ORIGINAL RESEARCH

You stay, but I Hop: Host shifting near and far co-dominated the evolution of Enchenopa treehoppers

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Abstract

The importance and prevalence of phylogenetic tracking between hosts and dependent organisms caused by co-evolution and shifting between closely related host species have been debated for decades. Most studies of phylogenetic tracking among phytophagous insects and their host plants have been limited to insects feeding on a narrow range of host species. However, narrow host ranges can confound phylogenetic tracking (phylogenetic tracking hypothesis) with host shifting between hosts of intermediate relationship (intermediate hypothesis). Here, we investigated the evolutionary history of the Enchenopa binotata complex of treehoppers. Each species in this complex has high host fidelity, but the entire complex uses hosts across eight plant orders. The phylogenies of E. binotata were reconstructed to evaluate whether (1) tracking host phylogeny; or (2) shifting between intermediately related host plants better explains the evolutionary history of E. binotata. Our results suggest that E. binotata primarily shifted between both distant and intermediate host plants regardless of host phylogeny and less frequently tracked the phylogeny of their hosts. These findings indicate that phytophagous insects with high host fidelity, such as E. binotata, are capable of adaptation not only to closely related host plants but also to novel hosts, likely with diverse phenology and defense mechanisms.

KEYWORDS
co-speciation, ecological speciation, host shifting, Membracidae, phylogenetic tracking, treehoppers

1 | INTRODUCTION

Elucidating patterns of species richness and mechanisms of speciation are major goals in the study of ecology and evolution. Ecological speciation occurs when two taxa evolve reproductive isolation (i.e., barriers to gene flow) due to divergent selection between environments (Nosil, 2012; Schluter & Rambaut, 1996) and has been proposed to be a major speciation mechanism (Schluter, 2009). For host-associated organisms, a change in host may result in novel environments for new adaptation. Changes in host can be caused by either divergence between hosts (i.e., co-evolution) or a shift between hosts (i.e., host shifting; Page, 2003; Carmona, Fitzpatrick, & Johnson, 2015; Soudi, Reinhold, & Engqvist, 2015). The timing of host divergence is critical for the timing of divergence of dependent organisms in co-evolution but not host shifting, but both mechanisms may result in phylogenetic tracking between hosts and their dependent organisms.

Two commonly considered concepts of phylogenetic tracking are Fahrenholz’s parallel cladogenesis and Szidat’s co-phylogeny (Eichler, 1948). Parallel cladogenesis occurs when the evolution of parasites matches the evolution of their hosts (Eichler, 1948; Fahrenholz, 1913; Timm, 1983). Previous studies have shown that both co-evolution and host shifting can result in parallel cladogenesis (Charleston &
Robertson, 2002). By contrast, Szidat’s co-phylogenetic concept focuses on phylogenetic tracking caused by co-evolution and postulates that ancestral hosts harbor more ancestral parasites (Eichler, 1948; Krasnov, Kiefer, Warburton, & Khokhlova, 2016; Szidat, 1939).

Although phytophagous insects have been a major focus in studies of host-associated speciation (Antwi, Sword, & Medina, 2015; Ehrlich & Raven, 1964; Knolhoff & Heckel, 2014; Matsubayashi, Ohshima, & Nosil, 2010), phylogenetic tracking between phytophagous insects and their host plants has rarely been tested (Winkler & Mitter, 2008; also reviewed in de Vienne et al., 2013; Suchan & Alvarez, 2015). de Vienne et al. (2013) reviewed 86 studies reporting co-phylogenetic analyses, of which only 12 examined phytophagous insects and their host plants. Similarly, only nine studies testing phylogenetic tracking between plants and insects were included in Suchan and Alvarez (2015)’s review. Both reviews concluded that there is a lack of support for phylogenetic tracking in insect–plant relationships.

However, in more than one-third of the studies of insect–plant interactions reviewed, the insects feed on only one plant order (or even one genus in several cases; de Vienne et al., 2013; Suchan & Alvarez, 2015). Such a narrow host range makes it difficult to distinguish phylogenetic tracking from host shifting between hosts of intermediate similarity. Alternatively, Nyman (2010) argued that a novel host of high similarity (usually a sister taxon) will not generate the disruptive selection required for speciation and that an insect is unlikely to colonize a novel host with little similarity to the original host. The intermediate hypothesis posits that the maximum probability of insect speciation occurs when alternative hosts are of intermediate similarity in resource space, as determined by the resource that is critical to the fitness of the focal insect (Nyman, 2010). Such resources may include the secondary chemical compounds, nutritional content, or phenotype of a plant, depending on the specific restrictions in each insect–plant interaction (Heard, 2012; Nyman, 2010). Comprehensive quantifications of the distance in resource space, however, are rare and not always applicable (Heard, 2012). Phylogenetically related plants often share similar physiological, morphological, and phenological characteristics due to phylogenetic conservatism (Cornwell et al., 2014; Davies et al., 2013; Liu et al., 2015). Therefore, the phylogenetic distance between host plants may represent the relative distance between host plants in the resource space to a certain degree.

