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ScienceDirect
Molecular Phylogenetics and Evolution 45 (2007) 750–756
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Short Communication

Phylogenetics and phylogeography of the oak treehopper *Platycotis vittata* indicate three distinct North American lineages and a neotropical origin

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Received 14 February 2007; revised 19 May 2007; accepted 6 June 2007
Available online 16 June 2007

1. Introduction

Ecological differences including habitat, pollinators, and temporal separation between species and populations are important isolation barriers that can impede gene flows among evolving lineages, and subsequently lead to population divergence or speciation (Coyne and Orr, 2004). Temporal or allochronic speciation occurs when members of co-existing species or populations differ in breeding period that causes reproductive isolation as in the famous example of 13- and 17-year periodical cicadas, *Magicicada* (Cooley et al., 2003). For insects like the apple maggot, *Rhagoletis pomonella* (Bush et al., 1989), the green lacewing, *Chrysoperla plorabunda* and *C. downesi* (Tauber and Tauber, 1977), and the treehopper, *Enchenopa binotata* (Wood, 1993a), the timing of reproduction can be greatly modified by their host-plant usage and the insect's reproduction is strongly correlated with the plant phenology. Host shifting that changes life history timing thus plays an important role in reproductive isolation and speciation of phytophagous insects (Berlocher and Feder, 2002; Drès and Mallet, 2002).

Treehoppers (Insecta: Hemiptera: Membracidae) are phytophagous insects that use their piercing and sucking mouthparts to feed on the phloem or xylem of plants (Wood, 1993b; Lin, 2006). The North American oak treehopper, *Platycotis vittata* (Fabricius) displays an extensive frontal horn dimorphism and color polymorphism in various life stages, between sexes, and throughout its geographic range (Dozier, 1920; Cook, 1955; Wood, 1993b; Lin, 2006). This host-specialist treehopper utilizes over 30 species of deciduous and evergreen oaks (*Quercus*, Fagaceae) and occurs in approximately 60% of the geographic range of its hosts (Keese and Wood, 1991; McKamey and Deitz, 1996). It is the only oak-inhabiting treehopper with a broad U-shaped geographic range spanning both the eastern and western coasts of North America. The eastern and southern distribution of *P. vittata* is at or below 40° latitudes. The oak treehopper is bivoltine and exhibits maternal care of eggs and nymphs (Wood, 1976; Wood et al., 1984). Throughout its North American range, the insect has discrete generations in the early spring and autumn (Wood, 1976). The spring generation hatches around the time of bud break from eggs deposited under the bark in late winter and early spring. Newly emerged female adults from spring generation enter a summer diapause where feeding and mating occur but no ovarian development (Keese and Wood, 1991). These females deposit eggs in late summer that give rise to the autumn generation. Adults of the autumn generation mate before the arrival of the winter. Females of autumn generation enter their over-wintering dormancy, and emerge in late winter to deposit the eggs that give rise to the next spring generation (Wood, 1976).

Platycotis vittata has been shown to exhibit latitudinal variation in life history timing along the eastern coastal plain of the United States (Keese and Wood, 1991). Populations in the northern and southern extremes (i.e., New Jersey vs. Florida) differ in eclosion times by two months in each generation. The occurrence of teneral adults in the populations decreases progressively with increasing

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1055-7903/$ - see front matter
doi:10.1016/j.ympev.2007.06.005

latitude (Keese and Wood, 1991). Transplant experiments that disrupted synchrony in life history timing by placing females from southern populations onto trees with a different northern phenology showed a reduced reproductive success (Keese and Wood, 1991). Therefore the latitudinal variation in life history timing appears to be the result of synchronization of life history of insects with the phenology of their host-plants. However, we do not know if such variation in life history timing mediated by plant phenology could provide temporal isolation barriers to gene flow among *P. vittata* populations. The temporal isolation model of speciation in phytophagous insects (Coyne and Orr, 2004) predicts that the divergences among populations with different life history timing can have no fixed genetic differences, and the reproduction isolation is a byproduct of developmental plasticity induced by plant phenology. Will this variation in life history timing lead to genetic differentiation of geographical lineages in the oak treehopper throughout its eastern US range? In addition, populations of *P. vittata* were described as separated species or subspecies using shapes of frontal horn and coloration (Dozier, 1920; Cook, 1955). However, these ambiguous characters make it difficult to access its species status in relating to geographical distribution (Wood, 1993b).

