	Hoedspruit, South Africa	<i>Rhus</i> sp. (1), <i>Commiphora</i> sp. (1)	NCI-JXB2000-0135
<i>B. undetermined</i> sp. 2	Cape Town Bot. Gardens, South Africa	<i>Rhigozum ebovatum</i> (Bignoniaceae) (?)	NCI-CDN2000-046
<i>B. undetermined</i> sp. 3	Bakubong, South Africa	<i>Commiphora mollis</i> (1)	NCI-JXB2000-0133
<i>Euplectroscelis</i>			
<i>E. xanti</i> Crotch	La Paz, BCS	<i>Bursera microphylla</i> (1,2)	USNM-Becerra09/15/1995
<i>Notozona</i>			
<i>N. histrionica</i> Chevrolat	Cancun, Qroo.	<i>Bursera simaruba</i> (1,2)	USNM-Becerra07/1996

Table 1 (continued)

Species location ^a	Collection site(s)	Host (b)	Voucher
<i>Podontia</i>			
<i>Podontia affinis</i>	Gröndal	Unknown	
<i>Diamphidia</i>			
<i>D. femoralis</i>	Gerstaecker	Bakubong, South Africa	<i>Sclerocarya birrea</i> (Anacardiaceae) (1) UA-JXB2000-0131
<i>D. nigro-ornata</i>	(Stahl)	Messina, South Africa	<i>Commiphora africana</i> (Burseraeaceae) (1), <i>C. glandulosa</i> (1) UA-JXB2000-0134
<i>D. vitatipennis</i>	Baly	Messina, South Africa	<i>Commiphora tenuipetiolata</i> (1) <i>C. africana</i> (1) UA-JXB2000-0130
<i>Timarcha</i>			
<i>Timarcha tenebricosa</i>	(F.)		

^a UA, University of Arizona Insect Collection; USNM: National Museum of Natural History, Smithsonian Institution; and NCI, National Collection of Insects, South Africa.

^b Evidence for host: (1) one or several adults found on that host; (2) larvae and/or adults observed feeding; (3) host relationship shown in captivity; (4) found on same host at different locations; (5) found on same host species and location on multiple years; and (?) one adult found, equivocal host.

$P = .47$), another analysis was performed with the two joined data sets using the same settings as the separate analyses. Bootstrap analyses were also implemented for the combined data set and consisted of 300 bootstrap searches with 25 replicates of random addition, TBR branch swapping and MULPARS in effect.

2.3.2. Maximum likelihood

Maximum likelihood (ML) is a consistent and robust estimator of phylogeny, often superior to parsimony, as long as an appropriate reconstruction model of evolution is selected (Sullivan and Swofford, 1997). The technique can be particularly inaccurate if they do not appropriately take site-to-site rate variation into account. Therefore, I examined four substitution models, each with four rate-heterogeneity models: Jukes–Cantor (JC; Jukes and Cantor, 1969), Felsenstein (F81; Felsenstein, 1981), Hasegawa–Kishino–Yano (HKY89; Hasegawa et al., 1985), and general time-reversible (GTR; (Yang, 1994a). In addition, four models of among-site rate heterogeneity were examined: (1) equal rates at all sites; (2) a proportion of sites estimated to be invariable, with equal rates at variable sites (I; Hasegawa et al., 1985); (3) rates at all sites assumed to follow a discrete approximation of the gamma distribution (Γ ; Yang, 1994b); and (4) some sites assumed to be invariable, with gamma-distributed rates at variable sites (I + Γ ; Waddell and Penny, 1996). Estimations of model parameters were done on the most parsimonious tree

following observation by Yang et al. (1995) that estimations are fairly insensitive to tree topology.

A likelihood-ratio test (Sullivan and Swofford, 1997; Yang et al., 1995) determined that the GTR + Γ was the model that fitted both the ITS2 and COI/COII data best (considering its number of parameters, Table 2). It was chosen to search for maximum likelihood trees using the combined ITS2 and COI/COII data matrices. This was done by invoking this model in PAUP using parameters estimated for the most parsimonious tree (empirical frequencies A = .304 C = .14, G = .20, T = .31; six substitution types; rate matrix = 0.733, 3.028, 2.24, 0.727, 2.11; gamma distribution of rates at variable sites; alpha shape parameter = .4; rate categories = 4). A heuristic search was performed using simple addition, TBR branch swapping and MulTrees in effect.

2.3.3. Bayesian analysis

Bayesian analysis with Markov Chain Monte Carlo sampling was also carried out with MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) for the combined data set. The analysis was run for 0.5 million generations, with three cold and one heated chains and a burn-in time determined by the time of convergence of the likelihood scores. One in every 100 trees was sampled to guard against autocorrelation. The model chosen was that used for the ML analysis. Clade support was estimated by computing the posterior probabilities of each node across the sampled trees after burn-in.