The *Enchenopa binotata* species complex of treehoppers in Eastern North America is one of the best-known examples of ecological speciation in phytophagous insects (Nosil, 2012; Wood, 1993). The host plants of *E. binotata* include eight plant orders (Wood, 1980; Wood & Gutman, 1982; Lin & Wood, 2002; Hamilton & Cocroft, 2009; Figure 1), with possible cases in another three plant orders (Cornus of Cornales, *Tilia* of Malvales, and *Ceanothus* of Rosales; Hamilton & Cocroft, 2009). The wide range of host plants for the *E. binotata* species complex provides a unique opportunity to test whether phylogenetic tracking or the intermediate hypothesis best explains insect–plant interactions.

It is not clear how interactions with host plants shaped the evolutionary history of the *E. binotata* species complex. Almost all *E. binotata* species are specialists, and each of them specialized in only one host plant species. The exceptions are *E. binotata* that feed on multiple species of *Viburnum* or *Carya*, but even these *E. binotata* are restricted to a single host genus (Lin & Wood, 2002). Many of the host plants used by the *E. binotata* species complex occur sympatrically, resulting in overlapping distributions of *E. binotata* species (Lin & Wood, 2002). The eggs of univoltine *E. binotata* species hatch asynchronously due to differences in water content among host plants in spring, but their subsequent life-history stages are similar in duration (Wood, 1993; Wood & Gutman, 1982). The asynchronous first mating dates caused by the different hatching dates therefore result in assortative mating through temporal segregation (Wood & Keese, 1990). In the rare cases where adult *E. binotata* from different host plants meet, they tend to mate with conspecifics as a result of female preference for male vibrational mating signals (Rodriguez, Sullivan, & Cocroft, 2004; Rodriguez, Rasamwamy, & Cocroft, 2006; Cocroft, Rodriguez, & Hunt, 2008; Cocroft, Rodriguez, & Hunt, 2010; but see Rodriguez, Haen, Cocroft, & Fowler-Finn, 2012 for a lack of male preference for signals of conspecific females).

In this study, we tested the concordance of the evolutionary histories of the *E. binotata* species complex and their host plants. The specific predictions derived from the phylogenetic tracking hypothesis were as follows. First, the phylogeny of the *E. binotata* species complex was predicted to match the host plant phylogeny (parallel cladogenesis). Second, more ancestral host plant species were predicted to harbor more ancestral *E. binotata* species (co-phylogenesis). Alternatively, according to the intermediate hypothesis, host shifting in *E. binotata* was expected to occur more often between host plant species with intermediate distance in resource space. As the major reproductive barrier between *E. binotata* species

![FIGURE 1](image-url)
is the difference in hatching dates caused by host plant phenology (Wood, 1980, 1993), which is phylogenetically conserved (Davies et al., 2013), we used the phylogenetic distance between host plants to represent the distance in resource space.

2 | METHODS

2.1 | Sample collection

A total of 61 Enchenopa individuals collected from across the species’ ranges were used for phylogenetic reconstruction (Table 1). Among the sampled specimens, 44 belonged to the E. binotata species complex and were collected from 15 host species of 10 genera from Eastern North America. We sampled the majority of E. binotata as 2nd–5th instar nymphs to permit accurate host association and species identification based on nymphal characteristics (Pratt & Wood, 1992). When nymphs were absent, we collected male adults and identified them by species-specific mating signals (Cocroft et al., 2010). The remaining 17 Enchenopa species were collected from Central America and used as outgroups in phylogenetic analyses. Most outgroup specimens were collected as adults without host plant information. All specimens were preserved in 95% EtOH at −20 to −80°C for long-term storage before DNA extraction.

2.2 | Molecular methods

For each sample, we extracted genomic DNA using a DNeasy animal tissue kit (Qiagen Inc., Valencia, CA, USA). We collected partial gene sequences from a nuclear intron of elongation factor 1 alpha (EF1α) and the mitochondrial cytochrome oxidase 1 (CO1) gene. We performed PCR amplification on an Eppendorf Mastercycler Gradient (Eppendorf North America, Westbury, NY, USA). The thermal cycling conditions for each primer set were as follows: 35 cycles of denaturation at 94°C for 50 s, annealing at 52°C (EF1α) or 50°C (CO1) for 1 min, and extension at 72°C for 1 min, with a final step at 72°C for 6 min. We used Sequencer v4.5 (Gene Codes Corp., Ann Arbor, MI, USA) to edit and align the resulting sequences.