Here, we report a phylogenetic and phylogeographic study of mitochondrial DNA variation among closely related *Platycotis* species and populations of *P. vittata* in the North America. Our objectives are (1) to determine the level of genetic variation among *P. vittata* populations; (2) to test the monophyly of the North American *P. vittata*, and its phylogenetic relationships with closely related *Platycotis* species; (3) to exam the phylogeographic patterns of *P. vittata* and its concordance with the prediction of no genetic structuring by a temporal isolation hypothesis.

2. Materials and methods

Individuals of *P. vittata* were collected as adults or nymphs in North America (Fig. 1, Table 1). Specimens of related treehopper species (*Platycotis* and *Umbonia*) were collected in Central and North America. *Umbonia*, which is a sister taxon of *Platycotis*, were used as outgroups for phylogenetic analyses (McKamey and Deitz, 1996; Lin et al., 2004). Field-collected treehoppers were immediately preserved in 95% ethanol, followed by short-term storage at −20 °C. Genomic DNA was obtained from the abdomen or thoracic muscles of a single individual leaving the rest of the specimen as a voucher preserved in 95% ethanol and a long-term storage of −80 °C.

Extraction of genomic DNA followed standard protocols outlined in Lin and Wood (2002). Polymerase chain reaction (PCR) was used to amplify a fragment approximately 900 bps of the mitochondrial cytochrome oxidase gene (COI) with Ron (C1-J-1751 in Simon et al., 1994) and Calvin primer (position 2725 of 5′ end of the mitochondrial sequence in *Drosophila yakuba*; Lin and Wood, 2002). The cycling profile began with one cycle of DNA denaturation of 45 s at 94 °C. This was followed by 35 cycles of amplification (DNA denaturation at 94 °C for 1 min, primer annealing at 50–58 °C for 1 min, sequence extension at 72 °C for 2 min). PCR products were purified and sequenced from both directions on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA). Sequences of DNA fragments were edited and assembled using EDITSEQ (DNASTAR, Madison,