Table 2

– ln Likelihoods of the most parsimonious tree under sixteen different evolution models, relative to the most parameter model for the ITS2 and COI/COII data matrices

Site-to-site rate variation model	ITS2				COI/COII			
	JC69	F81	HKY85	GTR	JC69	F81	HKY85	GTR
Equal rates	580	527	362	224	1531	1189	1100	841
%I	297	230	162	141	826	405	297	152
Γ	178	101	28	9	688	247	128	8
%I+ Γ	169	91	19	0	679	231	108	0

3. Results

Alignment of the ITS2 sequences resulted in a matrix of 880 characters, of which 408 (46%) were phylogenetically informative under maximum parsimony. The COI/COII matrix included 1464 characters, of which 385 (26%) were informative (GenBank accession numbers for ITS2 sequences: AY268494–AY268553; for COI/COII sequences: AY267869–AY267897).

3.1. Parsimony

Phylogenetic analyses of the ITS2 data matrix resulted in three equally parsimonious trees of 1442 steps (Fig. 1, CI = 0.55, RI = 0.71), while the COI/COII data

set generated six trees of 1614 steps (Fig. 2, CI = 0.47, RI = 0.50). Analysis of the two regions combined yielded six most-parsimonious trees of 3398 steps (Fig. 3, CI = 0.54, RI = 0.70). Bootstrap analysis of the combined data sets indicated that 79% of the individual clades had 50% or higher bootstrap support, 62% had 70% or higher support, and 36% of clades had 90% or higher bootstrap support.

3.2. Maximum likelihood

The heuristic search yielded one single tree with a score of $-\ln L = -17882.179$ (Fig. 4). There are some minor differences between the consensus tree generated under maximum parsimony and maximum likelihood,

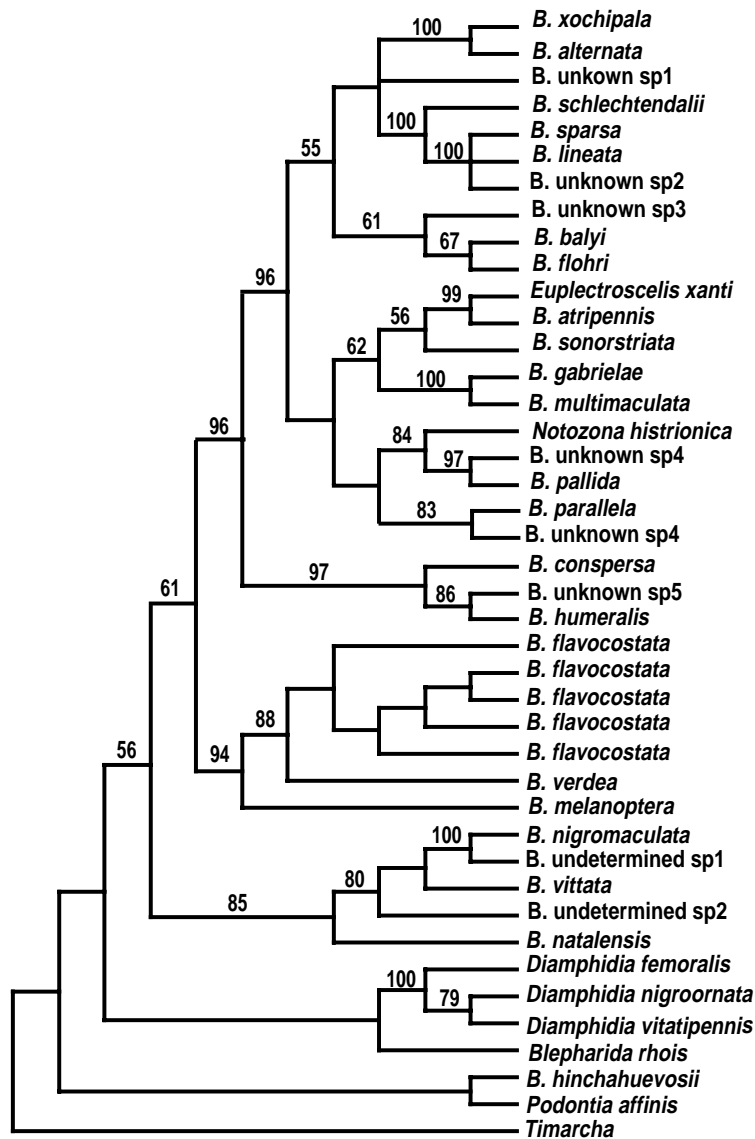


Fig. 1. Consensus tree of three equally most parsimonious trees resulting from analysis of the ITS2 data set. Numbers above branches are bootstrap percentage values for clades supported above a 50% bootstrap value.