The Sanger sequencing method was not compatible with some PCR products of the EF1α gene, and therefore, we cloned these PCR amplicons using a TOPO® Ta cloning kit (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) before sequencing. We isolated the plasmid DNA using a PureLink Quick Plasmid Mini Purification kit (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) and sequenced five colonies for each cloning reaction. We sequenced the inserted region of the vector using the universal primers supplied in the kit.

2.3 | Phylogenetic reconstruction of Enchenopa binotata

We first reconstructed the gene trees of CO1 and EF1α separately using the parameters of the substitution models described below. Both gene trees supported the clustering of E. binotata into two clades, and those feeding on Cercis and Liriodendron were sister groups (Figure S1). However, most tree branches had low support, suggesting that the phylogenetic information from each of the two genes alone was insufficient to resolve the relationships. We therefore combined CO1 (875 bp) and EF1α (870 bp) using a supermatrix approach in Sequence Matrix v1.8 (Vaidya, Lohman, & Meier, 2011). For maximum likelihood (ML) and Bayesian phylogenetic analyses, the best-fit nucleotide substitution model was selected in jModelTest v0.1.1 (Posada, 2008) using the Bayesian Information Criterion (BIC). The CO1 sequences were partitioned by each codon position, whereas the EF1α sequences were partitioned as introns and exons. ML trees were obtained using RAxML v8.2.8 (Stamatakis, 2014) under the GTRCAT1 model, followed by 10,000 bootstrap replicates to estimate the 95% credible intervals (95% CIs). Bayesian analyses were performed using MrBayes v3.2.6 (Ronquist et al., 2012) under the best-fit model for each partition (i.e., GTR for the 1st and 3rd codon positions in CO1, JC69 for the 2nd codon position in CO1 and exons in EF1α, and HKY for the introns in EF1α). In the Markov Chain Monte Carlo (MCMC) process, we ran four chains with 5 × 10⁷ generations. Convergence of the MCMC process was diagnosed when the average standard deviation of the split frequencies was 0. The first 25% of the MCMC samples were discarded as burn-in.

We reconstructed the species trees and estimated divergence times using BEAST v1.8.2 (Drummond, Suchard, Xie, & Rambaut, 2012) and the best-fit model for each partition (i.e., TN93 + I + G with three partitions for CO1, TN93 with two partitions for exons in EF1α, and HKY with no codon partitions for introns in EF1α). To estimate divergence times, we fit the lognormal relaxed clock (uncorrelated) with a range of mutation rates of CO1 in insects between the standard and revised rates (1.15 × 10⁻⁸ and 1.77 × 10⁻⁸, respectively, with an average of 1.46 × 10⁻⁸ mutations/site/year; Brower & DeSalle, 1998; Papadopoulou, Anastasiou, & Vogler, 2010). For exons and introns in EF1α, we applied a range of mutation rates between the highest and lowest mutation rates reported in insects (exons: 0.2942 × 10⁻⁸ and 0.558 × 10⁻⁸, respectively, with an average of 0.426 × 10⁻⁸ mutations/site/year; Brower & DeSalle, 1998; Papadopoulou, Anastasiou, & Vogler, 2010). For exons and introns in EF1α, we applied a range of mutation rates between the highest and lowest mutation rates reported in insects (exons: 0.2942 × 10⁻⁸ and 0.558 × 10⁻⁸, respectively, with an average of 0.426 × 10⁻⁸ mutations/site/year; reviewed in Lin & Danforth, 2004). For the MCMC settings, we ran three independent chains with the same set of parameters, each for 1 × 10⁷ generations. The convergence of each MCMC chain was diagnosed by the effective sample sizes of the parameters (ESS > 200). The results of these three chains were combined using LogCombiner v1.8.2 (Drummond et al., 2012), with the first 2.5 × 10⁷ MCMC samples of each chain discarded as burn-in.
TABLE 1  The collection locations of Enchenopa specimens, their associated host plants, and the GenBank accession numbers of their DNA sequences included in this study