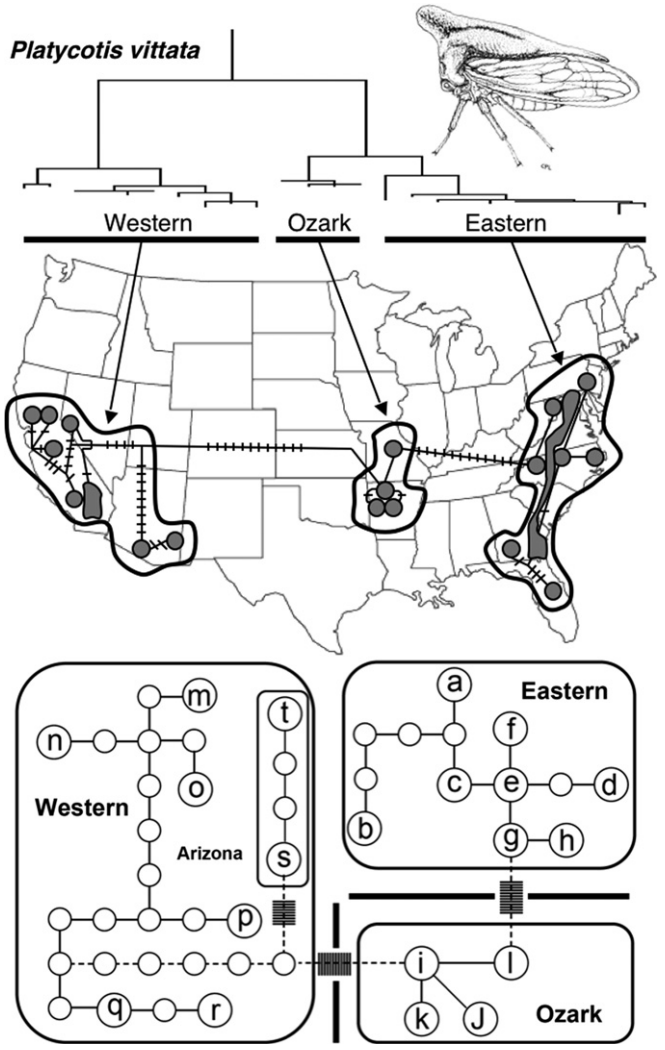

Fig. 1. Above: Sampling scheme and phylogeographic pattern based on COI in the oak treehoppers, *Platycotis vittata* (Fabricius). Geographic arrangement of maximum parsimony network for 20 distinct mtDNA haplotypes (grey dots). Slashes across network branches indicate inferred numbers of mutational steps. Heavy lines encompass phylogroups that differed by large number of mutational steps. Below: Maximum parsimony network. Circles encompassing letters a–t indicate observed COI haplotypes, open circles represent hypothetical and unobserved intermediate haplotypes. Solid branches connect haplotypes differed by one mutational step. Heavy lines encompass phylogroups where haplotypes are connected with 95% probability. Dashes connect phylogroups and haplotypes that differ by large number of mutational steps. Slashes across network branches indicate inferred large numbers of mutational steps between phylogroups and haplotypes.

Table 1
Locality of specimens with collecting information and mitochondrial haplotype codes of *P. vittata*

DNA Code	Genus	Species	Country	State/province	Locality	Haplotype	Collector
UMA	*Umbonia*	*ataliba*	Costa Rica	Puntarenas	Puntarenas		R. Cocroft
UC0601	*Umbonia*	*crassicornis*	United States	Florida	NA		Lab colony
URE	*Umbonia*	*redulta*	Costa Rica	Heredia	La Selva		R. Cocroft
UMBOSP	*Umbonia*	*spinosa*	Panama	Panama	Gamboa		R. Cocroft
PLAVOL1	*Platycotis*	sp. *1*	Panama	Chiriqui	Volcán Barú		C.-P. Lin
PLAVOL2	*Platycotis*	sp. *2*	Panama	Chiriqui	Volcán Barú		C.-P. Lin
BOPL	*Platycotis*	sp. *3*	United States	California	San Bernardino		R. Dowell
PLMI	*Platycotis*	*minax*	United States	California	San Bernardino		R. Dowell
PLMI2	*Platycotis*	*minax*	United States	California	San Bernardino		R. Dowell
PLATUB	*Platycotis*	*tuberculata*	Guatemala	Guatemala	Jocotenango		C.-P. Lin
M029	*Platycotis*	*tuberculata*	Honduras	Honduras	La Unión		C.-P. Lin
PLSB	*Platycotis*	*acutangula*	United States	California	Sacramento		R. Dowell
PLGAvd	*Platycotis*	*vittata*	United States	Georgia	Valdosta	a	M. Rothschild
PLGAwc	*Platycotis*	*vittata*	United States	Georgia	Waycross	c	M. Rothschild
PLFL	*Platycotis*	*vittata*	United States	Florida	Marion	b	K. Sime
PLVAlb	*Platycotis*	*vittata*	United States	Virginia	Lynchburg	c	T. Wood
PV0201	*Platycotis*	*vittata*	United States	Pennsylvania	Nottingham	c	D. Liu
P5	*Platycotis*	*vittata*	United States	Kentucky	Boyle	c	T. Wood
PLMDgs	*Platycotis*	*vittata*	United States	Maryland	Grantsville	d	R. Snyder
PLMDst	*Platycotis*	*vittata*	United States	Maryland	Swanton	h	R. Snyder
PLNCor	*Platycotis*	*vittata*	United States	North Carolina	Oak Ridge	e	T. Wood
PLNCpm	*Platycotis*	*vittata*	United States	North Carolina	Plymouth	f	M. Rothschild
P7	*Platycotis*	*vittata*	United States	North Carolina	Davie County	g	T. Wood
PLMOco	*Platycotis*	*vittata*	United States	Missouri	Columbia	i	R. Cocroft
PV6	*Platycotis*	*vittata*	United States	Arkansas	Magazine Mt.	k	T. Wood
PV7	*Platycotis*	*vittata*	United States	Arkansas	Magazine Mt.	j	T. Wood
PV3	*Platycotis*	*vittata*	United States	Arkansas	Magazine Mt.	l	T. Wood
PLSA	*Platycotis*	*vittata*	United States	California	Sacramento	m	R. Dowell
PLATNE	*Platycotis*	*vittata*	United States	California	Eagle Lake	n	R. Dowell
PLAVI2	*Platycotis*	*vittata*	United States	California	West Sacramento	o	T. Wood
PCLAC2	*Platycotis*	*vittata*	United States	California	Lake Arrowhead	p	R. Dowell
PV1	*Platycotis*	*vittata*	United States	California	Pyramid Peak	q	R. Dowell
PV4	*Platycotis*	*vittata*	United States	California	Orange County	r	G. Pratt
PVSB1	*Platycotis*	*vittata*	United States	California	San Bernardino	r	R. Dowell
PVSB2	*Platycotis*	*vittata*	United States	California	San Bernardino	r	R. Dowell
PLAVI26	*Platycotis*	*vittata*	United States	Arizona	Madera Canyon	s	T. Wood
PLAZch	*Platycotis*	*vittata*	United States	Arizona	Portal	t	T. Wood

