



# Host shifts and signal divergence: mating signals covary with host use in a complex of specialized plant-feeding insects

REGINALD B. COCROFT<sup>1\*</sup>, RAFAEL L. RODRÍGUEZ<sup>1,2</sup> and RANDY E. HUNT<sup>3</sup>

<sup>1</sup>*Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA*

<sup>2</sup>*Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI 53201, USA*

<sup>3</sup>*Biology Department, Indiana University Southeast, New Albany, IN 47150, USA*

*Received 29 April 2009; accepted for publication 3 August 2009*

A combination of divergent natural and sexual selection is a powerful cause of speciation. This conjunction of evolutionary forces may often occur when divergence is initiated by ecological differences between populations because local adaptation to new resources can lead to changes in sexual selection. The hypothesis that differences in resource use contribute to the evolution of reproductive isolation by altering the nature of sexual selection predicts that: (1) differences in sexual traits, such as signals and preferences, are an important source of reproductive isolation between species using different resources; (2) there are identifiable sources of selection on sexual traits that differ between species using different resources; and (3) signals vary between populations using different resources to a larger extent than between populations using the same resource at different localities. Testing these predictions requires a group of closely-related species or populations that specialize on different resources and for which the traits involved in mate choice are known. The *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae) are host plant specialists in which speciation is associated with shifts to novel host plants. Mating in this complex is preceded by an exchange of vibrational signals transmitted through host plant stems, and the signal traits important for mate choice have been identified. In the *E. binotata* complex, previous work has supported the first two predictions: (1) signal differences between species are important in mate recognition and (2) host shifts can alter both the trait values favoured by sexual selection and the evolutionary response to that selection. In the present study, we tested the last prediction by conducting a large-scale study of mating signal variation within and between the 11 species in the complex. We find that differences in host use are strongly associated with differences in signal traits important for mate recognition. This result supports the hypothesis that hosts shifts have led to speciation in this group in part through their influence on divergence in mate communication systems. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **99**, 60–72.

**ADDITIONAL KEYWORDS:** phytophagous insects – specialization – speciation – vibrational communication.

## INTRODUCTION

Ecological speciation occurs when populations become reproductively isolated as they adapt to different environments (Rice, 1987; Schluter, 2001; Rundle & Nosil, 2005). When the traits influenced by local adaptation include mating signals and preferences, the combination of ecological differences and

divergent sexual selection is an efficient cause of speciation (West-Eberhard, 1983; Kirkpatrick & Ravigné, 2002; Ritchie, 2007). There are many ways in which differences in resource use can lead to a change in mate communication systems (Cocroft, Rodríguez & Hunt, 2008). These include developmental plasticity (Landolt & Phillips, 1997; Rodríguez & Greenfield, 2003; West-Eberhard, 2003; Grace & Shaw, 2004; Schlichting, 2004; Etges *et al.*, 2007; Beckers & Schul, 2008; Rodríguez *et al.*, 2008);

\*Corresponding author. E-mail: cocroft@missouri.edu

**Table 1.** Tests of predictions of the hypothesis that adaptation to the use of different resources leads to reproductive isolation through changes in sexual communication

Prediction	Case study								
	Sticklebacks	Mosquitofish	Darwin's finches	Fruit flies	Leaf beetles	Walking sticks	Brown planthoppers	Green lacewings	<i>Enchenopa</i> treehoppers
(1)	✓ <sup>1</sup>		✓ <sup>5</sup>	✓ <sup>7</sup>	✓ <sup>9</sup>	✓ <sup>10</sup>	✓ <sup>12</sup>	✓ <sup>14</sup>	✓ <sup>16</sup>
(2)	✓ <sup>2</sup>	✓ <sup>4</sup>	✓ <sup>6</sup>	✓ <sup>8</sup>				✗ <sup>15</sup>	✓ <sup>17</sup>
(3)	✓ <sup>3</sup>					✓ <sup>11</sup>	✗ <sup>13</sup>		✓ <sup>18</sup>

Predictions: (1) Differences in signals/preferences are an important source of reproductive isolation; (2) Direct or indirect selection on signals/preferences differs among populations using different resources (3) Resource use is the main predictor of variation in signals/preferences.

<sup>1</sup>Vines & Schluter (2006).

<sup>2</sup>Boughman, Rundle & Schluter (2005); McKinnon *et al.* (2004).

<sup>3</sup>Rundle *et al.* (2000).

<sup>4</sup>Langerhans, Gifford & Joseph (2007).

<sup>5</sup>Huber *et al.* (2007).

<sup>6</sup>Podos (2001).

<sup>7</sup>Rice & Hostert (1993); Rundle *et al.* (2005).

<sup>8</sup>Greenberg *et al.* (2003), although the authors' findings could not be replicated independently (Coyne & Elwyn, 2006)).

<sup>9</sup>Funk (1998).

<sup>10</sup>Nosil *et al.* (2002, 2007); Sandoval & Nosil (2005); Arbuthnott & Crespi (2009).

<sup>11</sup>Nosil *et al.* (2007).

<sup>12</sup>Claridge *et al.* (1985); Butlin (1993, 1996).

<sup>13</sup>Claridge (1985, 1990); Claridge *et al.* (1988).

<sup>14</sup>Wells & Henry (1992).

<sup>15</sup>Henry & Wells (2004).

<sup>16</sup>Rodríguez *et al.* (2004).

<sup>17</sup>Rodríguez *et al.* (2006); McNett & Coccoft (2008).

<sup>18</sup>Present study.

selection for tuning of preferences and/or signals to environmental conditions (Endler, 1992; Fleishman, 1992; Boughman, 2002; Seehausen *et al.*, 2008); differences in suites of predators (Abrahamson *et al.*, 2001), some of which may eavesdrop on signals (Zuk & Kolluru, 1998); demographic changes that alter mating systems (Shuster & Wade 2003); and ecological adaptation that incidentally changes signal production or perception (Miyatake & Shimizu, 1999; Podos, 2001; Vines & Schluter, 2006; Nosil *et al.*, 2007). In addition, colonization of a new resource by a small number of individuals could lead to changes in sexual communication as a result of drift or through selection for less choosy females (Kaneshiro & Giddings, 1987). Each of these mechanisms has the potential to alter the relationship between variation in signals and in mating success (i.e. to change the nature of sexual selection).

The hypothesis that adaptation to the use of different resources leads to reproductive isolation and divergence through changes in sexual selection makes one main prediction: (1) differences in sexual traits, such as signals and preferences, should be an impor-

tant source of reproductive isolation between species using different resources. Understanding the link between changes in resource use and divergence in sexual traits requires testing two further predictions: (2) there should be identifiable sources of selection (whether direct or indirect) on sexual traits that differ among populations using different resources. (3) Sexual traits should vary between populations using different resources to a larger extent than between populations on the same resource at different localities, thus distinguishing the effect of differences in resource use *per se* from the reproductive isolation that is expected to arise merely because populations on different resources are also separated in space. There has been mixed support for these predictions, with the most support accruing to the first (Rundle & Nosil, 2005; Funk, Nosil & Etges, 2006; Funk & Nosil, 2008). Most studies have tested only one or two predictions, however (Table 1), and the relationship between changes in resource use and divergence in sexual traits remains poorly understood. This is in part because strong tests of predictions (2) and (3) ideally require a group of several closely-related

species or populations that specialize on different resources and for which the traits involved in mate choice have been identified (Etges, 2002).