| No | Species | Host plant | Locality | Collectors | Collection date | Accession number (CO1) | Accession number (EF1α) |
|----|---------|------------|----------|------------|----------------|------------------------|-------------------------|
| 1  | *E. binotata* | Sideroxylon lycioides | Moulton, AL | C.H. Dietrich | 26-May-2004 | KX791061 | KX791122 |
| 2  | *E. binotata* | Sideroxylon lanuginosum | Van Buren, MO | R.B. Cocroft | 30-Apr-2006 | KX791062 | KX791123 |
| 3  | *E. binotata* | Sideroxylon lanuginosum | Austin, TX | F.W. Stearns | 01-Apr-2007 | KX791063 | KX791124 |
| 4  | *E. binotata* | Carya | Mooresville, IN | R.L. Snyder | 11-Jun-2004 | KX791064 | KX791125 |
| 5  | *E. binotata* | Carya | Cloud Crossing, LA | C.P. Lin | 27-Apr-2003 | KX791065 | KX791126 |
| 6  | *E. binotata* | Celastrus scandens | Columbia, MO | R.B. Cocroft | 01-Jul-2002 | KX791066 | KX791127 |
| 7  | *E. binotata* | Celastrus scandens | Ithaca, NY | T.K. Wood & C.P. Lin | 06-Jul-2002 | KX791067 | KX791128 |
| 8  | *E. binotata* | Celastrus scandens | Stone Valley, PA | R.B. Cocroft & C.P. Lin | 04-Jun-2003 | KX791068 | KX791129 |
| 9  | *E. binotata* | Cercis Canadensis | Chinnabee, AL | C.P. Lin | 01-May-2003 | KX791069 | KX791130 |
| 10 | *E. binotata* | Cercis Canadensis | Rutledge, GA | R.B. Cocroft & C.P. Lin | 07-May-2003 | KX791070 | KX791131 |
| 11 | *E. binotata* | Cercis Canadensis | Effingham, IL | C.P. Lin | 21-May-2003 | KX791071 | KX791132 |
| 12 | *E. binotata* | Cercis Canadensis | Woodmont, MD | R.B. Cocroft & C.P. Lin | 07-Jun-2003 | KX791072 | KX791133 |
| 13 | *E. binotata* | Cercis Canadensis | Columbia, MO | R.B. Cocroft | 01-Jun-2002 | KX791073 | KX791134 |
| 14 | *E. binotata* | Cercis Canadensis | Tupelo, MS | C.P. Lin | 29-Apr-2003 | KX791074 | KX791135 |
| 15 | *E. binotata* | Cercis Canadensis | New Buffalo, PA | R.L. Snyder & N. Cai | 09-Jul-2002 | KX791075 | KX791136 |
| 16 | *E. binotata* | Cercis Canadensis | Parksville, TN | R.B. Cocroft & C.P. Lin | 03-May-2003 | KX791076 | KX791137 |
| 17 | *E. binotata* | Cercis Canadensis | Grafton, WV | R.B. Cocroft & C.P. Lin | 06-Jun-2003 | KX791077 | KX791138 |
| 18 | *E. binotata* | Dirca palustris | Bloomington, IN | R.E. Hunt | 15-Aug-2004 | KX791078 | KX791139 |
| 19 | *E. binotata* | Dirca palustris | White Lake, ON | R. Lee | 01-Jul-2004 | KX791079 | KX791140 |
| 20 | *Enchenopa* species | Unknown | Guatemala city, Guatemala | C.P. Lin | 20-Dec-1999 | KX791080 | KX791141 |
| 21 | *Enchenopa* species | Unknown | Gracias, Honduras | C.P. Lin & R.L. Snyder | 22-Jul-2001 | KX791081 | KX791142 |
| 22 | *Enchenopa* species | Unknown | Juticalpa, Honduras | C.P. Lin & R.L. Snyder | 24-Jul-2001 | KX791082 | KX791143 |
| 23 | *Enchenopa* species | Unknown | Lucerna, Honduras | C.P. Lin & R.L. Snyder | 21-Jul-2001 | KX791083 | KX791144 |
| 24 | *Enchenopa* species | Unknown | La Union, Honduras | C.P. Lin & R.L. Snyder | 25-Jul-2001 | KX791084 | KX791145 |
| 25 | *Enchenopa* species | Unknown | Bambito, Panama | T.K. Wood et al. | 18-Jan-2000 | KX791085 | KX791146 |
| 26 | *Enchenopa* species | Composite | Boquete, Panama | T.K. Wood & R.B. Cocroft | 06-Mar-1998 | KX791086 | KX791147 |
| 27 | *Enchenopa* species | Unknown | Panama | T.K. Wood et al. | 01-Jan-2000 | KX791087 | KX791148 |
| 28 | *Enchenopa* species | Diphyca robinoides | Pedasi, Panama | R.B. Cocroft | 13-Feb-2000 | KX791088 | KX791149 |
| 29 | *Enchenopa* species | Unknown | Gamboa, Panama | T.K. Wood et al. | 21-Jan-2000 | KX791089 | KX791150 |
| 30 | *Enchenopa* species | Unknown | La Union, Mexico | G. Moya Raygoza | 24-Oct-2001 | KX791090 | KX791151 |
| 31 | *Enchenopa* species | Unknown | La Huerta, Mexico | S.H. McKamey | 16-Oct-2001 | KX791091 | KX791152 |
| 32 | *Enchenopa* species | Unknown | Veracruz, Mexico | R.B. Cocroft & C.P. Lin | 2007 | KX791092 | KX791153 |
2.4 | Phylogeny of host plants