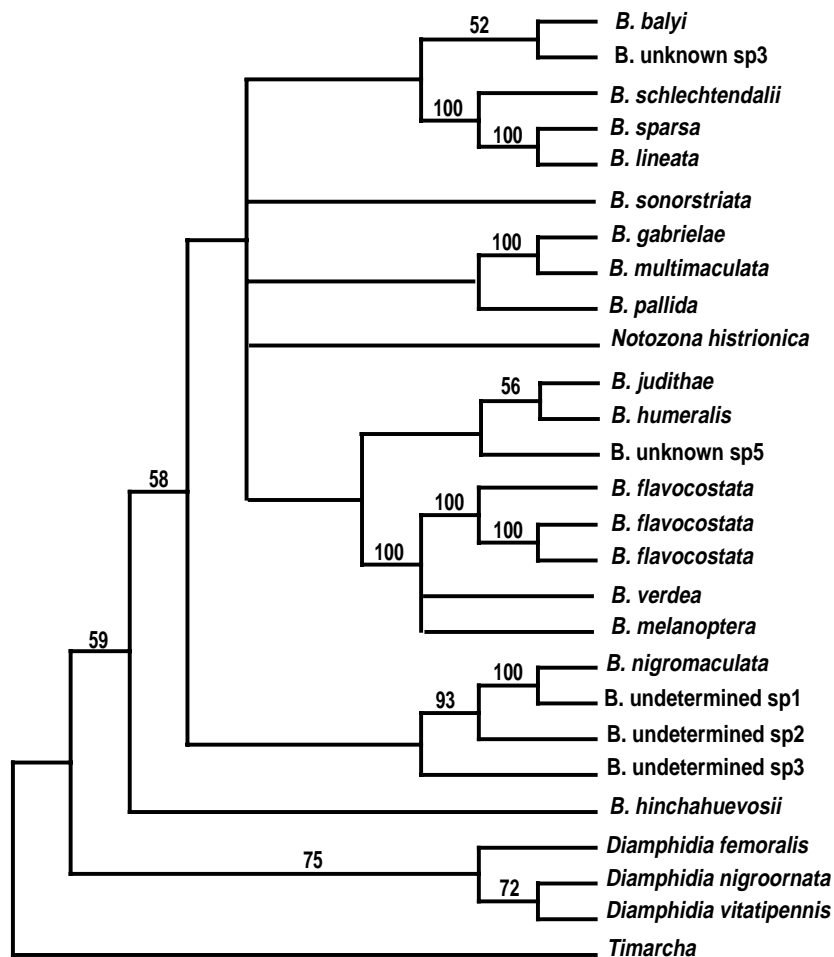


Fig. 2. Consensus tree of six most parsimonious trees generated by analysis of COI/COII sequences. Numbers above branches are bootstrap percentage values for clades supported above a 50% bootstrap value.

but in general, the topologies of these reconstructions are very similar. Maximum likelihood produced the same groups as parsimony for clades that had 50% or greater bootstrap support.

3.3. Bayesian analysis

The majority-rule consensus tree of 4557 sampled trees was almost identical to the MP tree and the ML tree (Fig. 5). This method provided higher support values for the trees and 72% of the nodes had a support of 95% or higher.

4. Discussion

4.1. Relationships between the New World *Blepharida* and related genera

Parsimony, maximum likelihood, and Bayesian analyses produced very similar topologies. The main differences were in the position of *B. balyi*, *B. sonor-*

striata, *B. natalensis*, and *B. hinchahuevosii*. The analyses suggest the existence several well-defined groups. One clade includes all of the New World *Blepharida* species, excepting *B. hinchahuevosii* and *B. rhois*, but includes *Euplectroscelis xanti* and *Notozona histrionica*. Two additional separate clades include the *Blepharidina* and the *Diamphidia* species. One last group, probably the weakest of all, includes *Podontia affinis*, *B. rhois*, and *B. hinchahuevosii*. Of the genera analyzed, *Diamphidia* is the most distantly related to the New World *Blepharida*, while *Blepharidina* constitutes a monophyletic, sister group to *Blepharida*.

The first interesting result is the position of *B. rhois*, the type species, completely outside of the New World *Blepharida* clade. However, this result is not completely unexpected. Bechyné (1968) had previously proposed that the “true” *Blepharida* included only three species, one of them being *B. rhois*, and the other two, *B. irrorata* and *B. haitiensis*. This claim was based on the presence of a transverse series of setiferous punctures along the anterior border of the clypeus and the lack of a transverse depression in the clypeus (Bechyné, 1968).