WI). DNA sequences used in this study were deposited in GenBank (Accession Nos. EF632113–EF632149). DNA sequences were aligned using EDITSEQ and MEGALIGN programs in Lasergene (DNA STAR, Inc.). The assignment of codon positions was confirmed by translating nucleotide sequences into amino acid sequences using MacClade (version 4.06, Maddison and Maddison, 2000) with reference to a mitochondrial genetic code of *Drosophila*.

Equally weighted parsimony analyses were done using PAUP* (version 4.0b10, Swofford, 1998). Heuristic tree searches were performed using 1000 random sequence additions and TBR branch swapping. Phylogenetic analyses were also carried out using the neighbor-joining method on log determinant distances (LogDet, Lockhart et al., 1994), with invariable sites excluded to assess the effect of nucleotide compositional bias on phylogenetic reconstruction. Nonparametric bootstrap (Felsenstein, 1985) values were calculated using 1000 replicates and 100 random taxon additions to evaluate branch support. Bremer support values (Bremer, 1988) were calculated using the TreeRot program (version 2c, Sorenson, 1999) based on 100 replicate heuristic searches with random addition of taxa.

To determine an appropriate model of sequence evolution for maximum likelihood and Bayesian analysis, we used the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) (Posada and Buckley, 2004; Alfaro and Huelsenbeck, 2006) in Modeltest (version 3.7, Posada and Crandall, 1998). The selected model was then used to find the maximum likelihood topology with a heuristic ML analysis implemented using PAUP*. To search for a maximum likelihood tree, the most parsimonious trees were used as starting trees, and heuristic searches were performed 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 a second round of TBR. At each iteration, the maximum likelihood parameters were re-estimated from the trees that were obtained from the previous round of branch swapping.

We performed Bayesian phylogenetic analyses using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). All Bayesian analyses were initiated with random starting trees, run simultaneous with four incrementally heated chains, and were run for the 2.0×10^6 generations. Ten independent analyses were run and the Markov chains were sampled at intervals of 50 generations for a total of 40,000 trees. Stability of the process was achieved when ln likelihood values approached equilibrium, as determined by plotting the ln likelihood scores of the sampled trees against generation time. All trees sampled before reaching stability are discarded as "burn in". After discarding burn-in samples, the remaining trees from each analysis were used to generate a 50% majority rule consensus tree with the percentage of trees recovering the node representing the node's posterior probability.

Network analysis was employed to examine the relationship of intraspecific genealogy in *P. vittata*. We first constructed haplotype networks of the COI sequences using the program TCS (version 1.21, Clement et al., 2000). The maximum numbers of mutational connections between pairs of haplotype sequences with 95% probability were calculated using the parsimony criterion. Ambiguous connections in the resulting haplotype networks were resolved using rules in Crandall and Templeton, 1993. The parsimony networks were superimposed over the geographic sources of collection in the North America to examine the overall phylogeographic pattern in the data.

3. Results

An alignment of 873 nucleotide sites without gaps was obtained for 37 taxa. Of 340 variable sites, 283 are parsimony informative (228 in *Platycotis*). Overall nucleotide frequencies for this COI gene fragment were biased toward A+T (69.3%), consistent with other insects (Simon et al., 1994; Lin and Danforth, 2004). Chi-square tests of base frequency stationarity of the third position show significant among-taxa variation in composition ($p < 0.0001$). The topology of neighbor-joining tree resulted from the LogDet analysis is congruent with that of the parsimony and likelihood/Bayesian analyses. Based on corrected pairwise distances of TIM+I+G model, sequence divergences within the North American *P. vittata* range from 0% to 8.5% (with the exception of haplotype t, 0.3–12.3%). Whereas the range between populations of *P. vittata* and their sister taxon, *P. acutangula* is from 10.7% to 16.6%.