We extracted the tree topology of the eight plant orders that cover the confirmed hosts of the *E. binotata* species complex (i.e., Celastrales, Dipsacales, Ericales, Fabales, Fagales, Magnoliaceae, Malvales, and Sapindales) according to the backbone of angiosperm phylogenies from Soltis et al. (2011) and Ruhfel, Gitzendanner, Soltis, Soltis, and Burleigh (2014). We then detangled the subtrees within the two orders with multiple branches: Fagales and Dipsacales. Three host taxa were included in the order Fagales: *Juglans nigra*, *J. cinerea*, and *Carya*. According to molecular and morphological data, *J. nigra* and *J. cinerea* are more closely related to each other than to *Carya* (Aradhya, Potter, Gao, & Simon, 2007; Manos et al., 2007). Among the four host species of Dipsacales, *Viburnum prunifolium*, and *V. rufidulum* are the most closely related species, followed by *V. lentago* and *V. cassinoides* (Winkworth & Donoghue, 2005). The final host plant phylogeny (Figure 1) included only host plants of the collected *E. binotata* in this study and encompassed nearly all host plant species that have been recorded more than once.

2.5 | Evaluating the major causes of speciation in *E. binotata*

We conducted an event-based parallel cladogenesis reconstruction analysis in *Jane* version 4 (Conow, Fielder, Ovadia, & Libeskind-Hadas, 2010) to evaluate whether the *E. binotata* phylogeny matches the

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**TABLE 1** (Continued)