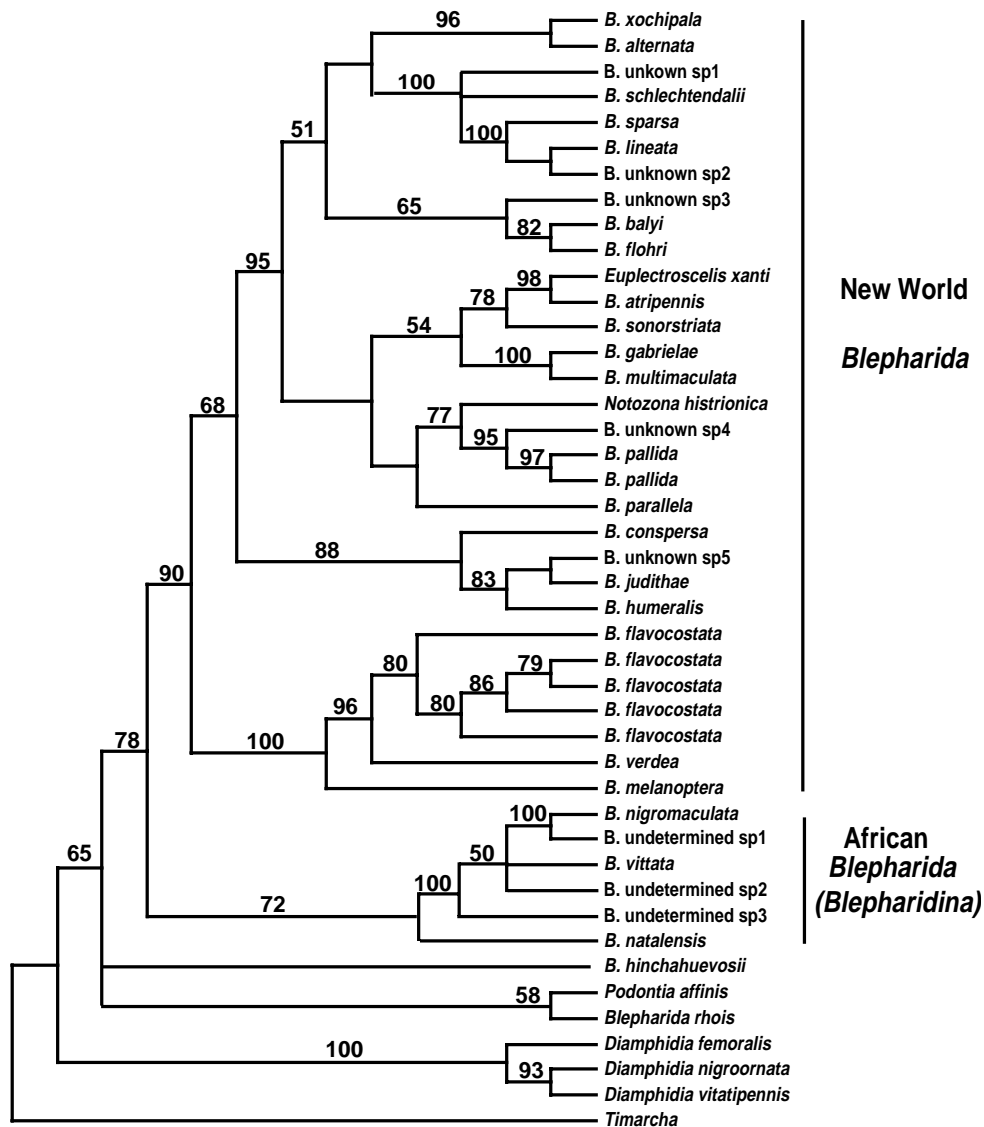


Fig. 3. Phylogeny of *Blepharida* based on maximum parsimony. Consensus tree of six most parsimonious trees resulting from analysis of combined ITS2 and COI/COII data sets. Numbers above branches are bootstrap percentage values for clades supported above a 50% bootstrap value.

B. rhois is also atypical in its geographic distribution which is paleotropical (North America). All of the other New World *Blepharida* species are distributed in Mexico and Central America. In addition, *B. rhois* is one of the very few New World species that feeds on Anacardiaceae instead of *Bursera* (Table 1). The other two Anacardiaceae-feeding species are *B. hinchahuevosii*, which also falls outside the New World *Blepharida* clade in the present analysis and *B. irrorata*, which was not analyzed here. Thus, this molecular analysis supports the position that *B. rhois* should be separated from the New World *Blepharida*.

