Molecular Phylogeny of the North American *Enchenopa binotata* (Homoptera: Membracidae) Species Complex

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ABSTRACT The North American *Enchenopa binotata* (Say) species complex is a model of sympatric speciation in which phytophagous insects are hypothesized to diverge through host-plant specialization resulting from changes in host plant usage that alter life history timing. A robust phylogeny is needed to evaluate the historical relevance of the prediction that sister taxa differ in critical life-history traits. Phylogenetic analysis using parsimony and likelihood criteria of 2305 nucleotides in sequences from mitochondrial COI, COII, tRNA-Leucine, and 12S genes revealed two pairs of sister taxa. Both pairs of sister taxa differ from each other in the timing of egg hatch in the spring that is mediated by differences in host-plant phenology. Host plant mediated timing of egg hatch results in asynchronous life histories among sister taxa facilitating reproductive isolation. Sister taxa of *Enchenopa* from *Celastrus* and from *Viburnum* differ in their diurnal and temporal spans during which mating occurs. Mating of *Enchenopa* from *Liriodendron* takes place after that of its sister species on *Cercis.* These results support the hypothesis that speciation could have been initiated through a shift to a host plant that alters life-history timing.

KEY WORDS Homoptera, *Enchenopa*, speciation, sympatric

CLADES OF SPECIES are interrelated through historical, genealogical, and geographical connections that influence how extant species complexes respond to evolutionary processes through time (Hillis 1997). Thus, the historical phylogenetic context of a species or its relationship within a clade is essential to interpreting the results of comparative studies dealing with evolutionary processes (Brooks and McLennan 1991, Brooks et al. 1995). One of the fundamental underlying processes of evolutionary biology is speciation. This is a difficult area because definitions of species are varied and often reflect underlying assumptions concerning the mode of speciation (Templeton 1989). For example, the historical debate over whether geographical isolation is a requisite for speciation or not has been controversial for many years (Mayr 1982). Many of the examples of purported sympatric speciation are difficult to evaluate from a phylogenetic perspective because organisms such as the host races of *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae) have not achieved species status regardless of definition (Bush 1969). In others, such as the *Enchenopa binotata* (Say) species complex, each species is demographically divergent and reproductively cohesive making cause and effect difficult to interpret (Wood 1993). Species in this complex not only have well-developed host plant preference/philopatry during mating and oviposition but also have been experimentally demonstrated in choice tests to mate by species. Females experimentally transferred during oviposition to inappropriate host plants do not successfully produce offspring (Wood 1980; Wood and Guttman 1982, 1983). Other differences such as substrate-borne mating signals, morphological and color pattern differences among nymphs have also been demonstrated (Wood 1980, Pratt and Wood 1992, Hunt 1994). However, discrete adult morphological differentiation in this complex of species has not developed (Pratt and Wood 1993). The challenge for species complexes like *E. binotata* is to find sufficient phylogenetically informative characters to provide a robust analysis of relationships within the clade to evaluate mechanisms of speciation.

The *E. binotata* species complex is a model of sympatric speciation in which phytophagous insects are hypothesized to diverge through host-plant specialization resulting from changes in host-plant usage (Wood 1980; Wood and Guttman 1981, 1982, 1983; Wood et al. 1990; Wood and Keese 1990; Wood 1993; Tilmon et al. 1998; Wood et al. 1999). The hypothesized speciation mechanism is that asynchronous mating is induced by differences in plant phenology which, interacting with philopatry during reproduction, allows divergence in host-plant associated performance traits (Wood et al. 1990; Wood and Keese

Nomenclatural acts or proposed reclassifications inferred in this article are not considered published within the meaning of the 1985 International Code of Zoological Nomenclature (Article 8b). This work will be published elsewhere in accordance with article eight of the Code.

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Table 1. Host plants of the *Enchenopa binotata* species complex (Wood 1993)

Genus	Species	Family
Ptelea	*trifoliata* (L.)	Rutaceae
Juglans	*nigra* (L.)	Juglandaceae
Juglans	*cinerea* (L.)	Juglandaceae
Carya	*illinoensis* (Wang) K. Koch	
	ovalis (Wang) Sarg.	
	cordiformis (Wang) K. Koch	
	laciniosa (Michx.) Loud.	
	ovata (Mill.) K. Koch	
Celastrus	*scandens* (L.)	Celastraceae
Liriodendron	*tulipifera* (L.)	Magnoliaceae
Robinia	*pseudoacacia* (L.)	Leguminosae
Cercis	*canadensis* (L.)	Leguminosae
Viburnum	*cassinoides* (L.)	Caprifoliaceae
	rufidulum (Raf.)	
	lentago (L.)	
	prunifolium (L.)	

1990; Wood 1993; Tilmon et al. 1998; Wood et al. 1999). The North American *E. binotata* species complex is comprised of nine species associated with eight plant genera distributed among six plant families (Table 1). Because the species in this complex have not been formally named, we refer to them by their host-plant genus or by host-plant species as in the case of the two species using *Juglans nigra* (L.) and *J. cinerea* (L.) (see footnote for nomenclatural disclaimer). The *E. binotata* species on *Carya* and *Viburnum* are polyphagous in that they occur on several species within their respective plant genera but the remaining species appear to be monophagous (Wood 1993). The *Enchenopa* species and their host plants are sympatric throughout the eastern United States (Wood 1993) (Fig. 1). There is substantial overlap of the distribution of *Enchenopa* species with that of its host plant (Little 1971).