| No | Species | Host plant | Locality | Collectors | Collection date | Accession number (CO1) | Accession number (EF1α) |
|----|---------|------------|----------|------------|----------------|------------------------|-------------------------|
| 33 | Enchenopa species | Unknown | Veracruz, Mexico | R.B. Cocroft & C.P. Lin | 2007 | KX791093 | KX791154 |
| 34 | Enchenopa species | Unknown | Tegucigalpa, Honduras | C.P. Lin & R.L. Snyder | 24-Jul-2001 | KX791094 | KX791155 |
| 35 | Enchenopa species | Unknown | Trinidad, Honduras | C.P. Lin & R.L. Snyder | 29-Jul-2001 | KX791095 | KX791156 |
| 36 | Enchenopa species | Unknown | Chiriqui Grande, Panama | T.K. Wood & R.B. Cocroft | 05-Mar-1998 | KX791096 | KX791157 |
| 37 | *E. binotata* | *Juglans cinerea* | Bangor, NY | T.K. Wood | 30-Aug-1997 | KX791097 | KX791158 |
| 38 | *E. binotata* | *Juglans cinerea* | Ithaca, NY | T.K. Wood | 16-Jun-1996 | KX791098 | KX791159 |
| 39 | *E. binotata* | *Juglans nigra* | Woodmont, MD | R.B. Cocroft & C.P. Lin | 07-Jun-2003 | KX791099 | KX791160 |
| 40 | *E. binotata* | *Juglans nigra* | Columbia, MO | R.B. Cocroft | 01-Jul-2002 | KX791100 | KX791161 |
| 41 | *E. binotata* | *Liriodendron tulipifera* | Ithaca, NY | C.P. Lin | 16-Jul-2002 | KX791101 | KX791162 |
| 42 | *E. binotata* | *Liriodendron tulipifera* | Oxford, OH | R.L. Snyder | 14-Jun-2004 | KX791102 | KX791163 |
| 43 | *E. binotata* | *Liriodendron tulipifera* | Harveysburg, OH | R.L. Snyder | 14-Jun-2004 | KX791103 | KX791164 |
| 44 | *E. binotata* | *Ptelea trifoliata* | Yorkville, IL | R.L. Snyder | 03-Jun-2004 | KX791104 | KX791165 |
| 45 | *E. binotata* | *Ptelea trifoliata* | — | R.E. Hunt | 01-Jun-2002 | KX791105 | KX791166 |
| 46 | *E. binotata* | *Ptelea trifoliata* | Columbia, MO | R.B. Cocroft | 01-Jun-2002 | KX791106 | KX791167 |
| 47 | *E. binotata* | *Robinia pseudoacacia* | Woodmont, MD | R.B. Cocroft & C.P. Lin | 07-Jun-2003 | KX791107 | KX791168 |
| 48 | *E. binotata* | *Robinia pseudoacacia* | Columbia, MO | R.B. Cocroft | 01-Jul-2002 | KX791108 | KX791169 |
| 49 | *E. binotata* | *Robinia pseudoacacia* | Ithaca, NY | T.K. Wood & C.P. Lin | 06-Jul-2002 | KX791109 | KX791170 |
| 50 | *E. binotata* | *Robinia pseudoacacia* | Stone Valley, PA | R.B. Cocroft & C.P. Lin | 04-Jun-2003 | KX791110 | KX791171 |
| 51 | *E. binotata* | *Viburnum cassinooides* | Cherry Lane, NC | R.L. Snyder & N. Cai | 05-Jun-2003 | KX791111 | KX791172 |
| 52 | *E. binotata* | *Viburnum cassinooides* | Davis, WV | R.B. Cocroft & C.P. Lin | 06-Jun-2003 | KX791112 | KX791173 |
| 53 | *E. binotata* | *Viburnum lentago* | Bernheim, KY | R.E. Hunt | 01-Jun-2002 | KX791113 | KX791174 |
| 54 | *E. binotata* | *Viburnum lentago* | Ridgeway, PA | R.B. Cocroft & C.P. Lin | 05-Jun-2003 | KX791114 | KX791175 |
| 55 | *E. binotata* | *Viburnum prunifolium* | Columbia, MO | R.B. Cocroft | 27-Jun-2002 | KX791115 | KX791176 |
| 56 | *E. binotata* | *Viburnum prunifolium* | Stone Valley, PA | R.B. Cocroft & C.P. Lin | 04-Jun-2003 | KX791116 | KX791177 |
| 57 | *E. binotata* | *Viburnum prunifolium* | Amherst, VA | R.L. Snyder & N. Cai | 06-Jun-2003 | KX791117 | KX791178 |
| 58 | *E. binotata* | *Viburnum rufidulum* | Rutledge, GA | R.B. Cocroft & C.P. Lin | 07-May-2003 | KX791118 | KX791179 |
| 59 | *E. binotata* | *Viburnum rufidulum* | Columbia, MO | 01-Jun-2003 | KX791119 | KX791180 |
| 60 | *E. binotata* | *Viburnum rufidulum* | Greenville, SC | C.P. Lin | 08-May-2003 | KX791120 | KX791181 |
| 61 | *E. binotata* | *Viburnum rufidulum* | Nashville, TN | R.B. Cocroft & C.P. Lin | 02-May-2003 | KX791121 | KX791182 |
angiosperm phylogeny. The high level of host fidelity in E. binotata together with differences in life-history timing caused by host phenology (Wood, 1980; Wood & Keese, 1990; Wood, Tilmon, Shantz, Harris, & Pesek, 1999) suggest that host shifting might be more costly than phylogenetic tracking with the original host species. We therefore set the cost of phylogenetic tracking at a lower level (0 units) and the cost of host shifting at three different levels (0, 1, and 2 units) to explore the sensitivity of the results to various weighting schemes. The costs of duplication, loss, and failure to diverge were set as the default settings (all equal to 1, with the exception of duplication cost = 0 when the cost of host shifting = 0 due to the software limitation). We ran each model for $5 \times 10^5$ iterations and checked for convergence (Gelman & Rubin, 1992). For each model, we report the means of the posterior distributions and their 95% CIs as the parameter estimates.

3 | RESULTS

3.1 | Enchenopa Phylogenetic trees

The combined sequence matrix used for phylogenetic reconstruction was 1645 bp (GenBank accession numbers: CO1, KX791061-KX791121; EF1a, KX791122-KX791182; Table 1), with 429 parsimony-informative sites. The phylogenetic trees indicated that among E. binotata species feeding on 10 different host plant genera, six were monophyletic (Carya, Dirca, Juglans, Liriodendron, Pelelea, and Robinia), with the first five having more than 70% branch support from both ML bootstrapping and Bayesian posterior probability (BPP) (Figure 2). For the remaining E. binotata, those feeding on Celastrus and Cercis were paraphyletic, and those feeding on Viburnum and Sideroxylon were polyphyletic, with various levels of branch support (from <50% to 100% BPP), suggesting a probable effect of incomplete lineage sorting in recently diverged species. The support values of the E. binotata species tree were generally low (BPP mostly <30%; Figure 3), raising doubts on the robustness of the phylogeny of E. binotata and thus the evolutionary history interpreted from it. However, this tree topology largely agrees with a previous E. binotata phylogeny reconstructed from mtDNA with limited geographical sampling (Lin & Wood, 2002), indicating congruence of different data sets.