Another important result is the position of *Notozona histrionica* deep within the New World *Blepharida* clade. Another *Notozona* species recently transferred to *Ble-*

pharida (*B. sparsa*; Furth, 1992) also falls in this clade. Separating New World species of *Blepharida* and *Notozona* on the basis of their morphology is difficult. Traditional dichotomous keys have often included traits that are not consistent for all species, such as the subapical tooth on the dorsal edge of the metatibiae in *Blepharida* and the more swollen metafemora with a ventral tooth in *Notozona*, or the filiform maxillary palps in *Blepharida* versus claviform in *Notozona* (Furth, 1992). Morphological characteristics often useful in separating other related genera, such as the tarsal claws, metaforal spring, and spermateca are quite similar in *Notozona* and *Blepharida*. Furthermore, *Notozona* species also feed on *Bursera* (Flores and Janzen, 1997). This lack of clear and consistent separating traits has been

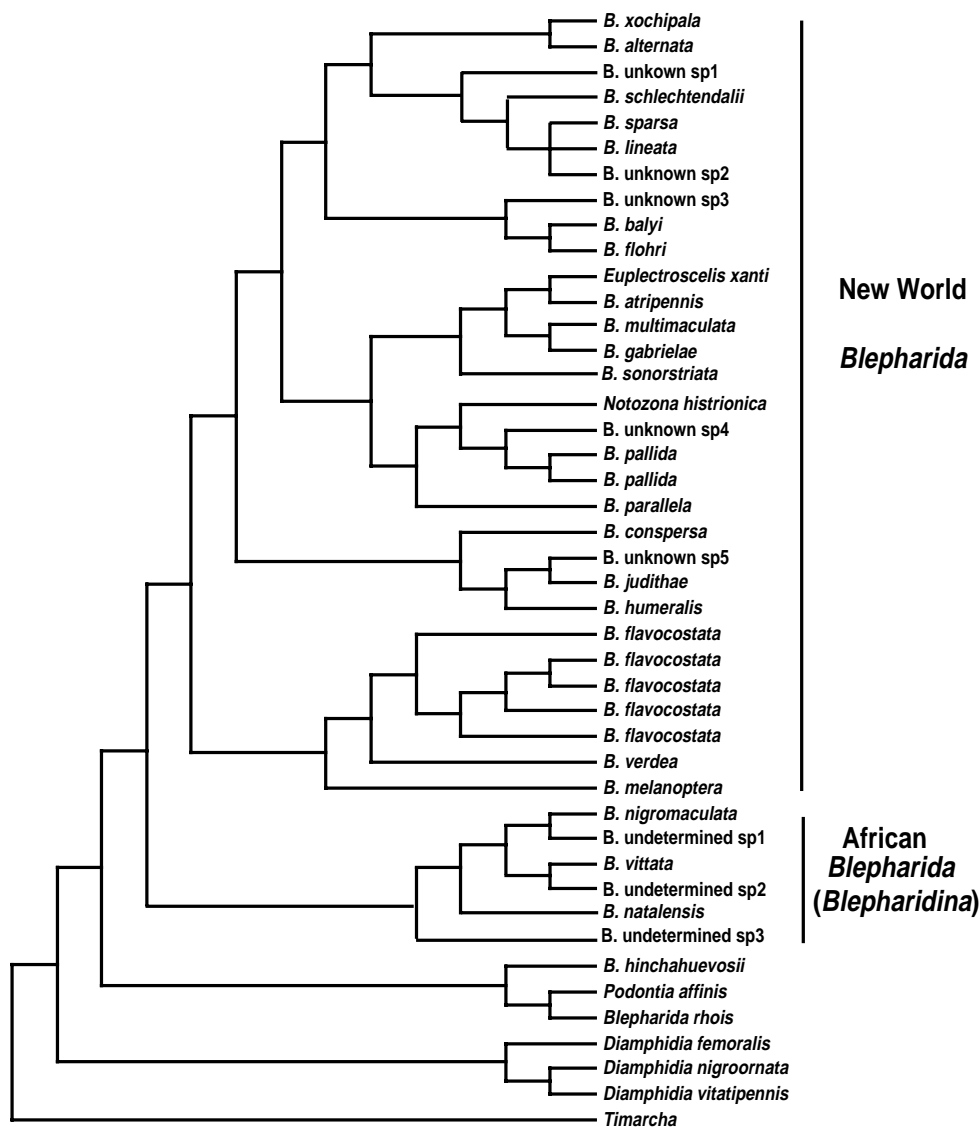


Fig. 4. Phylogeny of *Blepharida* based on maximum likelihood. Tree of highest likelihood found under the GTR + gamma model.

reflected in several species of *Blepharida* been initially described as *Notozona* (Furth, 1992). While my sampling of *Blepharida* was extensive, this analysis includes only one (current) species of *Notozona*. Nevertheless, *N. histrionica* has been used to illustrate the differences with respect to *Blepharida* (Furth, 1992). The fact that in my study *N. histrionica* falls in the *Blepharida* clade is thus concordant with previous arguments that the New World *Blepharida* is congeneric to *Notozona* (Bechyné, 1968).