In North America, the univoltine *E. binotata* species begin their life history in the spring when overwintered eggs hatch synchronously within a 10 d period on their host-plant. Nymphs mature to the adult stage ≈1 mo after egg hatch and males eclose 1–2 d before females. Mating begins ≈1 mo after adult eclosion. Males can mate several times, whereas females mate once (Wood 1993). Oviposition begins ≈1 mo after the onset of mating. Females insert eggs into woody stems, cover eggs with froth, and deposit multiple egg masses throughout the fall until mid-November (Wood and Patton 1971). Eggs of each of the nine *Enchenopa* species hatch at different times in the spring as a result of the differences in changes of water content among host plants (Wood et al. 1990). Asynchrony of egg hatch subsequently results in asynchrony of time of adult eclosion, mating and oviposition among *Enchenopa* species (Wood and Keese 1990). The consequence of life history coordinated with plant phenology is that the nine *Enchenopa* on phenologically different host plants have asynchronous life histories within the same locality across their geographic range. For an *Enchenopa* species, there are also latitudinal and longitudinal gradients in life history timing (Wood 1993).

The genus *Enchenopa* is placed within the tribe Membracini in the subfamily Membracinae on the basis of morphology (Metcalf and Wade 1965, Deitz 1975, McKamey 1998). There is debate over whether the tribe Membracini is monophyletic and its relationship to the other tribes within the subfamily (Dietrich and McKamey 1995). Because pronotal shape is a primary character for distinguishing genera, *Enchenopa* is not well delineated from other genera such as *Campylenchia* and *Enchophyllum.* Thus, it is possible that many species presently assigned to *Enchenopa, Campylenchia* and *Enchophyllum* are misplaced. Although adults of the nine species in the *E. binotata* complex differ in body length and pronotal shape, the genitalia (Pratt and Wood 1993) and other morphological features do not provide diagnostic characters suitable for the development of a rigorous phylogenetic hypothesis. Attempts have been made to understand relationships within the *E. binotata* species complex using allozymes, female pronotal shape and nymphal characters (Wood 1993).

Relationships within the *E. binotata* species complex were first inferred using allozyme data (Guttman et al. 1981, Wood 1993) using *Campylenchia latipes* (Say) as an outgroup to derive a distance matrix. There is considerable debate on whether allozymes provide appropriate information reflecting evolutionary history (Swofford et al. 1996). The two major concerns are whether or not to transform allozyme data to a genetic distance and how evolutionarily important is the presence/absence of alleles versus the frequency of alleles (Mickevich and Johnson 1976, Swofford and Berlocher 1987). Unless sample sizes are large, taxa that are in reality polymorphic for some alleles may be scored as fixed if one allele is rare. The same holds true if only a few populations within a species are sampled since the frequency of an allele can vary dramatically among populations over a geographic range (Swofford and Berlocher 1987). In the allozyme study of the *E. binotata* complex, sample size and number of localities appear to be adequate to counter these objections, but a distance matrix cannot be analyzed by modern character-based cladistic analysis, and homology assessments remain controversial. The only cladistically based analysis is a phylogeny using seven first and 24 fifth-instar nymphal characters (Pratt and Wood 1992).

In the above studies (Pratt and Wood 1992, Wood 1993), *C. latipes* was used as an outgroup because it was the only related North American genus (Metcalf and Wade 1965) where adequate fresh material was available for allozyme and nymphal character analysis. The phylogenetic relationship of *C. latipes* to the North American *E. binotata* complex is unknown and its use as an outgroup subjective. Regardless of the diversity of approaches, the results of previous studies are in general agreement that the *Enchenopa* on *Robinia, Liriodendron,* and *Carya* are basal, whereas *Enchenopa* on *Cercis, Ptelea, Celastrus,* and *Viburnum* are more apical (Wood 1993). *Enchenopa* on *J. nigra* and *J. cinerea* and those on *Ptelea* and *Celastrus* appear to be two sister groups but there are disagreements