Study of the relationship between host shifts and divergence in sexual communication in the *Enchenopa binotata* Say species complex of treehoppers provides an ideal opportunity to test all of the above predictions (Cocroft *et al.*, 2008). The *E. binotata* complex is a model for studies of speciation through host plant shifts (Wood, 1993; Coyne & Orr, 2004). Host shifts have occurred between distantly-related plants that constitute different selective environments, as demonstrated by reduced juvenile survivorship after reciprocal transplants (Wood, 1993). Host shifts also lead to ecological isolation. Because the insects' life history is tightly coordinated with the phenology of their host plant, shifts to hosts with differing phenologies lead to differences in the timing of egg hatch, adult eclosion, and mating (Wood & Keese, 1990; Wood, Olmstead & Guttman, 1990). The high host fidelity of these insects further reduces encounters between individuals on different hosts (Wood, 1980; Tilmon, Wood & Pesek, 1998).

Reproductive isolation resulting from differences in sexual selection may be even stronger than ecological isolation in the *E. binotata* complex. When multiple species were experimentally placed in close proximity, interspecific courtship was frequent but rarely led to mating (Wood, 1980). The traits underlying this assortative mating were revealed by Hunt (1994), who showed that male *E. binotata* court females using substrate-borne vibrational advertisement signals. Subsequently, we have tested prediction (1), that differences in sexual communication systems between host-associated species are a source of reproductive isolation. For one population of *E. binotata* on *Viburnum*, females strongly discriminated against the signals of males from all but one of the other host-associated species tested (Rodríguez, Sullivan & Cocroft, 2004). Furthermore, female preferences for several male signal traits (studied in four sympatric species) have diverged between species, and male signal variation appears to have been strongly shaped by sexual selection exerted by female choice (Rodríguez, Ramaswamy & Cocroft, 2006). Differences in signals between species represent evolutionary divergence rather than phenotypic plasticity because most aspects of signals remain unchanged when individuals develop (Rodríguez *et al.*, 2008) or signal (Sattman & Cocroft, 2003) on a nonhost plant.

We have also tested prediction (2), that there are causal links between host shifts and the evolution of communication. Sensory drive (Endler, 1992; Boughman, 2002) is one possibility (e.g., divergent selection arising from differences in the vibration-transmitting properties of the host plants). Signal transmission

characteristics have been examined for hosts of two species in the *E. binotata* complex [those using *Cercis canadensis* (Fabaceae) and *Ptelea trifoliata* (Rutaceae)]. Comparison of male signals and plant transmission properties suggests that signal frequency has evolved in response to divergent selection for efficient transmission through the communication channels provided by their respective hosts (McNett & Cocroft, 2008). If this signal–environment match turns out to be a general pattern, it would have important consequences for reproductive isolation because frequency is the most important signal trait for mate recognition (Rodríguez *et al.*, 2006). In addition, divergence of female preferences across host plants, including preferences for signal frequency (Rodríguez *et al.*, 2006), contributes to the differences in selection acting on signals. An additional factor, wind-generated noise, does not appear to have a role in signal divergence. Wind is a major source of abiotic noise for vibrationally-communicating insects on plants, although the spectral properties of wind-induced vibrations do not differ between four host plant species in which they were measured (McNett, 2007; G. D. McNett & R. B. Cocroft, unpubl. data).

Even where selection on signals does not change after a host shift, the evolutionary response to that selection may change. Host species constitute different environments for development, and experimental host shifts reveal genotype  $\times$  environment interactions ( $G \times E$ ) in signals and changes in genetic correlations among signal traits (Rodríguez *et al.*, 2008). Thus, the genetic backgrounds of attractive phenotypes may vary across host plant species, and colonizing a new host could alter the dynamics of sexual selection (Rodríguez *et al.*, 2008).  $G \times E$  in male attractiveness also occurs as a consequence of host shifts in *Drosophila mojavensis* (Etges *et al.*, 2007, 2009).

Having shown that differences in sexual communication cause reproductive isolation, and that use of different host species can favour such differences, we now test prediction (3), that signals in the *E. binotata* complex vary between populations using different resources to a larger extent than between populations using the same resource at different localities. The *E. binotata* complex offers a powerful test of this prediction because it affords a categorical measure of variation in ecological factors (host species) that varies independently of geographical distance. If factors associated with host shifts are the main cause of signal diversification in *Enchenopa*, then differences in host plant use should explain a greater proportion of signal variation than differences in locality for populations using the same host plant. If this diversification is important in speciation, it should occur in the signal traits that most contribute to behavioural

isolation. If this prediction is not met, factors other than developing, signalling and selecting a mate on a new host may be responsible for signal diversification.

## MATERIAL AND METHODS

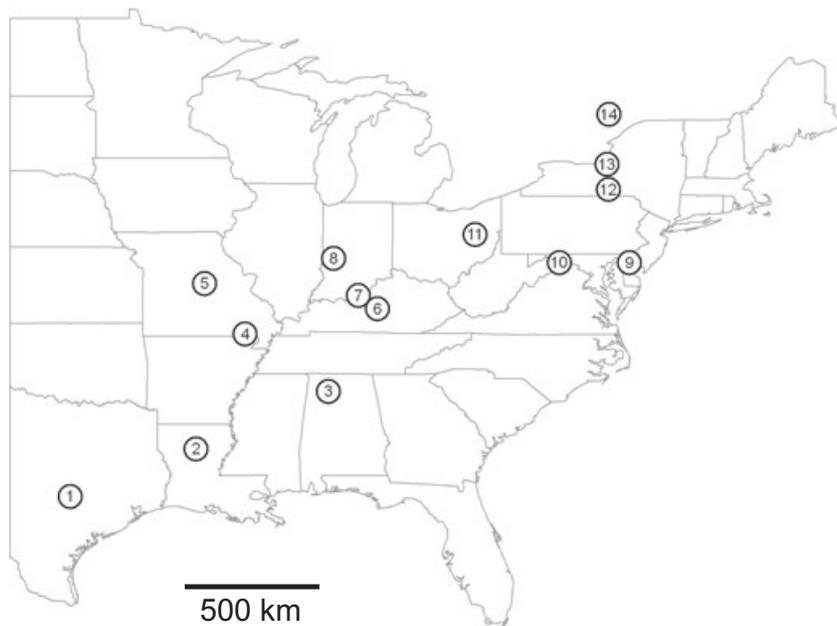
### SAMPLING

The *E. binotata* complex occurs throughout eastern North America (Lin & Wood, 2002). The present study aimed to record three populations spanning the distribution of each species, although, for rarer species, this was not possible (Fig. 1; for locality and sample size information, see Appendix). We collected nymphs and/or teneral adults in the field and reared them on potted host plants in the greenhouse [at both University of Missouri (MU) and Indiana University Southeast (IUS)]. This procedure ensured a reliable association between individuals and host plants because the presence of immatures indicates that reproduction has occurred on that plant, while adults can sometimes be encountered on nonhosts. Furthermore, species in the *E. binotata* complex are similar as adults, but distinctive in morphology and coloration during the nymphal stage (Pratt & Wood, 1992). The species in this complex have not yet been formally described, and we refer to them using their host plant genus.