The monotypic genus *Euplectroscelis* also attaches to the New World *Blepharida* clade. *Euplectroscelis* is endemic to Baja California where it feeds on *Bursera microphylla*. Morphologically and behaviorally it is very similar to *Blepharida* (Furth, 1992). Its position close to other Baja Californian *Blepharida* species in the phylogeny strongly suggests that it is not a separate genus.

4.2. Relationships within New World *Blepharida* species

This new analysis is in general concordant with a previous molecular analysis (Becerra, 1997). According to both, the New World *Blepharida* includes two main clades. The first one includes *B. flavocostata*, *B. verdea*, and *B. melanoptera*. These species have a distinctive combination of two morphological characters: the procoxal cavity is open and there is a deep transverse impression in the clypeus. The second group includes all of the remaining New World *Blepharida* species. With the exception of *B. humeralis*, *B. conspersa*, and the closely related undescribed species 5, all of the species in this group share the characteristic of having the procoxal cavity closed. This group is further divided into three main clades, although support for two of them is not high. As in the past, it is difficult to find

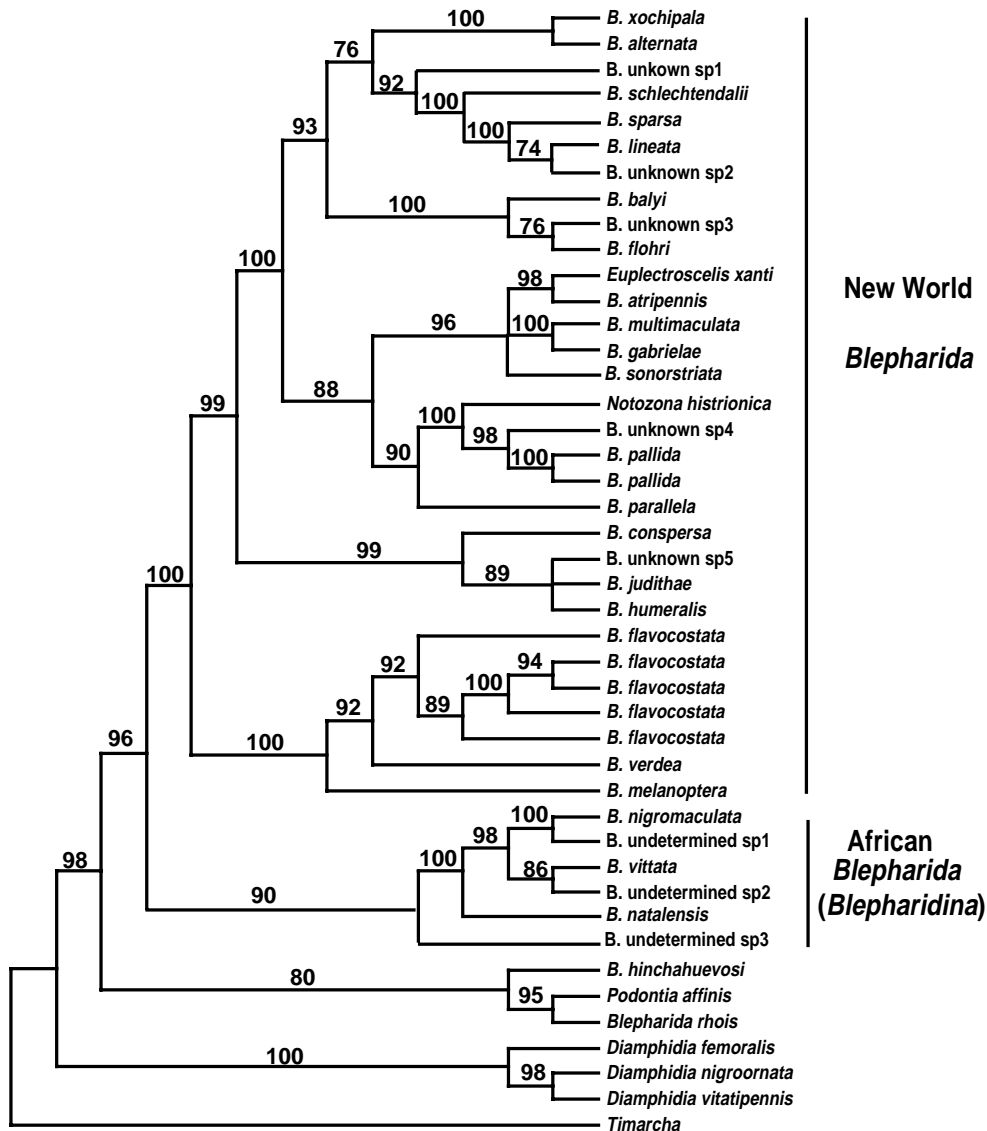


Fig. 5. Majority-rule consensus tree of 4557 sampled trees obtained with Bayesian analysis. Numbers above branches are Bayes posterior probabilities.

morphological synapomorphies supporting these groups and only a few patterns can be discerned. In the most ancestral group, which includes *B. judithae* and *B. humeralis*, species are smaller in size and their elytra are striate. With the exception of *B. judithae*, they have the procoxal cavity open. The vaginal palpi of *B. judithae* and *B. humeralis* are also very similar in shape. The group including *E. xanti* and *B. multimaculata* consists of species with all black or dark elytra with small spots.