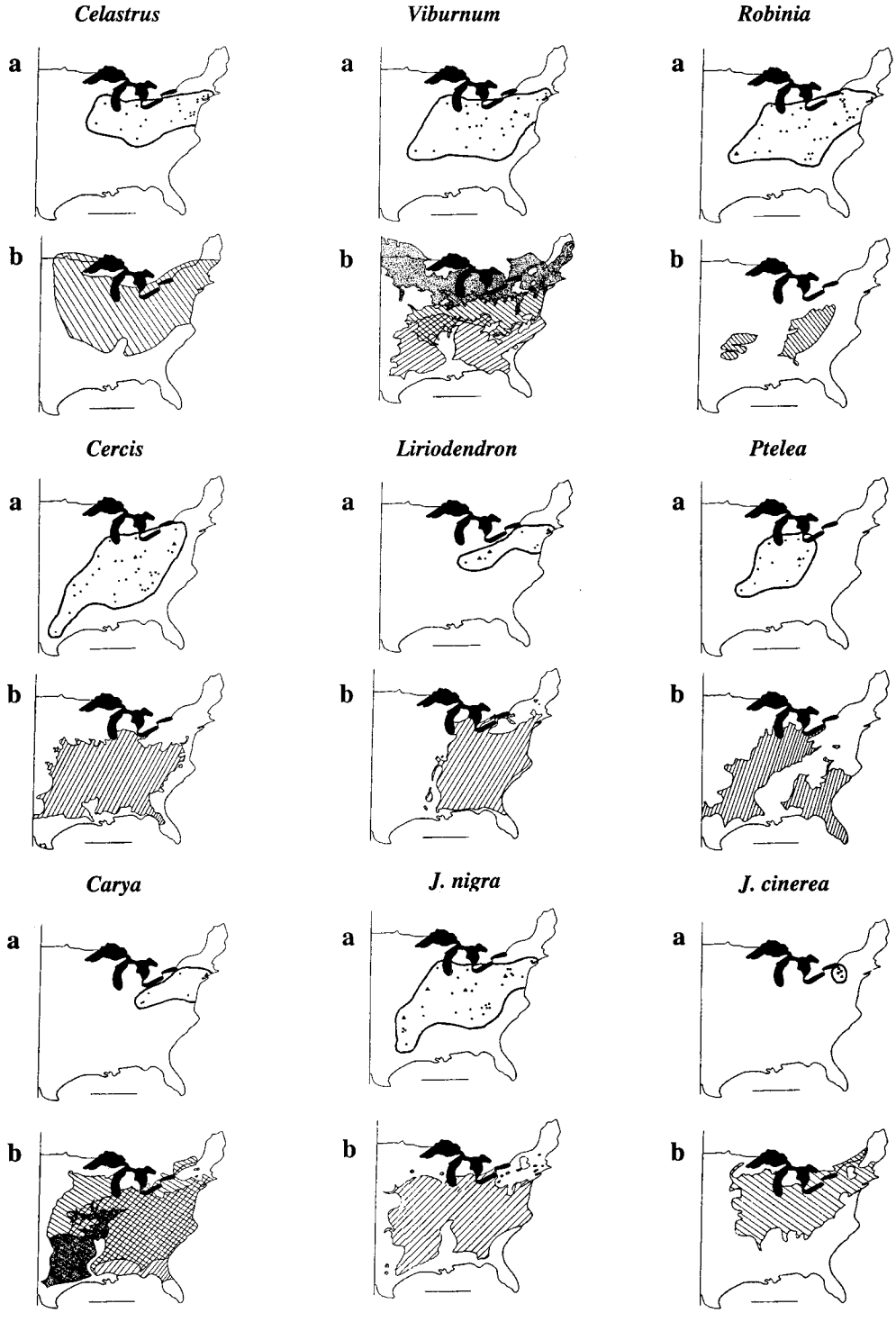

Fig. 1. Distribution of the North American *Enchenopa* species and their respective host plants. Distribution of *Enchenopa* species (a) was redrawn from line drawings of Pratt et al. (unpublished data). Additional collecting records of *Enchenopa* species are from Guttman and Weigt (1989) and T.K.W. (unpublished data). Distribution of the host plant of an *Enchenopa* species is presented in (b), which has been redrawn from Little (1971).

Table 2. Locality data of specimens examined

Species	Locality	Date	Collector	Code
Campylenchia latipes	Newark, DE	30/8/95	T. Wood	LCL
E. binotata on *Carya*	Cecil County, MD	26/8/96	C. P. Lin	LE24-1
E. binotata on *Carya*	Cecil County, MD	29/6/96	M. Adams/C. P. Lin	E14-1
E. binotata on *Carya*	Ottawa County, OK	3/9/96	M. Adams	E27
E. binotata on *Celastrus*	Cecil County, MD	25/8/96	C. P. Lin	LE15-2
E. binotata on *Celastrus*	Cecil County, MD	25/8/96	C. P. Lin	LE15-3
E. binotata on *Celastrus*	Cecil County, MD	25/8/96	C. P. Lin	LE15-4
E. binotata on *Cercis*	Newark, DE	24/8/96	C. P. Lin	LE20-1
E. binotata on *Cercis*	Wilmington, OH	18/6/96	K. Tilmon/T. Wood	E3-1
E. binotata on *Cercis*	Wilmington, OH	18/6/96	K. Tilmon/T. Wood	E3-2
E. binotata on *J. cinerea*	Ithaca, NY	16/6/96	K. Tilmon/T. Wood	E4
E. binotata on *J. cinerea*	Ithaca, NY	16/6/96	K. Tilmon/T. Wood	E4-2
E. binotata on *J. cinerea*	Ithaca, NY	16/6/96	K. Tilmon/T. Wood	E4-3
E. binotata on *J. nigra*	Ithaca, NY	6/7/96	K. Tilmon/T. Wood	LE12-2
E. binotata on *J. nigra*	Ithaca, NY	6/7/96	K. Tilmon/T. Wood	E12-2
E. binotata on *J. nigra*	Ithaca, NY	6/7/96	K. Tilmon/T. Wood	E12-3
E. binotata on *Liriodendron*	Cecil County, MD	8/25/96	C. P. Lin	LE22-1
E. binotata on *Liriodendron*	Cecil County, MD	6/29/96	M. Adams/C. P. Lin	E10-1
E. binotata on *Liriodendron*	Cecil County, MD	6/29/96	M. Adams/C. P. Lin	E10-2
E. binotata on *Ptelea*	Wilmington, OH	6/18/96	K. Tilmon/T. Wood	LE6-1
E. binotata on *Ptelea*	Wilmington, OH	6/18/96	K. Tilmon/T. Wood	E6-2
E. binotata on *Ptelea*	Wilmington, OH	6/18/96	K. Tilmon/T. Wood	E6-3
E. binotata on *Robinia*	Ithaca, NY	6/16/96	K. Tilmon/T. Wood	E26
E. binotata on *Robinia*	Ithaca, NY	6/16/96	K. Tilmon/T. Wood	E2-1
E. binotata on *Robinia*	Ithaca, NY	6/16/96	K. Tilmon/T. Wood	E2-2
E. binotata on *Viburnum*	Newark, DE	8/25/96	C. P. Lin	E23
E. binotata on *Viburnum*	Newark, DE	8/25/96	C. P. Lin	K7
E. binotata on *Viburnum*	Newark, DE	8/25/96	C. P. Lin	K8
Enchenopa sp. 1	Panama City, Panama	2/7/98	R. Cocroft	ENCA
Enchenopa sp. 2	Guanacaste, Costa Rica	5/7/96	R. Cocroft	LE31-1