Equal weights parsimony analyses found two equally parsimonious trees which are identical except the placement of the *P. vittata* from North Carolina (haplotype g) and Maryland (haplotype h). The well-resolved strict consensus tree recovered the monophyly of the North American *P. vittata* and its three major geographic lineages (Ozark Plateau, Eastern, and Western US clades) with moderate to strong bootstrap and Bremer support values (Fig. 2). The Western US clade including populations from California and Arizona is the basal lineage within the North American *P. vittata*. Californian populations were grouped into mainly the northern and southern clade, in which northern populations are inferred to be more derived lineages. Populations from the geographic proximity within the Eastern US clade did not cluster together. Phylogenetic relationships of *P. vittata* and closely related species are well resolved, and *P. acutangula* is the sister taxon of *P. vittata*.

Using both AIC and BIC, the best TIM+I+G model was chosen for likelihood and Bayesian analyses. One tree resulted from the likelihood analysis is identical to that of parsimony trees with the exception of placing *P. tuberculata* as sister to *P. vittata* (Fig. 2). Ten independent Bayesian analyses converged on similar likelihood scores and reached stability no later than 5×10^5 generations. The first 20,000 trees of each run were discarded and a majority rule consensus tree was constructed using the pooled 2×10^5 trees from 10 analyses. The Bayesian tree is well resolved and contains a majority of ingroup nodes with posterior probability >95% (Fig. 2). The tree topologies of the Bayesian and likelihood analysis are identical.

Mitochondrial COI haplotypes separated by up to 11 mutational steps have a probability $\geqslant$95% of being connected into a single network using a parsimony criterion. Within these limits, four disjoint networks (Eastern US, Ozark Plateau, Arizona, and Western US) are obtained, each with no internal ambiguous nodes (Fig. 1). These four haplotype networks are connected by a minimum of 20 mutational steps. A single widespread haplotype c of the Eastern US clade was found in Pennsylvania, Virginia, Kentucky, and Georgia. The TCS analysis placed the haplotype e from North Carolina, the haplotype i from Missouri, and the haplotype q from Pyramid Peak of Northern California as ancestral for the Eastern US, Ozark Plateau, and Western US clade, respectively. However, parsimony, likelihood, and Bayesian phylogenetic analyses placed the Western US haplotype clade as the basal lineage within the North American *P. vittata* (Fig. 2). The overall haplotype network was then polarized using the results from the phylogenetic analyses in which the haplotype s from Arizona are the basal/ancestral within the North American *P. vittata*. Superimposing the networks onto the collecting sites yielded a close match between the sampled haplotypes and broad scale geography of North America (Fig. 1). Three major geographical clades were apparent: the Eastern US clade covering Pennsylvania, Maryland, Virginia, North Carolina, Kentucky, Georgia, and Florida; the Ozark Plateau clade centered in Missouri and Arkansas; the Western US clade containing ancestral haplotype s radiating out from Arizona, through the southern California, and then onto the Northern California.

4. Discussion

The well-supported phylogenies clearly show a monophyletic North American *P. vittata* with respect to con-generic Central and North American species. Sequence