Thus, the analysis presented here has some important implications for the systematics and coevolution of *Blepharida*. It suggests that New World *Blepharida* is a monophyletic group that has evolved entirely on *Bursera* hosts and that it has a separate evolutionary history from the African *Blepharida* (*Blepharidina*). However, as

defined here, it includes members of several related genera and excludes the type species.

Acknowledgments

The author thanks C. Duckett, F. Vencl, and B. Farrell for specimen and DNA donations and B. Grobelaar and D. Furth for specimen identifications. Thanks also to L. Venable and to L. de Jager for assistance with field collections in Mexico and South Africa, to M. Kaplan, A. Agellon, and B. Farrell for advise with molecular work, and to W. Moore for help with Bayesian analysis. Research was founded by NSF Grants DEB-9815648 and DEB-0237545. Writing of this work was done while a Sabbatical Fellow at the

National Center for Ecological Analysis and Synthesis, a Center funded by NSF (DEB-0072909), the University of California, and the Santa Barbara campus. This work is dedicated to Leon de Jager, an outstanding naturalist.

References

- Baldwin, B.G., 1992. Phylogenetic utility of the internal transcribed spacer of nuclear ribosomal DNA in plants: an example from the Compositae. *Mol. Phylogenet. Evol.* 1, 3–16.
- Becerra, J.X., 1997. Insects on plants: macroevolutionary chemical trends in host use. *Science* 276, 253–256.
- Becerra, J.X., 1994. Squirt-Gun defense in *Bursera* and the Chrysomelid counterploy. *Ecology* 75, 1991–1996.
- Becerra, J.X., 2003. Evolution of Mexican *Bursera* (Burseraceae) inferred from ITS, ETS, and 5S nuclear ribosomal DNA sequences. *Mol. Phylogenet. Evol.* 26, 300–309.
- Becerra, J.X., 1993. Adaptations to ecological interactions. Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, pp. 176.
- Becerra, J.X., Venable, D.L., 1999a. Macroevolution of insect–plant associations: the relevance of host biogeography to host affiliation. *Proc. Natl. Acad. Sci. USA* 96, 12626–12631.
- Becerra, J.X., Venable, D.L., 1999b. Nuclear ribosomal, DNA phylogeny and its implications for evolutionary trends in Mexican *Bursera* (Burseraceae). *Am. J. Bot.* 86, 1047–1057.
- Becerra, J.X., Venable, D.L., 1990. Rapid-terpene-bath and squirt-gun defense in *Bursera schlehtendalii* and the counterploy of Chrysomelid beetles. *Biotropica* 22, 320–323.
- Becerra, J.X., Venable, D.L., Evans, P.H., Bowers, W.S., 2001. Interactions between chemical and mechanical defenses in the plant genus *Bursera* and their implications for herbivores. *Am. Zool.* 41, 865–876.
- Bechné, J., 1968. Contribution a la fauna du Congo (Brazzaville). Mission A. Villiers et A. Descarpentries. LXXXI. Coleopteres Alticinae. *Bull. Inst. Franc. Afrique Noire* 30, 1687–1728.
- Evans, P.H., Becerra, J.X., Venable, D.L., Bowers, W.S., 2000. Chemical analysis of squirt-gun defense in *Bursera* and counterdefense by chrysomelid beetles. *J. Chem. Ecol.* 26, 745–754.
- Farris, J.S., Källersjö, M., Kluge, A., Bult, C., 1994. Testing significance of incongruence. *Cladistics-Int. J. Willi Hennig Soc.* 10, 315–447.
- Felsenstein, J., 1981. Evolutionary trees from DNA-sequences—a maximum-likelihood approach. *J. Mol. Evol.* 17, 368–376.
- Flores, R.W., Janzen, D.H., 1997. Feeding records of Costa Rican leaf beetles (Coleoptera: Chrysomelidae). *Fla. Entomol.* 80, 334–366.
- Frost, S.W., 1973. Hosts and eggs of *Blepharida dorothea* (Coleoptera: Chrysomelidae). *Fla. Entomol.* 52.
- Furth, D.G., 1982. *Blepharida* biology, as demonstrated by the sacred sumac flea beetle *B. sacra* (Weise) (Coleoptera: Chrysomelidae: Alticinae). *Spixiana* 7, 43–52.