among trees in the placement of *Enchenopa* on *Liriodendron.*

Recently Liu (1996) examined five individuals of each of two treehopper species (*Atymna querci* Fitch and *E. binotata*) using partial sequences of mitochondrial cytochrome oxidase II (COII) gene. Only one nucleotide in five *A. querci* individuals and two nucleotides in five *E. binotata* individuals were found to differ (intraspecific variations are equal or $<0.3\%$) among 379 nucleotides in each sequence. In addition to low intraspecific variation, Liu's study (1996) demonstrated that interspecific sequence differences exist in the partial COII gene between these two species of treehoppers suggesting the mitochondrial COI and COII genes could be a source of phylogenetic characters to resolve the nine *E. binotata* species.

Compared with mitochondrial protein coding genes such as COI and COII, the small subunit ribosomal gene (12S), which has a critical role in protein assembly, evolves more slowly as a result of its structural conservation (Simon et al. 1994). Preliminary work showed that partial sequences of 12S were phylogenetically useful for tribal levels in treehoppers (Liu 1996) and could be used to determine the placement of the nine *Enchenopa binotata* species within the tribe Membracini of the subfamily Membracinae, and to facilitate the selection of closely related species or genera for outgroups.

A host-shift field experiment to directly test the assumptions of the sympatric speciation hypothesis is in progress, but a robust phylogeny is necessary to evaluate the historical relevance of this mechanism to the extant North American *E. binotata* species complex. The objectives of this study are as follows: (1) to determine whether partial DNA sequences for four mitochondrial genes provide sufficient phylogenetically informative characters to infer a phylogeny, (2) to determine monophyly of the North American *E. binotata* species complex, and (3) to determine the concordance between phylogeny and a host shift hypothesis of speciation.

Materials and Methods

Intraspecific Variation. Before DNA sequences can be used for phylogenetic analysis of a cryptic species complex, it is important to determine whether nucleotide characters are polymorphic among different individuals within a species. Therefore, for each of the partial sequences of the three genes, COI (397 bp), COII (357 bp), and 12S (339 bp), we sampled three individuals from each of nine North American *E. binotata* species to determine if intraspecific variation exists within each species (total 27 individuals or 81 sequences).

Outgroup Taxa. To choose appropriate outgroup taxa to polarize the characters among the nine species of *E. binotata*, 31–63 treehopper taxa representing five tribes in the subfamily Membracinae and *Centrodontus atlas* (subfamily Centrodontinae) were sequenced for the small COI (391 bp), COII (347 bp), and 12S (347 bp) fragments (Lin 2000). Parsimony analyses of these data showed two Central American *Enchenopa* species (*Enchenopa* sp.1 and *Enchenopa* sp. 2, Table 2) are the most closely related taxa to the North American

Table 3. Oligonucleotide primers

Name	Position[a]	Sequence
Ron[b] (C1-J-1751)	1729	5′ GGATCACCTGATATAGCATTYCC 3′
Nancy[b] (C1-N-2191)	2216	5′ CCCGGTAAAATTAAAATATAAACTTC 3′
Dick[b] (C1-J-2441)	2410	5′ CCAACAGGAATTAAAATTTTTTAGATGATTAGC 3′
Rick[b] (C1-J-2441)	2410	5′ CCAACAGGAATTAAAGTTTTTTAGATG 3′
Calvin[c]	2725	5′ GGRAARAAWGTTAARTTWACTCC 3′
A-298[d] (C2-J-3400)	3380	5′ ATTGGACATCAATGATATTGA 3′
Barb[b] (C2-N-3661)	3684	5′ CCACAAATTTCTGAACATTGACCA 3′
B-tLYS[d] (TK-N-3785)	3804	5′ GTTTAAGAGACCAGATACTTG 3′
12Sbi[e] (SR-J-14233)	14214	5′ AAGAGCGACGGGCGATGTGT 3′
12Sai [e] (SR-N-14588)	15179	5′ AAACTAGGATTAGATACCCTATTAT 3′

The standardized primer names are in parentheses (Simon et al. 1994).
[a] Position number refer to 5′ end of primer sequence in *Drosophila yakuba* (Clary and Wolstenholme 1985).
[b] Designed by Harrison Laboratory at Cornell University.
[c] Designed by C. Keeler at the University of Delaware.
[d] Designed by Liu and Beckenbach (1992).
[e] Designed by Kocher et al. (1989).