Because the topologies of the reconstructed phylogenetic trees based on different sets of mutation rates were similar, we only present the phylogenetic tree reconstructed with the average mutation rates (Figure 3). According to this phylogenetic tree, the Eastern North American E. binotata complex was estimated to have diverged from Central American Enchenopa species approximately 17.7 million years ago (Mya) in the early Miocene (Figure 3). The E. binotata feeding on Juglans diverged from the rest of E. binotata ~0.62 Mya in the middle Pleistocene, with a BPP of 100%. The remaining E. binotata species were inferred to diverge from each other more recently, between 29 and 117 thousand years ago in the late Pleistocene.

3.2 | Evaluating the major causes of speciation in E. binotata

The three different cost combinations of phylogenetic tracking and host shifting all resulted in more host shifting than phylogenetic tracking (Table 2). Therefore, we only present detailed results for the moderate setting, in which the cost of host shifting was 1. A total of 100,000 solutions were obtained from the event-based parallel cladogenesis analyses. All solutions suggested that the E. binotata complex and their host plants were estimated to undergo more host-shifting events ($n = 8$) than phylogenetic-tracking events ($n = 5$; Figure 4). The randomization analysis reported that the cost combinations from...
our results were significantly better than random cost combinations ($p < .01$). In addition, the estimated host shifting occurred regardless of the host plant relationships in the phylogenetic tree (Figure 4), indicating that host shifting was not restricted to plant species with intermediate relationships.

According to the test of co-phylogenesis, the clade ranks of the host plant orders were not significantly associated with the clade ranks of *E. binotata* (posterior mean = 0.06, 95% CI = −0.05 to 0.18). The estimated posterior mean of the phylogenetic heritability ($H^2$) was 50.37%, indicating a significant phylogenetic signal for the distribution of speciation events in the phylogeny.

4 | DISCUSSION

We found that the degree of phylogenetic association between the *Enchenopa binotata* species complex and their host plants does not support phylogenetic tracking as the major mechanism of speciation of *E. binotata*. In contrast, our results suggested that host shifting dominated the evolutionary history of the *E. binotata* complex. However, the existence of a few estimated events indicates that phylogenetic tracking might have occurred, although at a much lower frequency than host shifting. These results indicate that the plant–insect evolutionary interactions may have been the product of multiple mechanisms. Furthermore, because the host shifting between *E. binotata* species was not limited to immediately related host plants but also occurred across several plant orders (e.g., shifting from *Cercis* in Fabales to *Liriodendron* in Magnoliales), our results are also inconsistent with the intermediate hypothesis of host shifting.

Of the two causes of phylogenetic tracking, co-evolution has been the focus of extensive attention for decades (reviewed in Suchan & Alvarez, 2015). Vigorous discussions of co-evolution in recent years (Althoff, Segraves, & Johnson, 2014; Carmona et al., 2015; Hembry, Yoder, & Goodman, 2014; Martínez-Aquino, 2016; Suchan & Alvarez, 2015; Thompson, 2014) have suggested that event-based analyses may be inadequate because even if co-evolution dominated the evolutionary history of host plants and associated insects, their phylogenies may still be incongruent due to other events (e.g., occasional host shifting, lineage duplications, and/or lineage extinction; de Vienne et al., 2013). It is therefore advisable to determine the age of each node in the host and insect phylogenies to establish a more reliable test of temporal congruence in co-evolution events (Hafner et al., 1994; Page, 1996). This method cannot be formally applied in the current study due to the lack of divergence times among the host plants. However, the relatively newly evolved host plant genera (e.g., *Juglans* and *Carya*)
**Enchenopa binotata**

Our results instead suggest that host shifting between distantly related host plants plays a dominant role in the divergence of the *E. binotata* species complex. This conclusion is not surprising given that current evidence of co-evolution mostly comes from parasites or symbionts living inside their hosts (e.g., endosymbionts or ectosymbionts on internal surfaces of hosts; reviewed in de Vienne et al., 2013). In contrast to parasites and symbionts, *E. binotata* treehoppers are mobile and can freely move between plants, providing ample opportunities to encounter novel hosts.