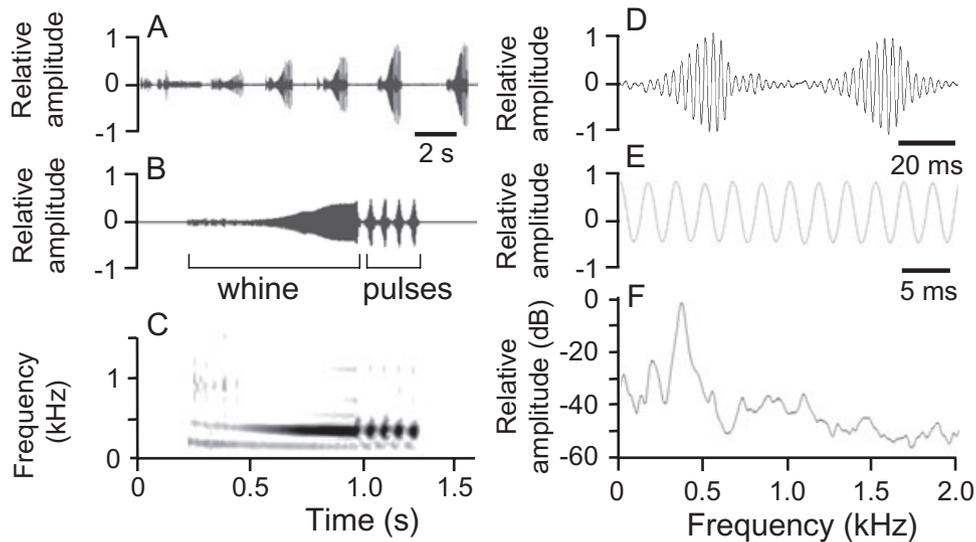
### SIGNAL MEASUREMENTS

Male signals in the *E. binotata* complex are produced in bouts in which the first few signals are lower in amplitude (Fig. 2A). Each signal, which is produced by abdominal tremulation, consists of a frequency-modulated whine followed by one or more pulses (Fig. 2B, C, D). Both whine and pulses are dominated by a single frequency (Fig. 2C, D, E, F), which is sometimes accompanied by lower-amplitude harmonics (Fig. 2C). At high densities, males may form stationary choruses (R. B. Cocroft, R. L. Rodriguez & R. E. Hunt, pers. observ.). We recorded individual males producing a single bout, as in Figure 2A. Comparisons among species are facilitated by the structural similarity of their signals (Fig. 3).

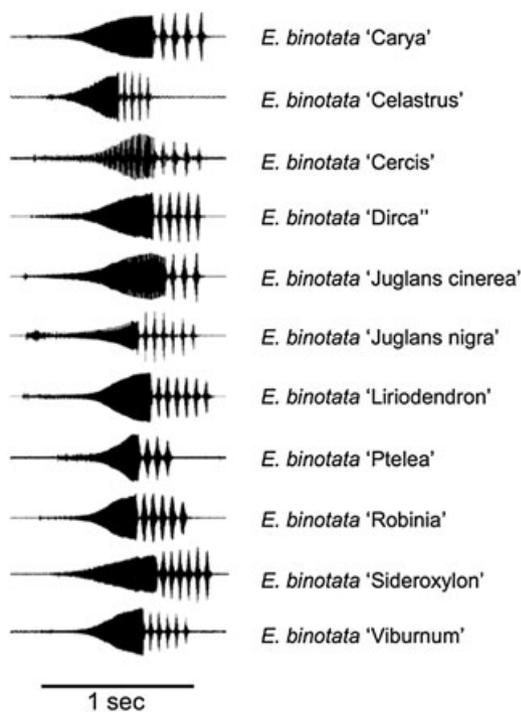
For each male, we measured the number of signals per bout, signal rate within a bout, whine length, frequency at the end of the whine, number of pulses, and pulse rate. To characterize signal frequency, we used time-domain rather than frequency-domain analysis. This was possible because of the relatively pure-tone nature of *Enchenopa* male signals (Fig. 2E). The frequency falls over the course of the whine, with most of the frequency change occurring during the initial low-amplitude portion (Fig. 2C). Although the frequency change is usually slight during the latter high-amplitude portion of the whine, measurements



**Figure 1.** Localities at which collections were made for recording male signals. Names given are the host plant genus from which *Enchenopa binotata* were collected. The localities are (with numbers corresponding to numbers on map): *Carya* (2,9); *Celastrus* (5, 7, 12); *Cercis* (5, 7, 10); *Dirca* (7, 8, 14); *Juglans cinerea* (13); *Juglans nigra* (5, 7, 10); *Liriodendron* (11); *Ptelea* (5, 7); *Robinia* (5, 7, 12); *Viburnum* (5, 6, 10); *Sideroxylon* (1, 3, 4). For locality and sample size information, see Appendix.



**Figure 2.** Representative substrate-borne male advertisement signal in *Enchenopa*; example is from *Enchenopa binotata* 'Robinia' in Indiana. A, waveform, showing one bout of six signals. B, waveform showing whine and pulse components of last signal from bout shown in (A). C, spectrogram of the same signal. D, waveform showing two pulses. E, waveform of the signal near the end of the whine, showing its tonal, sinusoidal nature. F, amplitude spectrum of the entire signal, showing single prominent frequency peak with other components 20 dB or more below.



**Figure 3.** Waveforms (with relative amplitude on the y-axis) of male advertisement signals of 11 species in the *Enchenopa binotata* complex.

made from amplitude spectra (as in Fig. 2F) would likely be influenced to some extent by host plant filtering properties. By measuring frequency from ten cycles at a consistent 'landmark', comprising the end

of the whine where amplitude reaches a peak (Fig. 2B), we avoid the influence of plant filtering on our measurements (Cocroft *et al.*, 2006). The frequency during the highest-amplitude portion of the signal is likely the most relevant for female choice: it has more energy than other frequencies in the signal, and synthetic signals containing only this frequency were approximately as attractive to females as natural signals in four species tested (R. L. Rodríguez & R. B. Cocroft, unpubl. data).

Recordings were made from 2001–06, both at IUS and at MU. The same protocols were used at each institution. Males were recorded 2–4 weeks after adult eclosion; a study of age-related variation in one species in the complex revealed no effect of age on signal variation over this time scale (Sattman & Cocroft, 2003).

**MU:** Each male was placed on the stem of a potted host plant, located on a vibration isolation table (Vibraplane, Kinetic Systems). Recordings were made by focusing the beam of a laser vibrometer (Polytec CLV 1000 with a CLV M030 decoder module; Polytec Inc.) on the host plant stem within 5 cm of the male. A small piece of reflective tape was attached to the stem to increase laser reflectance. The output was high-pass filtered at 60 Hz using a Krohn-Hite 3202 filter (Krohn-Hite Corporation) and sent to a Macintosh G4 computer through an Edirol UA-5 USB interface (Roland Corporation) and recorded with SoundEdit 16 Version 2 (Macromedia, Inc.) at 44.1-kHz sampling rate. We monitored male signals with a Radio Shack MPA-45 amplifier connected to an RCA

loudspeaker and a Hameg HM 203–7 20 MHz oscilloscope (Hameg Instruments). For one population (*E. binotata* ‘Dirca’ from Ontario), signals were recorded in the field using a phono cartridge, but measurements from these recordings are directly comparable with the other measurements in the study.