Enchenopa species complex and that *C. latipes* is an appropriate outgroup (Lin 2000).

After an outgroup was chosen, COI and COII fragments that encompass an additional 822 bp in COI, 69 bp in t-RNA-Leucine and an additional 335 bp in COII were sequenced for both outgroup and the ingroup *Enchenopa* species complex. We analyzed the North American *E. binotata* species using a "total evidence" approach by reconstructing the phylogeny with all available DNA sequences.

Specimen Treatment. Specimens were collected as adults or nymphs at various localities in North and Central America (Table 2). Field-collected treehoppers were immediately preserved in 95% ethanol, followed by long-term storage at −20°C. DNA extraction followed protocols outlined in Danforth (1999), with the remainder of the specimen preserved as vouchers in 95% ethanol at −20°C.

Primers. Initially the small partial mitochondrial COI, COII, and small ribosomal subunit gene (12S) fragments were amplified via polymerase chain reaction (PCR) with three sets of primers (see Table 3 for primer sequences and locations). Ron-Nancy primers produced a PCR product of ≈400 bp in the COI gene. A combination of A-298 and B-LYS primers produced a PCR product of nearly 350 bp between the 3′ half of COII and tRNA-Lys gene. The 12Sbi and 12Sai primers produced a PCR product of ≈330 bp in 12S gene. Once appropriate outgroups were determined another two sets of PCR products of ≈1,000 and 1,200 bp in the COI, tRNA-leucine, and COII regions were amplified for *Enchenopa* and *Campylenchia* using the new primer combinations of Ron-Calvin and Rick (Dick)-Barb.

Sequencing Protocols. A Perkin-Elmer thermal cycler (GeneAmp PCR System 2400, Foster City, CA) was used for double-stranded amplifications of the COI, COII, and 12S gene. The cycling profile began with one cycle of DNA denaturation at 94°C for 2 min and followed by 35–45 cycles of sequence amplification (DNA denaturation at 94°C for 30 s, primer annealing at 50–53°C for 30 s and sequence extension at 72°C for 1 min). The PCR products were purified by a gel purification method provided by J. McDonald (University of Delaware). Sequences were obtained from both sense and antisense strands using the Applied Biosystems 373A DNA sequencer (Foster City, CA). The chromatograph of each sequence was first examined using the 373 DNA Data Analysis Program (Foster City, CA) to determine the quality of each sequence and subsequently edited in SeqEd (version 1.0.3 Applied Biosystems 1992) by manually comparing the aligned chromatograph of both sense and antisense strands to confirm ambiguous bases. Sequences used in this study can be obtained from GENBANK (accession number AY057846-AY057857).

Sequence Alignment. DNA sequences of each species were transferred to Editseq files and aligned with EDITSEQ and MEGALIGN programs in Lasergene (DNASTAR, Madison, WI). The Clustal method in MEGALIGN was used with the pairwise alignment parameter Ktuple set to 2. For COI and COII protein coding genes, the multiple alignment parameter gap penalty was set to 100 to minimize gap formation. Sequence alignment for protein-coding gene sequences like COI and COII was straightforward because codon reading frames could be determined by alignment with *Drosophila yakuba* (Burla) (Clary and Wolstenholme 1985). Alignment of ribosomal gene sequences of distantly related species may be difficult because of insertion and deletion events. For *Enchenopa* species, the sequence alignments of both protein-coding and ribosomal genes are relatively unambiguous because of low sequence divergence. For the 12S ribosomal gene, sequences were manually aligned with reference to the secondary structure of the third domain using Cicadidae as a model (Kjer 1995, Hickson et al. 1996). Each data matrix was subsequently saved as MEGALIGN and PAUP (NEXUS format) files. Combining data matrices from different sequence fragments was done using MacClade (version 3.05, Maddison and Maddison 1992).

Phylogenetic Analysis. Maximum parsimony and likelihood analyses were done by using PAUP 4.0.0 d64 (Swofford 1998). As a result of outgroup analyses, two Central American *Enchenopa* species were included in the ingroup and *C. latipes* was chosen as outgroup (Lin

Table 4. Nucleotide composition of 2305 bp of COI, COII, t-RNA-Leucine and 12S of *Enchenopa binotata* species complex and outgroup taxon

Gene	Codon	A	C	G	T	A+T	Chi-square	*P*-value
COI	nt1	30.7	12.9	24.1	32.3	63	2.8	1
	nt2	19.5	21.5	15.8	43.2	62.7	0.71	1
	nt3	42.5	7	4.2	46.2	88.7	29.35	0.65
	Overall	30.9	13.8	14.7	40.5	71.4	9.38	0.99
COII	nt1	39.2	11.3	10.5	38.9	78.1	3.1	1
	nt2	27.8	17.9	10.5	43.7	71.5	0.73	1
	nt3	45.9	5.7	3.2	45.2	91.1	23.26	0.89
	Overall	35.9	12.9	10.4	40.7	76.6	4.11	1
COI+COII	nt1	32.7	13.5	13.2	40.6	73.3	6.97	0.99
	nt2	22.4	20.2	13.9	43.4	66.1	0.54	1
	nt3	43.7	6.6	3.9	45.9	89.6	24.5	0.86
	Overall	32.7	13.5	13.2	40.6	73.3	6.97	0.99
tRNA-Leu		39.2	11.3	10.5	38.9	78.1	3.1	1
12S		32.5	6.8	12.9	47.8	80.3	5.84	0.99
Total		32.9	12.5	13.1	41.6	74.5	9.32	0.99