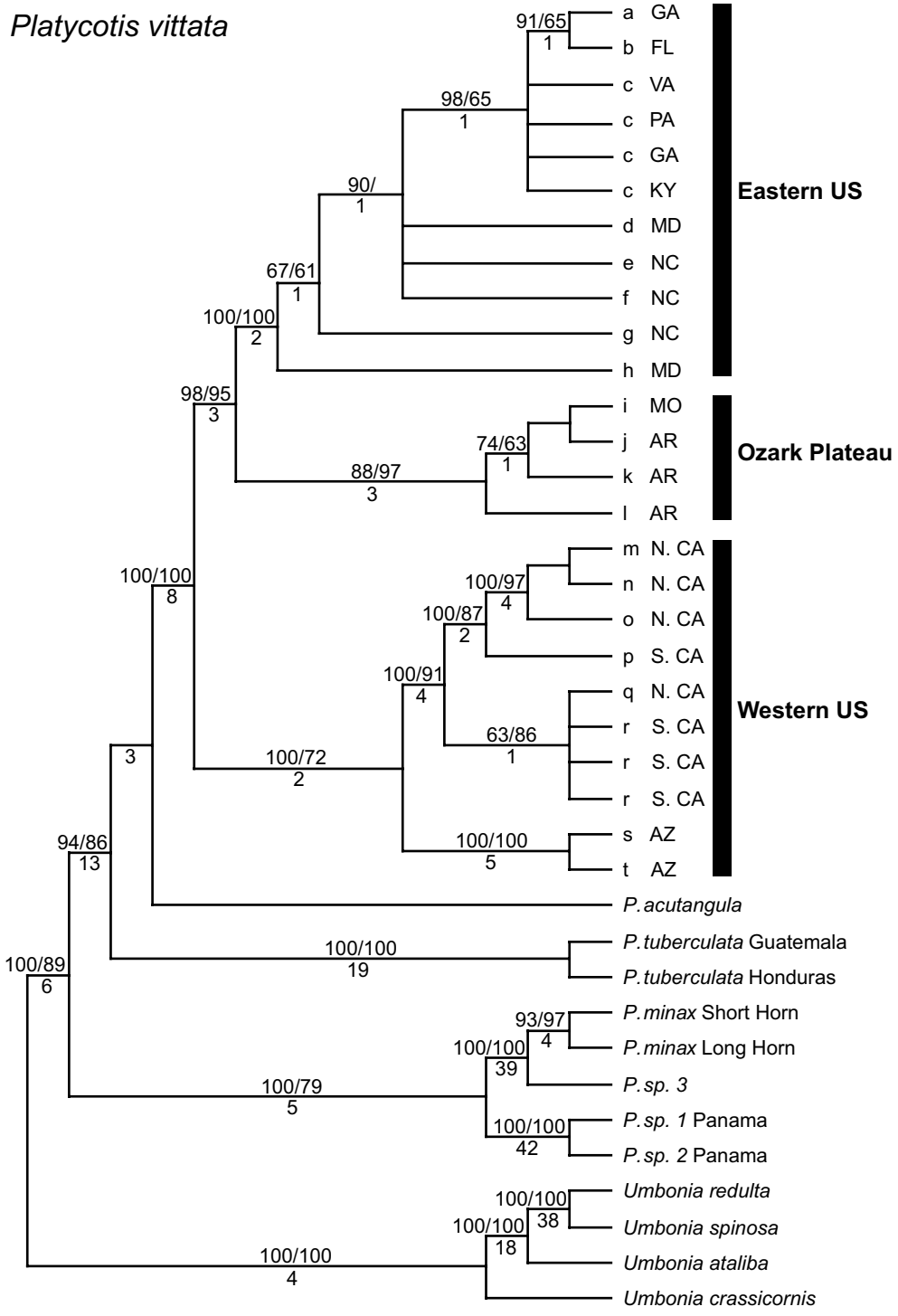

Fig. 2. The 50% majority rule consensus tree from the Bayesian analyses of COI data based on a TIM+I+G model. Numbers above the branches to the left and to the right of the slashes are posterior probability and parsimony bootstrap values of the nodes, respectively. Numbers below the branches are Bremer support values. This tree topology is identical to that of parsimony and likelihood analyses except for one node (see text for Results).

divergences within populations of *P. vittata* are smaller than that of between *P. vittata* and sister *P. acutangula*. These results suggest that *P. vittata* from across the North America constitutes a single "true" phylogenetic species. The phylogenetic analyses also support the monophyly of the genus *Platycotis*, and resolve species relationships among members of the genus. However, to fully test these phylogenetic hypotheses, exemplars of *P. vittata* populations from Mexico and less sampled North American distribution would have to be included in the analysis.