*IUS*: Except for the following differences in equipment, procedures were the same as those followed at MU. The vibration isolation table used was a TMC Model 63–541. Recordings were made using a Polytec OFV 353 laser vibrometer and OFV 2602 decoder module. The output was sent to a Macintosh G4 with an Audiomedia III (Digidesign) digital interface and recorded with PEAK, version 3.0 (BIAS). Male signals were monitored using a TASCAM DA-3-MK II digital tape recorder connected to headphones.

The recording room at each location was maintained at approximately 24 °C. Because there was some temperature variation within the recording rooms, all signal variables were examined for temperature-related variation within each population; in most cases there was no influence of temperature, although, for four populations, it was necessary to adjust pulse rate and frequency to a common temperature of 24 °C using the slope of the regression of the signal trait on temperature.

When characterizing variation in vibrational signals it is important to minimize variation as a result of substrate effects. This effort is critical when the goal is to evaluate differences among species that signal on different substrates. We conducted two studies evaluating the influence of differences among plant individuals and species on variation in substrate-borne signals. For *E. binotata* (Sattman & Cocroft, 2003), none of the features measured here were affected by differences among individual plants of the same species. Although basic signal structure also did not change when the same individual male signalled on a nonhost plant, males produced fewer, shorter signals on a nonhost (Sattman & Cocroft, 2003; Rodríguez *et al.*, 2008). Substrate-related variation can be minimized by making recordings close to the signaler on its own host species, as was performed in the present study, but it is not necessary to record all males of a given species on the same individual host plant (Cocroft *et al.*, 2006).

#### STATISTICAL ANALYSIS

For the variance component analysis, measurements for the signals within a bout were first averaged for each individual. We conducted a nested analysis of variance using PROC GLM in SAS, version 6.12 (SAS Institute). This analysis requires at least two populations per host, so we excluded two rare species (*E. binotata* ‘Juglans cinerea’ and *E. binotata* ‘Lirioden-

dron’) for which we obtained recordings for only one population. We used a model that corrects for the effects of the unbalanced design on the denominator degrees of freedom and mean square, with the effect of host species tested over the population-within-host species term, and the effect of population tested over error (Steel *et al.*, 1997). To test the prediction that host plant species should more strongly influence signal differences than geographic differences, we compared the relative magnitude of the variance components. We used PROC VARCOMP to partition variation in signal traits among host plant species, populations within host species, and individuals within populations (*sensu* Steel *et al.*, 1997).

#### RESULTS

There were significant differences among host-associated species in all signal traits (Table 2). There were also differences among populations within species (Table 2). We illustrate these findings with the two signal traits, namely frequency and whine length (Fig. 4), for which females in four species studied show the strongest preferences. Frequency and whine length preferences are closed (i.e. intermediate values are preferred) and the preferred values differ among species (Rodríguez *et al.*, 2006). For the other signal traits, females showed weak open preferences or no preference (Rodríguez *et al.*, 2006), so variation in these traits is unlikely to cause reproductive isolation. For frequency and whine length, there was a graded spectrum of geographic variation within species. In most species, there was little or moderate variation among populations (Fig. 4). However, for one species (*E. binotata* ‘Viburnum’), variation among populations was comparable to that among species.

To assess the relative contribution of host shifts and geographical distance to signal divergence, we compared the magnitude of the variance components of these two factors. In all cases, the variance component as a result of species differences was substantially larger than the variance component as a result of population differences (Fig. 5). The variance component as a result of individual differences within populations was substantial for all parameters except signal frequency.

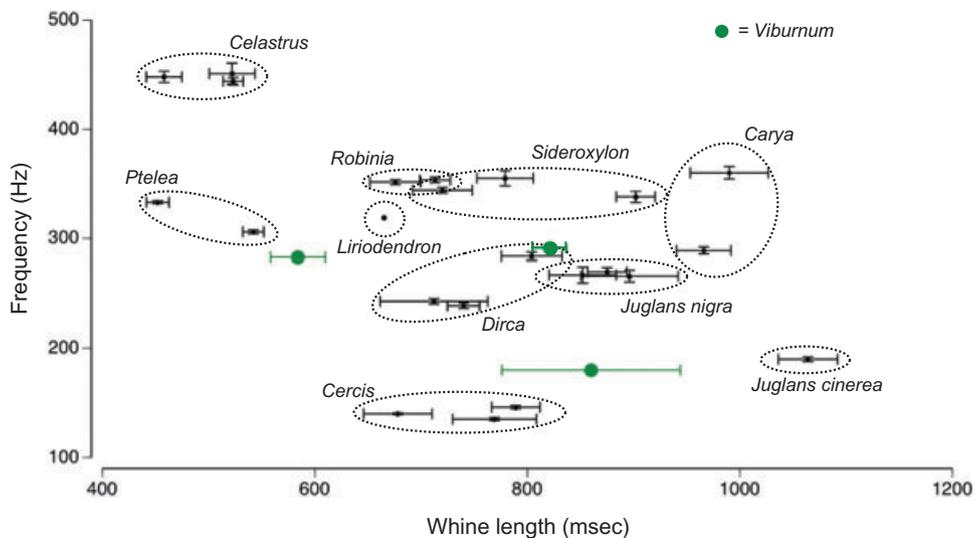
#### DISCUSSION

We tested a critical prediction of the hypothesis that host shifts have led to divergence in mate communication systems. Supporting this hypothesis, differences in male advertisement signals of *Enchenopa* treehoppers covary with differences in resource use. For most traits, geographical separation between populations using the same host plant species also

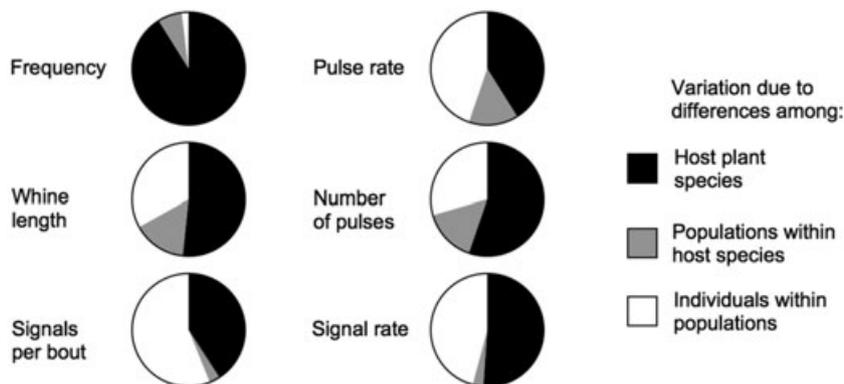
**Table 2.** Tests of hypotheses from mixed model analysis of variance, assessing variation due to use of different host plants (HOST), and to populations at different localities (LOC) using the same host

	Source	d.f.	MS	<i>F</i>	<i>Pr &gt; F</i>
Frequency	HOST	6	414986.75	32.74	0.0001
	Error	14.87	12676.82		
	LOC(HOST)	15	9471.75	58.00	0.0001
Whine length	HOST	6	1044027.84	8.53	0.0005
	Error	14.08	122376.90		
	LOC(HOST)	15	93966.00	8.21	0.0001
Signals/bout	HOST	6	232.94	18.67	0.0001
	Error	11.01	12.48		
	LOC(HOST)	15	10.75	1.83	0.0292
Signal rate	HOST	6	11752600.55	7.23	0.0012
	Error	13.74	1624873.81		
	LOC(HOST)	15	1273446.33	5.84	0.0001
Number of pulses	HOST	6	61.50	25.56	0.0001
	Error	11.33	2.41		
	LOC(HOST)	15	2.05	1.98	0.0155
Pulse rate	HOST	6	143.44	9.19	0.0003
	Error	14.16	15.60		
	LOC(HOST)	15	11.98	8.91	0.0001
	Error	356	1.34		

Note that model corrects for effects of unbalanced design on denominator d.f. and MS (see text).