2000). Because of the small number of taxa (1 outgroup and 11 ingroup taxa), parsimony tree searches were performed using the more exhaustive branch and bound search method with equally weighted characters. In separate analyses, gap coded characters in tRNA-Leucine and 12S gene were treated as missing data or as a new (fifth) state. To assess the level of branch support, bootstrap values were calculated based on 1000 replications using the branch and bound search method (Felsenstein 1985). Bremer support (Bremer 1988) was calculated using the TreeRot program (Sorenson 1999) based on 20 replicate heuristic searches with random addition of taxa.

For maximum likelihood analyses, equally weighted trees obtained from parsimony analysis were used to estimate the log likelihood of each tree under 20 distinct models of sequence evolution (Huelsenbeck and Crandall 1997). The four basic models were Jukes-Cantor (JC), which has single substitution type and equal base frequency, Kimura two-parameter (K2P), which has two substitution types (transition and transversion) and equal base frequency, Hasegawa-Kishino-Yano (HKY), which has two substitution types (transition and transversion) and nonequal base frequency and General Time Reversible (GTR), which has six substitution types and nonequal base frequency. Within each model there were four methods of accounting for rate heterogeneity: no rate heterogeneity, gamma distributed rates (G), proportion of invariant sites (I), gamma + invariant sites (I+G) and site-specific rates (SSR). For the site-specific rate model (SSR), we assigned five different rate categories: the first, second, third codon positions, t-RNA-Leu gene and 12S gene. After likelihood scores were calculated for each model, we used the equally weighted parsimony trees as starting trees and performed searches using increasingly exhaustive branch swapping methods in the following order: Nearest neighbor interchange (NNI), subtree pruning and regrafting (SPR), second round of SPR, tree bisection and reconnection (TBR), and second round of TBR. At each iteration, the maximum likelihood parameters were reestimated from the trees which were obtained from the previous round of branch swapping.

Results

Intraspecific Variation. Of these 1093 bp from small fragments of COI, COII, and 12S, only one nucleotide (insertion or deletion of Thymine in the 12S sequence) difference was found among three individuals of *Enchenopa* on *J. cinerea.* This nucleotide difference needs to be confirmed by sampling additional individuals. The remaining sequences of all three genes are identical among the three individuals within each of the other eight *E. binotata* species. With this one possible exception the nucleotide differences among the nine *E. binotata* species are fixed and useful as phylogenetic characters. Of these 1,093 nucleotide sites examined, 1,018 (93%) are constant, 39 (3.5%) are uninformative and 36 (3.3%) are phylogenetically (parsimony) informative characters.

Nucleotide Composition and Codon Bias. A total of 2305 bp of aligned sequences for the four genes was obtained for 11 *Enchenopa* species and the outgroup taxon. The distribution of nucleotides is as follows: 1219 (position 1–1219) in COI, 65 (1220–1288) and four gap coded sites (1235–38) in tRNA-Leucine, 681 (1289–1969) in COII, 329 (1970–2305) and seven gap coded sites (2091–92, 2123–38, 2233, 2248 and 2256) in 12S. The overall base composition was A+T biased (74.5%, Table 4) as in other insect mitochondrial genomes (60–80%, Simon et al. 1994). Codons for amino acids were inferred by alignment with mitochondrial DNA sequences of *D. yakuba* (Clary and Wolstenholme 1985). The base composition of protein coding genes varies among codon positions and between the two genes. The A+T bias is highest in the third codon of both COI (88.7%) and COII (91.1%) whereas the second codon of the COI has the least A+T bias (62.7%). Chi-square tests show no significant deviation from homogeneity of base frequencies across taxa ($P > 0.65$).

Parsimony Analyses. The *E. binotata* species complex was analyzed using *C. latipes* as an outgroup with the expanded character of 2305 nucleotides. Of 249 (70 for ingroup) informative characters, most are found in COI and COII protein-coding genes (223, 90%) with 182 or 82% (56, 90% for ingroup) in the third

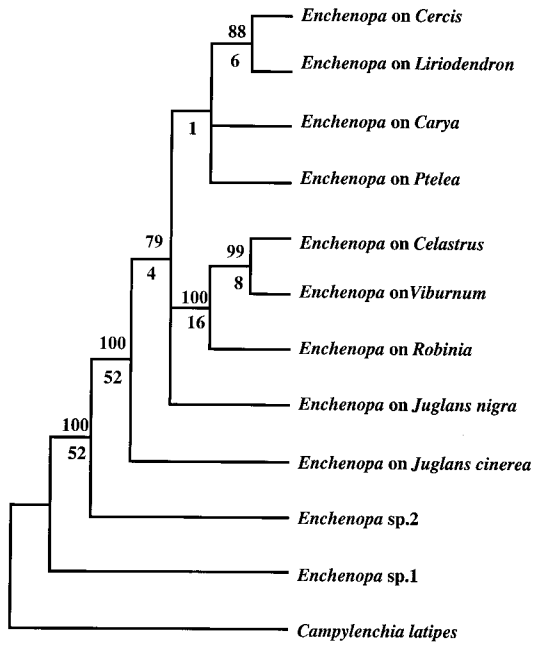

Fig. 2. Strict consensus of four equally parsimonious trees from 2305 bp of mitochondrial COI, tRNA-Leucine, COII and 12S gene (tree length = 890, CI = 0.849, RI = 0.680). Numbers above branch are bootstrap scores of 1,000 replicates (bootstrap values <50% not shown). Numbers below branch are the decay index of 20 replicate heuristic searches with random addition of taxa.