- Furth, D.G., 1989. Metaphorical spring studies of some Neotropical genera of Alticinae. *Entomography* 6, 497–510.
- Furth, D.G., 1992. The New-World *Blepharida* Group, with a key to genera and description of a new species (Coleoptera, Chrysomelidae). *J. N.Y. Entomol. Soc.* 100, 399–414.
- Furth, D.G., 1998. The New World *Blepharida* Chevrolat 1836 (Coleoptera: Chrysomelidae). *Mem. Ent. Soc. Wash.*
- Furth, D.G., Young, D.A., 1988. Relationships of herbivore feeding and plant flavonoids (Coleoptera, Chrysomelidae and Anacardiaceae, Rhus). *Oecologia* 74, 496–500.
- Gomez-Zurita, J., Juan, C., Petitpierre, E., 2000a. The evolutionary history of the genus *Timarcha* (Coleoptera, Chrysomelidae) inferred from mitochondrial COII gene and partial 16S rDNA sequences. *Mol. Phylogenet. Evol.* 14, 304–317.
- Gomez-Zurita, J., Juan, C., Petitpierre, E., 2000b. Sequence, secondary structure and phylogenetic analyses of the ribosomal internal transcribed spacer 2 (ITS2) in the *Timarcha* leaf beetles. *Ins. Mol. Biol.* 9, 591–604.
- Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human–ape spitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 21, 160–174.
- Heikertinger, F., Csiki, C., 1940. *Coleoptera catalogus*, Chrysomelidae: Halticinae. pars 169, 335–635.
- Hillis, D.M., 1997. Biology recapitulates phylogeny. *Science* 276, 218–219.
- Huelsenbeck, J.P., Ronquist, F.R., 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17, 754–755.
- Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), *Mammalian Protein Metabolism*. Academic Press, New York.
- Kim, J., 1993. Improving the accuracy of phylogenetic estimation by combining different methods. *Syst. Biol.* 42, 331–340.
- Koch, C., 1958. Preliminary notes on the coleopterological aspect of the arrow poison of the bushmen. Pamphlet S. Afr. Biol. Soc. 20, 49–54.
- Maddison, D.R., Maddison, W.P., 2000. MacClade—Analysis of Phylogeny and Character Evolution. Sinauer, Sunderland.
- Mitchell-Olds, T., Bergelson, J., 2000. Biotic interactions—Genomics and coevolution—Editorial overview. *Curr. Opin. Plant Biol.* 3, 273–277.
- Scherer, G., 1983. Diagnostic key for the Neotropical Alticinae genera. *Entomol. Arbeiten Mus. G. Frey* 31, 1–89.
- Seeno, T.N., Wilcox, J.A., 1982. Leaf beetle genera (Coleoptera: Chrysomelidae). *Entomography* 1, 1–221.
- Simon, C.F., Frati, A., Beckenbach, B., Crespi, H., 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.
- Sullivan, J., Swofford, D.L., 1997. Are guinea pigs rodents? The importance of adequate models in molecular phylogenetics. *J. Mamm. Evol.* 4, 77–86.
- Swofford, D.L., 2000. PAUP*: phylogenetic analysis using parsimony (*and other methods). Sinauer, Sunderland.
- Takizawa, H., 1978. Notes on Taiwanese Chrysomelid larvae. *V. Ent. Rev. Jpn.* 31, 75–84.
- Terrazas, T., Chase, M., 1996. A phylogenetic analysis of Anacardiaceae based on morphology and rbcL sequence data. *Am. J. Bot.* 83, 1977–1983.
- Thomson, J.D., Higgins, D.G., Gibson, T.J., 1996. Clustal W. 16.
- Waddell, P.J., Penny, D., 1996. Evolutionary trees of apes and humans from DNA sequences. In: Lock, A.J., Peters, C.R. (Eds.), *Handbook of Symbolic Evolution*. Clarendon Press, Oxford.
- Weise, J., 1897. Eine neue *Podontia aus Jericho*. *Weiner Ent. Zeit.* 16, 81–82.
- Wicox, J.A., 1975. Checklist of the Chrysomelidae of Canada, United States, Mexico, Central America, and the West Indies. Family No. 104 N. Am. Beetle Fauna Proj.
- Yang, Z., 1994a. Estimating the pattern of nucleotide substitution. *J. Mol. Evol.* 39, 105–111.
- Yang, Z., 1994b. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J. Mol. Evol.* 39, 306–314.
- Yang, Z., Goldman, N., Friday, A.E., 1995. Maximum likelihood trees from DNA sequences: a peculiar statistical estimation problem. *Syst. Biol.* 44, 384–399.