**Figure 4.** Variation in frequency and whine length in the *Enchenopa binotata* complex. Shown are mean  $\pm$  95% confidence interval for each population, indicated by the name of their host plant; dotted lines surround populations of a given species, labelled according to host plant, except for the species on *Viburnum*, which is indicated by green (grey in print) symbols.



**Figure 5.** Relative magnitude of variance components in signal measurements from male *Enchenopa binotata* as a result of variation among host-associated species, among populations using the same host, and among individuals within populations.

has promoted changes in signals. The findings obtained in the present study thus provide support both for host shifts and host-independent effects as mechanisms of signal divergence. However, host shifts have a much stronger role, especially for signal frequency, the most important trait for mate recognition (Rodríguez *et al.*, 2006). Once differences in signal traits have appeared (especially in signal frequency and whine length, for which females show strong closed preferences), female mate choice is likely to reduce gene flow and promote further divergence. In the *E. binotata* complex, differences in mating signals and preferences are an important source of reproductive isolation (Rodríguez *et al.*, 2004, 2006), and there are causal links between host shifts and signal divergence (McNett & Cocroft, 2008; Rodríguez *et al.*, 2008). Together with the finding from the present study that signal variation is linked with resource use, these studies of sexual communication in *E. binotata* strongly support the hypothesis that host shifts contribute to speciation partly through their influence on the evolution of sexual communication.

Ecological and behavioural isolation are both important in the current coexistence of species in the *E. binotata* complex, and both may have been important during their initial divergence. Host shifts lead immediately to ecological isolation because sympatric individuals developing on different hosts eclose and mate at different times (Wood & Guttman, 1982; Wood & Keese, 1990; Wood *et al.*, 1990) and because these relatively sedentary insects show high host fidelity in mating and oviposition (Wood, 1980). Host shifts also impose divergent natural selection, leading to specialization on the new host (Wood & Guttman, 1983; Tilmon *et al.*, 1998). This combination of assortative mating and divergent selection may be sufficient to initiate speciation (Wood, 1993). However, we

suggest that divergence in communication systems acts in synergy with ecological isolation and may have been important for completing the speciation process. Ecological isolation among *E. binotata* populations on different hosts is leaky: there is considerable overlap of mating periods between individuals on some hosts (Wood & Guttman, 1982), and mate-searching males can sometimes be found on hosts of other species in the complex during the mating period (R. B. Cocroft, unpubl. data). The importance of behavioural isolation was made clear when breeding adults from multiple host-associated species, maintained on potted host plants, were placed in close proximity: mixed-species courtship was frequent but heterospecific matings were rare (Wood, 1980). Divergence in signals and preferences (Rodríguez *et al.*, 2004, 2006) contributes to this behavioural isolation.

The signals of male *Enchenopa* differ not only between populations using different hosts, but also to some extent among populations using the same host. This geographic variation could have its roots in interactions among *E. binotata* species because mate-searching males can sometimes be found on the 'wrong' host during the breeding season (R. B. Cocroft, unpubl. data). Host plants of the 11 species in the complex co-occur in a mosaic in which the set of common species differs among localities (Lin & Wood, 2002). As a consequence, processes such as gene flow among species on different hosts, or reinforcement in response to the cost of mismating, could promote different patterns of signal divergence at different localities. Signal variation could also be related to geographic variation in host plant characteristics, such as those that affect signal transmission, or to random factors such as genetic drift. Finally, in one case (*E. binotata* 'Viburnum'), recent work suggests the presence of two genetically distinct lineages with different signal frequencies (Snyder, 2008).

*Viburnum*-associated *E. binotata* populations use four closely-related hosts (*Viburnum cassinoides*, *Viburnum lentago*, *Viburnum prunifolium*, and *Viburnum rufidulum*), but both lineages occur on each host species.

Although we examined host-associated variation only in male signals, female preferences can also change after a host shift. Such changes can occur as a byproduct of adaptation to different environments (Rundle *et al.*, 2005); as a consequence of selection favouring the avoidance of mating with individuals adapted to different hosts (Nosil *et al.*, 2003); because of selection favouring sensitivity at the frequencies that best transmit through the environment (Boughman, 2002; McNett & Cocroft, 2008); through Fisherian dynamics (Kokko *et al.*, 2002; Mead & Arnold, 2004; Rodríguez *et al.*, 2008); through developmental effects on preferences (Rodríguez & Greenfield, 2003); or because different signal traits or values are associated with male viability or condition on different host plants (Proulx, 1999; Lorch *et al.*, 2003). Given that any or all of these factors may lead to changes in mating preferences, and that, once differences in preferences appear, the strength of sexual selection would facilitate reproductive isolation and divergence (West-Eberhard, 1983), it is perhaps surprising that there are so few examples of closely-related, host-specific insects for which signals and preferences have been investigated for their role in ecological speciation (Claridge, 1985; Claridge *et al.* 1988; Percy, Taylor & Kennedy, 2006; Etges *et al.*, 2007; Nosil *et al.*, 2007).

How general are the patterns seen in the *E. binotata* complex? Evidence that differences in host use promote differences in sexual communication also comes from the study of chemical signals in populations of *Timema* walkingsticks (Nosil *et al.*, 2007). Pairs of allopatric populations are more likely to be sexually isolated if they use different hosts than if they use the same host, as demonstrated by behavioural responses of males, and differences in olfactory cues likely explain these responses (Nosil *et al.*, 2007).

Another host-specific phytophagous insect in which the hypothesis that host shifts contribute to divergence through their effects on sexual selection has been examined in detail is the brown planthopper, *Nilaparvata lugens*. This species is a pest of rice (*Oryza*) and may be the result of two independent host shifts from *Leersia* plants (Jones *et al.*, 1996). The rice and *Leersia* forms occur syntopically over a broad geographic range in India, south-east Asia and Australia, and Claridge *et al.* (1985, 1988) examined geographic variation in mating signals over much of that range. The first prediction, that there is assortative mating based on differences in sexual commu-

nication, is met: variation in pulse repetition rate in male signals influences mate choice and contributes to assortative mating between the two forms (Claridge *et al.*, 1985, 1988; Butlin, 1993, 1996). Although prediction (2) has not been tested, Claridge *et al.* (1988) suggested that plant signal transmission characteristics were unlikely to account for the signal differences contributing to reproductive isolation. Butlin & Tregenza (1998) suggested that changes in signals were largely a result of drift, although a quantitative genetic analysis revealed a signature consistent with directional selection (Butlin, 1996). The third prediction, that signal variation is more strongly correlated with host use than with geography, is not met: pulse repetition rates of the *Leersia* form are faster than those of the rice form in some areas, but slower in others (i.e. geography appears to explain more variation in pulse rate than does host use) (Claridge *et al.*, 1988). Whatever the causes of rapid signal evolution in *Nilaparvata*, the evolution of behavioural isolation is important for species coexistence (Claridge *et al.*, 1988) and may have been important during speciation.