codon position. The t-RNA-Leu and 12S genes combined had 26 or 10% (8, 11% for ingroup) of the informative characters.

Four equally parsimonious trees of length 890 were obtained. Treating gap-coded characters either as missing data or as a fifth state yielded the same result. The strict consensus (Fig. 2) of four equally parsimonious trees shows support (bootstrap value of 100%) for the basal position of the two *Enchenopa* species from Central America. This tree strongly suggests the monophyly of the North American *E. binotata* species complex with bootstrap value of 100%. Two pairs of sister species within the complex: *Enchenopa* from *Celastrus* and from *Viburnum* (99%) and *Enchenopa* from *Cercis* and from *Liriodendron* (88%) are also strongly supported by this tree. However, the relationships among all of the nine species of the North American *Enchenopa binotata* complex were not completely resolved.

Likelihood Analyses. Log likelihood scores of 20 models are shown in Fig. 3. Allowing for variable transition/transversion ratios and non-equal base frequencies (HKY model) greatly improved the likelihood scores among the four basic models (Fig. 3, arrow 1). Within HKY models, accounting for rate heterogeneity among sites (SSR) improved the likelihood scores compared with the other four different methods of accommodating rate heterogeneity (Fig. 3, arrow 2). Therefore, we chose HKY model with SSR

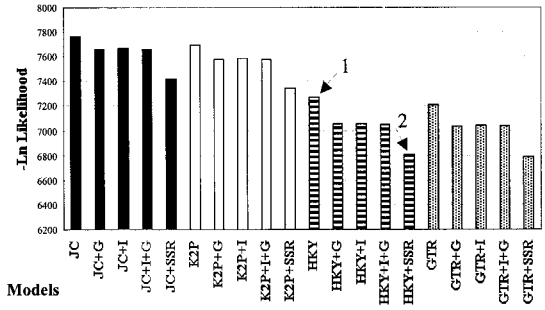

Fig. 3. Log likelihood scores of 20 models of sequence evolution (JC, Jukes and Cantor; K2P, Kimura two-parameter; HKY, Hasegawa-Kishino-Yano; GTR, General Time Reversible; G, Gamma distribution rates; I, Proportion of invariant sites; SSR, Site-specific rates).

for maximum likelihood analysis because it required the least assumptions, while substantially improving the likelihood scores.

One tree (Fig. 4) was obtained after branch swapping using the HKY+SSR model that had the same tree topology as the more complex GTR+SSR and less complex HKY+I+G model. The topologies of these trees were congruent with that of strict consensus tree derived from the four equal parsimony trees. In ad-

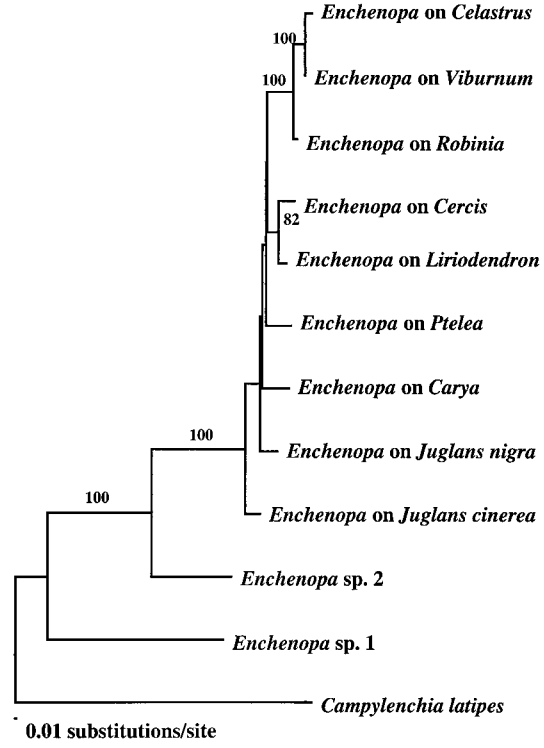

Fig. 4. Maximum likelihood tree based on HKY+SSR model (-Ln likelihood = 6824.81164). The numbers above branch are bootstrap scores of 100 replicates (bootstrap values <50% not shown).

dition, they revealed a basal relationship of *Enchenopa* species from *J. cinerea, J. nigra* and *Carya* (all in the Juglandaceae) relative to the remaining six North American *Enchenopa* species.