Our analyses revealed the presence of substantial genetic variation within the North American *P. vittata* that

matches closely to a large scale geographic distribution of the Eastern US, Ozark Plateau, and Western US lineages. The Western US populations were further subdivided into Arizona and Californian lineages. Within the California clade, northern and southern phylogeographic substructures are also present. This broad scale phylogeographic pattern of genetic differentiation across the eastern and western coasts of North America suggests either long-term extrinsic barriers to genetic exchange, an accumulation of *de novo* mutation after population separation, and/or ancestral lineage sorting of polymorphic mitochondrial haplotypes are responsible (Avise, 2000). On the contrary, within the Eastern US lineage, there is a widely distributed haplotype across the eastern coast but no apparent phylogeographic subdivisions existed among haplotypes of northern and southern populations. Most or all haplotypes from the Eastern US are related closely (few mutational steps) and geographically localized. This phylogeographic pattern implies that the current gene flow has been low enough to allow ancestral lineage sorting and random genetic drift to promote genetic divergence among populations (Avise, 2000).

Latitudinal variation in life history timing was hypothesized to provide temporal isolation barriers to gene flow, and subsequently lead to genetic divergence in the Eastern US populations of *P. vittata* (Keese and Wood, 1991). One of the unique characteristics of temporal isolation model in phytophagous insects is that it can be entirely nongenetic and reproduction isolation is a byproduct of developmental plasticity induced by plant phenology (Coyne and Orr, 2004). This has been shown in the *Enchenopa binotata* complex of treehoppers using transplant experiments (Wood, 1993a; Wood et al., 1999). Our results of no apparent phylogeographic substructuring and a low level of gene flow among northern and southern populations of the Eastern US clade are in concordance with this hypothesis of temporal isolation without fixed genetic changes. Latitudinal variation in life history timing mediated by host-plant phenology could provide temporal isolation barriers among *P. vittata* populations with low contemporary gene flow. This variation evidently did not lead to substantial genetic differentiation of geographical lineages in the oak treehopper throughout its Eastern US range. Nevertheless, our conclusion of no clear genetic structuring may also be due to the lack of resolution in mitochondrial genes. Additional variable markers such as microsatellite loci or SNPs are needed.

Of the North American oak-inhibiting treehoppers, *P. vittata* is one of the most remarkable species with a broad geographic distribution spanning both the eastern and western coasts of North America (Keese and Wood, 1991; McKamey and Deitz, 1996). Members of the genus *Platycotis* are largely Neotropical treehoppers with restricted geographic ranges except *P. vittata* being one of a few species having extensive North American distribution. It can be inferred from our molecular phylogeny that the North American *P. vittata* diverged from sister taxa, *P. acutangula* and *P. tuberculata*, indicating that their origin was from the Central America. The phylogeny and haplotype network clearly identified three major derived *P. vittata* lineages, Eastern US, Ozark Plateau, and Western US. These phylogroups separated by relatively large mutational steps are likely to represent discrete dispersal events in the North America, or alternatively fragmented populations isolated from a broader geographic range from the Rockies to the Appalachians of the Eastern United States. Presuming the dispersal scenario, the ancestral Central American population may have expanded and colonized host populations along the Gulf of Mexico and the Pacific Coast. This range expansion toward the east and west coasts of the North America may have caused the initial split of the ancestral population into the Eastern and Western US clade. The ancestral Western US population then expanded northward into the highlands of the Southwest US and formed the basal southern Arizona lineage. The ancestral population continued to expand toward northwest, and eventually reached firstly the southern California and later the Northern California. The ancestral Eastern US population expanded into the Ozark Plateau and then toward the Appalachians of the Eastern US. The use of mitochondrial DNA and the number of sampling populations in this study limit our ability to discern fine scale population genetic structures and historical demographies necessary to test alternative hypotheses of diversification in *P. vittata* (i.e., vicariance with long-term geographical barriers to dispersal versus postglacial range expansion events in continental biota) (Avise, 2000, 2004). Our results nevertheless revealed a unique broad spatial scale phylogeographic structuring in these treehoppers, and serve to provide a basis for further investigation.

Acknowledgments

We thank R. Gill for identification of Californian specimens, R. Cocroft, R. Dowell, D. Liu, G. Pratt, M. Rothschild, K. Sime, and R. Snyder for providing treehopper specimens, and P. O'Grady and two anonymous reviewers for helpful comments on the manuscript. Funding for this research came from a National Science Foundation Grant (DEB-9509125) to T.K. Wood and National Science Council Grant (94-2311-B-029-007) to C.-P. Lin.

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