Another group providing an instructive contrast with the *E. binotata* complex is the *Chrysoperla carnea* species complex of lacewings. In both cases, closely-related species differ more in signals than in morphology, especially in the *C. carnea* complex where some species have been recognized solely on the basis of song differences (Henry, 1985). Lacewings are predators that use plant stems to transmit their substrate-borne signals, but are not host plant specialists like *Enchenopa*. Some lacewing species, however, are restricted to broad classes of substrates, such as woody versus herbaceous plants. Henry & Wells (2004) tested the hypothesis that the signals of conifer-dwelling and grass/herbaceous plant-dwelling lacewings are adapted to these substrates. This hypothesis makes the untested though reasonable assumption that variation in signal-filtering properties within these broad classes of plants is less than that between them, although Cokl *et al.* (2007) showed that structurally similar plant species can differ in vibration-transmitting properties. There did not appear to be a strong effect of substrate type on signal transmission, measured either as propagation fidelity or as preferences for signals propagated through native versus foreign substrates. On the basis of those results, and the pattern that some closely-related species in the *C. carnea* complex differ in song but not ecology, it was suggested that environment-independent mechanisms explain signal differences.

Host-specific phytophagous insects constitute a large fraction of terrestrial diversity, and provide many likely examples of ecological speciation (Tauber

& Tauber, 1989; Mallet, 2001; Price, 2002; Bush & Butlin, 2004). In these insects, diversification often occurs in concert with shifts to new host plants (Berlocher & Feder, 2002). After a host shift, ecological factors promote reproductive isolation between populations on ancestral and novel hosts (Tauber & Tauber, 1989; Wood, 1993; Berlocher & Feder, 2002; Funk *et al.*, 2002). However, ecological isolation is often partial, and speciation may require additional sources of reproductive isolation (Claridge *et al.*, 1988). A recent review of divergence in host-associated insects (Dres & Mallet, 2003) showed that gene flow was more restricted between closely-related taxa that experienced both ecological and behavioural isolation than between those that experienced ecological isolation alone. The latter were considered to be 'host races'; if these represent an intermediate stage of speciation, as was suggested by Dres & Mallet (2003), then the evolution of behavioural isolation may often be important during speciation in phytophagous insects. Host specialist insects provide an especially useful model for investigating the relationship between communication and speciation. They exhibit a great diversity of communication signals (Cocroft & Rodríguez, 2005), and the intimate relationship between insect and plant facilitates experimental study of the processes leading to divergence in mating signals and preferences. In this regard, the *E. binotata* complex (with its many members specializing on a diverse set of hosts, substantial signal divergence, and strong mate preferences) showcases an important and widespread process: the interaction between local adaptation and the evolution of reproductive isolation.

#### ACKNOWLEDGEMENTS

We thank Peter Dunn, Gerlinde Höbel, Robert Snyder, Linda Whittingham, and three anonymous reviewers for comments on the manuscript. We also thank Robert Lee, Chung Ping Lin, Robert Snyder, Frank Stearns, and Kelley Tilmon for assistance in collecting males for recording, and Barb Sonderman for help in maintaining insects and plants. This research was supported by NSF IBN 0132357 to R.B.C. and REH; NSF IBN 0318326 to R.B.C., R.L.R., and R.E.H.; and the University of Missouri Research Board.

#### REFERENCES

- Abrahamson WG, Eubanks MD, Blair CP, Blair P, Whipple AV. 2001.** Gall flies, inquilines, and goldenrods: a model for host-race formation and sympatric speciation. *American Zoologist* **41**: 928–938.
- Arbuthnott D, Crespi BJ. 2009.** Courtship and mate discrimination within and between species of *Timema* walking-sticks. *Animal Behaviour* **78**: 53–59.
- Beckers O, Schul J. 2008.** Developmental plasticity of mating calls enables acoustic communication in diverse environments. *Proceedings of the Royal Society of London Series B, Biological Sciences* **275**: 1243–1248.
- Berlocher S, Feder JL. 2002.** Sympatric speciation in phytophagous insects: moving beyond controversy? *Annual Review of Entomology* **47**: 773–815.
- Boughman JW. 2002.** How sensory drive can promote speciation. *Trends in Ecology and Evolution* **17**: 571–577.
- Boughman JW, Rundle HD, Schluter D. 2005.** Parallel evolution of sexual isolation in sticklebacks. *Evolution* **59**: 361–373.
- Bush GL, Butlin RK. 2004.** Sympatric speciation in insects. In: Dieckmann U, Doebeli M, Metz JAJ, Tautz D, eds. *Adaptive speciation*. Cambridge: Cambridge University Press, 229–248.
- Butlin RK. 1993.** The variability of mating signals and preferences in the brown planthopper, *Nilaparvata lugens*. *Journal of Insect Behavior* **6**: 125–140.
- Butlin RK. 1996.** Co-ordination of the sexual signalling system and the genetic basis of differentiation between populations in the brown planthopper, *Nilaparvata lugens*. *Heredity* **77**: 369–377.
- Butlin RK, Tregenza T. 1998.** Levels of genetic polymorphism: marker loci vs. quantitative traits. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* **353**: 187–198.
- Claridge MF. 1985.** Acoustic behavior of leafhoppers and planthoppers: species problems and speciation. In: Nault LR, Rodríguez JG, eds. *The leafhoppers and planthoppers*. Brisbane: John Wiley & Sons, 103–125.
- Claridge MF. 1990.** Acoustic recognition signals: barriers to hybridization in Homoptera Auchenorrhyncha. *Canadian Journal of Zoology* **68**: 1741–1746.
- Claridge MJ, Den Hollander J, Morgan JC. 1985.** The status of weed-associated populations of the brown planthopper, *Nilaparvata lugens* (Stål) – host race or biological species? *Zoological Journal of the Linnean Society* **84**: 77–90.
- Claridge MJ, Den Hollander J, Morgan JC. 1988.** Variation in hostplant relations and courtship signals of weed-associated populations of the brown planthopper, *Nilaparvata lugens* (Stål), from Australia and Asia: a test of the recognition species concept. *Biological Journal of the Linnean Society* **35**: 79–93.
- Cocroft RB, Rodríguez RL. 2005.** The behavioral ecology of insect vibrational communication. *BioScience* **55**: 323–334.
- Cocroft RB, Rodríguez RL, Hunt RE. 2008.** Host shifts, the evolution of communication and speciation in the *Enchenopa binotata* species complex of treehoppers. In: Tilmon KJ, ed. *Speciation, specialization and radiation: the evolutionary biology of insect and plant interactions*. Berkeley, CA: University of California Press, 88–100.
- Cocroft RB, Shugart HJ, Konrad KT, Tibbs K. 2006.** Variation in plant substrates and its consequences for insect vibrational communication. *Ethology* **112**: 779–789.