Discussion

The expanded DNA sequences from COI (1219 bp), COII (681 bp), t-RNA-Leucine (69 bp including gaps) and 12S (336 bp including gaps) provide sufficient characters to resolve the relationships of closely related North American *Enchenopa* species with the exception of two internal nodes in the parsimony analyses (Fig. 2). This lack of complete resolution may be due to character conflicts or simply a result of not enough informative characters. Additional sequences from other mitochondrial genes may provide more informative characters for complete resolution of this species complex. With the exception of one nucleotide difference, the mitochondrial sequences from three individuals of each of nine North American *Enchenopa* species are identical. However, the extent of intraspecific variation needs to be expanded beyond the limited data present here to reflect the geographic ranges. For future study answering the question of whether there is intra or interspecific genetic structure throughout the geographic range of the extant nine *Enchenopa* species requires more variable mitochondrial genes and extensive geographic sampling of each species throughout the eastern North America.

Because the topologies of maximum parsimony and strict consensus of maximum likelihood trees are in concordance, we chose the maximum likelihood tree (Fig. 4) as a working phylogenetic hypothesis for the *E. binotata* species complex. Despite the relatively short branch lengths of internodes in the maximum likelihood tree, applying likelihood analysis provides a useful method of investigating regions of the cladogram which lack parsimony resolution. Choosing among models of DNA substitution is among the most important steps when applying likelihood criterion in phylogenetic analysis. Applying the HKY+SSR model for likelihood analyses is appropriate because there is a highly unequal base frequency (A+T bias, Table 4). These data also had an unequal transition/transversion ratio of 1.2–2.8 depending on the model of sequence evolution. Accounting for rate heterogeneity is also reasonable because the rate of evolution varies not only among three codon positions of protein coding genes but also in transfer RNA and ribosomal genes (Simon et al. 1994).

The phylogenetic hypothesis derived from molecular characters is not in concordance with that of nymphal morphology. These two hypotheses suggest different sets of sister taxa relationships. The tree based on nymphal characters suggests three sets of sister taxa: *Enchenopa* from *Cercis* and *Viburnum*, *Enchenopa* from *Ptelea* and *Celastrus* and *Enchenopa* from *J. nigra* and *J. cinerea* (Pratt and Wood 1992). However, the mitochondrial tree suggests another two sets of sister taxa: *Enchenopa* from *Cercis* and *Liriodendron* and *Enchenopa* from *Celastrus* and *Viburnum*. The nymphal character based tree suggests that *Enchenopa* from *Robinia* is basal to the remaining North American *Enchenopa* species whereas the mitochondrial based tree suggests *Enchenopa* from *J. cinerea* is basal. Several technical, gene or organismal level factors may account for the discordance among trees derived from different sources of characters (Wendel and Doyle 1998). It is likely that the discordance between nymphal and mitochondrial trees is due to difference in taxon sampling and the nature of coding continuous nymphal characters. Although both trees use the same outgroup, *C. latipes*, the mitochondrial tree includes two more Central American *Enchenopa*. However, the discordance of the two trees cannot be resolved until the nymphal data are reanalyzed including the two additional Central American *Enchenopa* species.

The hypothesis that the nine extant species of North American *E. binotata* are monophyletic is supported by this mitochondrial phylogeny because the two *Enchenopa* species from Central America are basal to the remaining *E. binotata* species complex and these two ingroup nodes are strongly supported by bootstrap values (Figs. 2 and 4). This result suggests that the North American *Enchenopa* species were derived from a common ancestor in Central or South America and subsequently speciated through host shifts in North America. Additional taxon sampling of Mexico, Central and South American *Enchenopa* species is required to fully test the hypothesis.

Sympatric speciation is one of the more controversial subjects in evolutionary biology. Sympatric speciation could occur if biological traits (e.g., life history timing, philopatry) impeded gene flow between populations in the absence of geographic isolation. Except for polyploidy in plants, where speciation events take place almost instantaneously, most examples of sympatric speciation require ecological or habitat differences to promote reproductive isolation. In addition to the *E. binotata* species complex, examples of sympatric speciation through shifts in host use or prey specialization can be found in other insect groups like *Rhagoletis* (Bush 1969) and Chrysopidae (Tauber and Tauber 1982). The *E. binotata* species complex is hypothesized to diverge through host-plant specialization resulting from changes in host-plant use. This sympatric hypothesis of speciation predicts that sister taxa should differ in critical life-history traits, like time of egg hatch, length of development to mating, temporal span of mating, which are hypothesized to have initiated divergence (Wood 1993). Therefore, sister taxa relationships revealed by a phylogenetic analysis and the differences in life history traits among species together could be used to test the validity of this prediction. Sequences from mitochondrial DNA provide fixed characters for reconstructing a robust phylogenetic hypothesis. Well-supported sister taxa of the *Enchenopa* from *Celastrus* and *Viburnum* in the mitochondrial tree differ both in their diurnal and temporal spans during which mating occurs. Although the complete life-history data of the *Enchenopa* from *Liriodendron* is limited, the available data suggest that

mating of this species takes place after that of its sister taxon the *Enchenopa* from *Cercis* (Wood and Guttman 1985). Thus, this mitochondrial phylogeny supports the hypothesis that shifts to host plants that disrupt life history synchrony could have initiated speciation. The relatively few informative characters (70 in 2,305 sites) found along with little adult morphological differentiation (Pratt and Wood 1993) suggest the North American *Enchenopa* species complex have speciated recently.

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