The secondary chemical compounds, nutritional content, and phenology of plants (i.e., the contents of the resource space for each host plant) often constrain their utilization by insects and thus are frequently associated with insect divergence (especially the phenology of host plants in the divergence of *E. binotata*; Wood & Keese, 1990; Bruce, 2015). Closely related plant species frequently share similarities in chemical compounds, nutrition, and phenology (Davies et al., 2013; Prasad et al., 2012). Therefore, host shifting is traditionally expected to occur more often between closely related host plants. Given the lack of support in this study for phylogenetic tracking, this is unlikely to be the case between *E. binotata* and their host plants. Alternatively, the intermediate hypothesis predicts that the maximum probability of host shifting in *E. binotata* occurs between intermediately related host plants because these plants tend to share intermediate similarity.

### FIGURE 3

The Bayesian species tree of *Enchenopa binotata* from the software BEAST v1.8.2. The number at each node indicates the estimated divergence time with their 95% credible intervals, whereas the number above each branch presents the Bayesian posterior probability (BPP <40% not shown).

### TABLE 2

| Cost settings (Phylogenetic tracking, host shifting) | Estimated number of phylogenetic-tracking events | Estimated number of host-shifting events |
|---------------------------------------------------|-----------------------------------------------|---------------------------------------|
| (0, 0)                                            | 1                                             | 13                                    |
| (0, 1)                                            | 5                                             | 8                                     |
| (0, 2)                                            | 5                                             | 8                                     |
FIGURE 4 The host-shifting and co-evolution history of *Enchenopa binotata* and their host plants estimated from the event-based parallel cladogenesis reconstruction analysis.
with each other in the resource space. However, this hypothesis is not supported because the results suggest that E. binotata shifts between both intermediately related species (e.g., Viburnum in Dipsacales and Dirca in Malvales; Figures 1 and 4) and distantly related ones (e.g., Carya in Fagales and Liriodendron in Magnoliidae). These results suggest that distance in resource space does not explain the patterns of host shifting in E. binotata. As a phytophagous insect with high host fidelity (Wood et al., 1999), it is fascinating that E. binotata remains sufficiently evolutionarily flexible to shift between distant host plant species regardless of host phylogeny or positions in the resource space. These results also indicate that although plant phenology is largely responsible for the divergence between E. binotata, these treehoppers exhibit a range of plasticity to overcome potentially strong host selection, adapt to temporal differences between novel host plants, and successfully shift between distant host plants (Wood, 1993; Wood et al., 1999).

Once the restrictions of host plant use are relaxed, the temporal and spatial distributions of host plants and insects may become major determinants of host shifting (de Vienne et al., 2013). Specifically, the timing of the overlapping distribution of E. binotata species might be a key factor in host-shifting events. The recent overlapping area between Carya and Juglans is larger than that between Carya and Ptelea, similar to the distributions of E. binotata feeding on these plants (reviewed in Lin & Wood, 2002). Therefore, if the present distribution reflects past range to some extent, host shifting between Carya and Juglans is expected to be more likely than that between Carya and Ptelea. However, our results indicate that the recent range shifts of host plants and E. binotata are insufficient to explain host shifting by E. binotata. Alternatively, Enchenopa might have shifted to a novel host when these plant species expanded their distributions in Eastern North America after the last glacial period (~26,000 to 19,000 years ago; Clark et al., 2009). According to available data on the paleo-distribution of Carya and Juglans, both were distributed in Eastern North America ~20,000 years ago and reached 20% of dominance in at least half of this area 10,000–12,000 years ago (Delcourt & Delcourt, 1987). However, the E. binotata feeding on these plants are estimated to have diverged much earlier (114,000 and 189,000 years ago, respectively; Figure 3), which does not support this potential explanation. More detailed analyses, such as ecological niche modeling of both Enchenopa and their host plants, are required to further evaluate whether the paleo-distribution of the host plants was associated with host shifting of the E. binotata complex, which may have facilitated the speciation of the latter.

In conclusion, we have demonstrated that both phylogenetic tracking and host shifting played roles in the evolutionary history of the E. binotata species complex, with greater importance of host shifting compared to phylogeographic tracking. These results suggest multiple modes of insect–plant evolutionary interactions. Furthermore, Enchenopa treehoppers are capable of shifting between distantly related host plants, which is inconsistent with the general assumption of host shifting. This capability indicates that the evolution of E. binotata with high host fidelity could surprisingly be relaxed from the constraints set by the phenologies and defense mechanisms of their host plants. Such evolutionary plasticity suggests that, in addition to the dependence of host choice on host characteristics, recent and historical changes in the distributions of host plants and Enchenopa are key factors shaping the host-shifting history of E. binotata. Testing these hypotheses will require more detailed information on the distributions of both Enchenopa and their host plants across different time frames.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

CPL and RBC designed the study; CPL, RBC, and RLS collected samples; CPL and RLS extracted DNA and conducted sequencing; YHH conducted phylogenetic and statistical analyses; YHH wrote the draft with input from CPL and RBC.

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