- Cokl A, Zorovic M, Millar J. 2007.** Vibrational communication along plants by the stink bugs *Nezara viridula* and *Murgantia histrionica*. *Behavioural Processes* **75**: 40–54.
- Coyne JA, Elwyn S. 2006.** Does the *desaturase-2* locus in *Drosophila melanogaster* cause adaptation and sexual isolation? *Evolution* **60**: 279–291.
- Coyne JA, Orr HA. 2004.** *Speciation*. Sunderland, MA: Sinauer Associates.
- Dres M, Mallet J. 2003.** Host races in plant-feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* **357**: 471–492.
- Endler JA. 1992.** Signals, signal conditions, and the direction of evolution. *American Naturalist* **139**: S125–S153.
- Etges WJ. 2002.** Divergence in mate choice systems: does evolution play by rules? *Genetica* **116**: 151–166.
- Etges WJ, Cardoso de Oliveira C, Gragg E, Ortíz-Barrientos D, Noor MAF, Ritchie MG. 2007.** Genetics of incipient speciation in *Drosophila mojavensis*. I. Male courtship song, mating success, and genotype  $\times$  environment interactions. *Evolution* **61**: 1106–1119.
- Etges WJ, Cardoso de Oliveira C, Ritchie MG, Noor MAF. 2009.** Genetics of incipient speciation in *Drosophila mojavensis*: II. Host plants and mating status influence cuticular hydrocarbon QTL expression and G  $\times$  E interactions. *Evolution* **63**: 1712–1730.
- Fleishman LJ. 1992.** The influence of the sensory system and the environment on motion patterns in the visual displays of anoline lizards and other vertebrates. *American Naturalist* **139**: S36–S61.
- Funk DJ. 1998.** Isolating a role for natural selection in speciation: host adaptation and sexual isolation in *Neochlamisus bebbianae* leaf beetles. *Evolution* **52**: 1744–1759.
- Funk DJ, Filchak KE, Feder JL. 2002.** Herbivorous insects: model systems for comparative study of speciation ecology. *Genetica* **116**: 261–267.
- Funk DJ, Nosil P. 2008.** Comparative analyses of ecological speciation. In: Tilmon KJ, ed. *Speciation, specialization and radiation: the evolutionary biology of insect and plant interactions*. Berkeley, CA: University of California Press, 117–135.
- Funk DJ, Nosil P, Etges WJ. 2006.** Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 3209–3213.
- Grace JL, Shaw KL. 2004.** Effects of developmental environment on signal-preference coupling in a Hawaiian cricket. *Evolution* **58**: 1627–1633.
- Greenberg AJ, Moran JR, Coyne JA, Wu C-I. 2003.** Ecological adaptation during incipient speciation revealed by precise gene replacement. *Science* **302**: 1754–1757.
- Henry CS. 1985.** Sibling species, call differences, and speciation in green lacewings (Neuroptera: Chrysopidae: Chrysoperla). *Evolution* **39**: 965–984.
- Henry CS, Wells MLM. 2004.** Adaptation or random change? The evolutionary response of songs to substrate properties in lacewings (Neuroptera: Chrysopidae: Chrysoperla). *Animal Behaviour* **68**: 879–895.
- Huber SK, De León LF, Hendry AP, Bermingham E, Podos J. 2007.** Reproductive isolation of sympatric morphs in a population of Darwin's finches. *Proceedings of the Royal Society of London Series B, Biological Sciences* **274**: 1709–1714.
- Hunt RE. 1994.** Vibrational signals associated with mating behavior in the treehopper, *Enchenopa binotata* Say (Hemiptera: Homoptera: Membracidae). *Journal of the New York Entomological Society* **102**: 266–270.
- Jones PL, Gacesa P, Butlin RK. 1996.** Systematics of brown planthopper and related species using nuclear and mitochondrial DNA. In: Symondson WOC, Liddell JE, eds. *The ecology of agricultural pests: biochemical approaches*. London: Chapman & Hall, 133–148.
- Kaneshiro KY, Giddings LV. 1987.** The significance of asymmetrical sexual isolation and the formation of new species. In: Hecht MK, Wallace B, Prance G, eds. *Evolutionary biology*. New York, NY: Plenum Publishing Co., **21**: 29–43.
- Kirkpatrick M, Ravigné V. 2002.** Speciation by natural and sexual selection: models and experiments. *American Naturalist* **159**: S23–S35.
- Kokko H, Brooks R, McNamara JM, Houston AI. 2002.** The sexual selection continuum. *Proceedings of the Royal Society of London Series B, Biological Sciences* **269**: 1331–1340.
- Landolt PJ, Phillips TW. 1997.** Host plant influences on sex pheromone behavior of phytophagous insects. *Annual Review of Entomology* **42**: 371–391.
- Langerhans RB, Gifford ME, Joseph EO. 2007.** Ecological speciation in *Gambusia* fishes. *Evolution* **61**: 2056–2074.
- Lin CP, Wood TK. 2002.** Molecular phylogeny of the North American *Enchenopa binotata* (Homoptera: Membracidae) species complex. *Annals of the Entomological Society of America* **95**: 162–171.
- Lorch PD, Proulx S, Rowe L, Day T. 2003.** Condition-dependent sexual selection can accelerate adaptation. *Evolutionary Ecology Research* **5**: 867–881.
- Mallet J. 2001.** The speciation revolution. *Journal of Evolutionary Biology* **14**: 887–888.
- McKinnon JS, Mori S, Blackman BK, David L, Kingsley DM, Jamieson L, Chou J, Schluter D. 2004.** Evidence for ecology's role in speciation. *Nature* **429**: 294–298.
- McNett GD. 2007.** Noise and signal transmission properties as agents of selection in the vibrational communication environment. PhD Dissertation, University of Missouri.
- McNett GD, Cocroft RB. 2008.** Host shifts favor vibrational signal divergence in *Enchenopa binotata* treehoppers. *Behavioral Ecology* **19**: 650–656.
- Mead LS, Arnold SJ. 2004.** Quantitative genetic models of sexual selection. *Trends in Ecology and Evolution* **19**: 264–271.
- Miyatake T, Shimizu T. 1999.** Genetic correlations between life-history and behavioral traits can cause reproductive isolation. *Evolution* **53**: 201–208.

- Nosil P, Crespi BJ, Gries R, Gries G. 2007.** Natural selection and divergence in mate preference during speciation. *Genetica* **129**: 309–327.
- Nosil P, Crespi BJ, Sandoval CP. 2002.** Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* **417**: 440–443.
- Nosil P, Crespi BJ, Sandoval CP. 2003.** Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270**: 1911–1918.
- Percy DA, Taylor GS, Kennedy M. 2006.** Psyllid communication: acoustic diversity, mate recognition and phylogenetic signal. *Invertebrate Systematics* **20**: 431–445.
- Podos J. 2001.** Correlated evolution of morphology and vocal structure in Darwin's finches. *Nature* **409**: 135–140.
- Pratt G, Wood TK. 1992.** A phylogenetic analysis of the *Enchenopa binotata* species complex (Homoptera: Membracidae) using nymphal characters. *Systematic Entomology* **17**: 351–357.
- Price PW. 2002.** Resource-driven terrestrial interaction webs. *Ecological Research* **17**: 241–247.
- Proulx SR. 1999.** Mating systems and the evolution of niche breadth. *American Naturalist* **154**: 89–98.
- Rice WR. 1987.** Speciation via habitat specialization: the evolution of reproductive isolation as a correlated character. *Evolutionary Ecology* **1**: 301–314.
- Rice WR, Hostert EE. 1993.** Laboratory experiments on speciation: what have we learned in 40 years? *Evolution* **47**: 1637–1653.
- Ritchie MG. 2007.** Sexual selection and speciation. *Annual Review of Ecology, Evolution and Systematics* **38**: 79–102.
- Rodríguez RL, Greenfield MD. 2003.** Genetic variance and phenotypic plasticity in a component of female mate choice in an ultrasonic moth. *Evolution* **57**: 1304–1313.
- Rodríguez RL, Ramaswamy K, Cocroft RB. 2006.** Evidence that female preferences have shaped male signal evolution in a clade of specialized plant-feeding insects. *Proceedings of the Royal Society of London Series B, Biological Sciences* **273**: 2585–2593.
- Rodríguez RL, Sullivan LE, Cocroft RB. 2004.** Vibrational communication and reproductive isolation in the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). *Evolution* **58**: 571–578.
- Rodríguez RL, Sullivan LM, Snyder RL, Cocroft RB. 2008.** Host shifts and the beginning of signal divergence. *Evolution* **62**: 12–20.
- Rundle HD, Chenoweth SF, Doughty P, Blows MW. 2005.** Divergent selection and the evolution of signal traits and mating preferences. *PLoS Biology* **3**: 1988–1995.
- Rundle HD, Nagel L, Boughman JW, Schluter D. 2000.** Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287**: 306–308.
- Rundle HD, Nosil P. 2005.** Ecological speciation. *Ecology Letters* **8**: 336–352.
- Sandoval CP, Nosil P. 2005.** Counteracting selective regimes and host preference evolution in ecotypes of two species of walking-sticks. *Evolution* **59**: 2405–2413.
- Sattman DA, Cocroft RB. 2003.** Phenotypic plasticity and repeatability in the mating signals of *Enchenopa* treehoppers, with implications for reduced gene flow among host-shifted populations. *Ethology* **109**: 981–994.
- Schlichting CD. 2004.** The role of phenotypic plasticity in diversification. In: DeWitt TJ, Scheiner SM, eds. *Phenotypic plasticity: functional and conceptual approaches*. Oxford: Oxford University Press, 191–200.
- Schluter D. 2001.** Ecology and the origin of species. *Trends in Ecology and Evolution* **16**: 372–380.
- Seehausen O, Terai Y, Magalhaes IS, Carleton KL, Mrosso HDJ, Miyagi R, van der Sluijs I, Schneider MV, Maan ME, Tachida H, Imai H, Okada N. 2008.** Speciation through sensory drive in cichlid fish. *Nature* **455**: 620–626.
- Shuster SM, Wade MJ. 2003.** *Mating systems and strategies*. Princeton, NJ: Princeton University Press.
- Snyder RL. 2008.** Diversification in plant feeding insects: patterns of host-plant specialization and mating signal evolution inferred from species-level phylogeny and population genetics. PhD Dissertation.
- Steel RGD, Torrie JH, Dickey DA. 1997.** *Principles and procedures of statistics*, 3rd edn. Toronto: McGraw-Hill.
- Tauber CA, Tauber MJ. 1989.** Sympatric speciation in insects: perception and perspective. In: Otte D, Endler JA, eds. *Speciation and its consequences*. Sunderland, MA: Sinauer Associates, 307–344.
- Tilmon KJ, Wood TK, Pesek JD. 1998.** Genetic variation in performance traits and the potential for host shifts in *Enchenopa* treehoppers (Homoptera: Membracidae). *Annals of the Entomological Society of America* **91**: 397–403.
- Vines TH, Schluter D. 2006.** Strong assortative mating between allopatric sticklebacks as a by-product of adaptation to different environments. *Proceedings of the Royal Society of London Series B, Biological Sciences* **273**: 911–916.
- Wells MM, Henry CS. 1992.** The role of courtship songs in reproductive isolation among green lacewings of the genus *Chrysoperla* (Neuroptera: Chrysopidae). *Evolution* **46**: 31–42.
- West-Eberhard MJ. 1983.** Sexual selection, social competition, and speciation. *Quarterly Review of Biology* **58**: 155–183.
- West-Eberhard MJ. 2003.** *Developmental plasticity and evolution*. New York, NY: Oxford University Press.
- Wood TK. 1980.** Divergence in the *Enchenopa binotata* Say complex (Homoptera: Membracidae) effected by host plant adaptation. *Evolution* **34**: 147–160.
- Wood TK. 1993.** Speciation of the *Enchenopa binotata* complex (Insecta: Homoptera: Membracidae). In: Lees DR, Edwards D, eds. *Evolutionary patterns and processes*. New York, NY: Academic Press, 299–317.
- Wood TK, Guttman SI. 1982.** Ecological and behavioral basis for reproductive isolation in the sympatric *Enchenopa*

- binotata* complex (Homoptera: Membracidae). *Evolution* **36**: 233–242.
- Wood TK, Guttman SI. 1983.** *Enchenopa binotata* complex: sympatric speciation? *Science* **220**: 310–312.
- Wood TK, Keese MC. 1990.** Host–plant–induced assortative mating in *Enchenopa* treehoppers. *Evolution* **44**: 619–628.
- Wood TK, Olmstead KL, Guttman SI. 1990.** Insect phenology mediated by host–plant water relations. *Evolution* **44**: 629–636.
- Zuk M, Kolluru GR. 1998.** Exploitation of sexual signals by predators and parasitoids. *Quarterly Review of Biology* **73**: 415–438.

## APPENDIX

List of populations by host plant, number on map in Fig. 1, and locality (by state abbreviation in the USA), with number of males recorded from that host at that locality (*Dirca palustris*: ONT = Ontario, Canada)

Host plant	Map number	Locality	Number of males recorded
<i>Carya</i> spp.	2	LA: Winn Co.	4
	9	MD: Cecil Co.	2
<i>Celastrus scandens</i>	5	MO: Boone Co.	14
	7	IN: New Albany	8
	12	NY: Ithaca	13
<i>Cercis canadensis</i>	5	MO: Columbia	31
	7	IN: New Albany	20
	10	MD: Green Ridge	12
<i>Dirca palustris</i>	14	ONT: White Lake	15
	8	IN: New Harmony	7
	7	IN: Harrison Co.	4
<i>Juglans cinerea</i>	13	NY: Oswego	3
<i>Juglans nigra</i>	5	MO: Columbia	14
	7	IN: Greenville	5
	10	MD: Little Orleans	8
<i>Liriodendron</i>	11	OH: Wooster	1
<i>Ptelea trifoliata</i>	5	MO: Columbia	51
	7	IN: Crawford Co.	25
<i>Robinia pseudoacacia</i>	5	MO: Columbia	19
	7	IN: New Albany	20
<i>Sideroxylon Lycioides</i>	3	AL: Moulton	14
	4	MO: Van Buren	6
<i>S. lanuginosum</i>	1	TX: Austin	4
<i>Viburnum lentago</i>	6	KY: Bernheim Forest	24
<i>V. prunifolium</i>	5	MO: Columbia	45
	10	MD: Little Orleans	6