Divergent host-plant use promotes reproductive isolation among cynipid gall wasp populations

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Ecological speciation occurs when reproductive isolation evolves as a consequence of divergent natural selection among environments. A direct prediction of this process is that ecologically divergent pairs of populations will exhibit greater reproductive isolation than ecologically similar pairs of populations. By comparing allopatric populations of the cynipid gall wasp Belonocnema treatae infesting Quercus virginiana and Quercus geminata, we tested the role that divergent host use plays in generating ecological divergence and sexual isolation. We found differences in body size and gall structure associated with divergent host use, but no difference in neutral genetic divergence between populations on the same or different host plant. We observed significant assortative mating between populations from alternative host plants but not between allopatric populations on the same host plant. Thus, we provide evidence that divergent host use promotes speciation among gall wasp populations.

Keywords: ecological speciation; host race; Cynipidae

1. INTRODUCTION

Understanding the mechanisms contributing to speciation is a fundamental question in biology. Ecological speciation, a process in which reproductive isolation evolves as a by-product of divergent natural selection among environments, may be an important mechanism of speciation [1,2]. Geographically separated (allopatric) pairs of populations in ecologically similar habitats may diverge owing to genetic drift, sexual conflict and certain forms of sexual selection, whereas populations in ecologically different habitats may diverge owing to any of these processes as well as divergent natural selection [3–5]. Thus, ecological speciation theory predicts that ecologically divergent pairs of populations will exhibit greater reproductive isolation than ecologically similar pairs of populations, ceteris paribus [3,4]. This experimental framework isolates the role of natural selection in generating reproductive isolation while controlling for other host-independent factors and assumes populations of a similar age, yet explicit tests of hypotheses using the powerful same-environment/different-environment comparative method remain rare [3,5–7; reviewed in [8]]. Much of the support for ecological speciation comes from studies of herbivorous insects inhabiting different host plants where pairs of populations on the same host plant species offer ecologically similar comparisons (hereafter, ‘same-host’ pairs) and pairs of populations on different host plants offer ecologically different comparisons (hereafter, ‘different-host pairs’) [3–5,9].

In the southeastern United States, the cynipid gall-forming wasp, Belonocnema treatae, attacks two sister species of live oak within the series Virentes, Quercus virginiana and Quercus geminata, which overlap in geographical range, but occupy different habitats. Specifically, Q. virginiana occurs in moister, more nutrient-rich, and higher pH sites than Q. geminata [10]. The two species differ in leaf morphology, flowering time, and growth and photosynthetic rates [10]. Thus, populations of B. treatae on these two oak species may experience divergent natural selection.

Here we test a main prediction of ecological speciation theory: allopatric populations of B. treatae inhabiting different host plant species will exhibit greater differences in ecologically important traits (e.g. body size [11] and gall morphology [12]) and express greater sexual isolation than do allopatric populations on the same host plant. To conduct our same-host and different-host comparisons, we used gall wasps from six allopatric populations (three Q. geminata and three Q. virginiana), yielding 15 pairwise comparisons (nine different-host and six same-host). Patterns of divergence among populations are best understood within a historical context addressing the relative age of geographical separation and restricted gene flow. Here, we estimate relative divergence times among populations using sequence divergence from mtDNA collected from each study population.

2. MATERIAL AND METHODS

(a) Study system and sampling
Belonocnema treatae exhibits a heterogonous life cycle [13]. The asexual generation develops within leaf galls during the summer and autumn and emerges in the winter, whereas the sexual generation develops within root galls and emerges during the spring. We collected root galls from six allopatric populations (three Q. geminata and three Q. virginiana) in April 2010 (figure 1a). Galls were husbanded under common conditions (12 L: 12 D, 23°C), and upon emergence, root galls were dissected to count the number of chambers per gall on each host plant.

(b) Body size and gall morphology
The body size of sexual generation adults reared from each collection site was indexed by measuring the length of the right hind tibia. Sexes were pooled, as tibia lengths did not differ between males and females (ANOVA: F1,185 = 1.9, p > 0.15). Root galls of B. treatae are typically multi-chambered, with one adult developing per chamber [13]. Following emergence, root galls were dissected to count the number of chambers per gall on each host plant.

(c) Sexual isolation assays
No-choice mating trials were conducted to assess assortative mating as a function of host plant and collection site. Trials took place within 25 × 8 cm clear plastic cups provisioned with a dried, defoliated twig from a neutral host (Prunus serotina). For each trial, one male and one female each from a different collecting site were aspirated into the cup and then observed at 5 min intervals for 1 h (ν = 12 observations, sample sizes in table 1 and figure 2). For each observation, we recorded whether the pair was copulating.
(d) **Genetic analysis**

To control for the confounding effects of time since divergence among allopatric populations, sequence divergence among *B. treatae* populations was evaluated for, in total, 44 individual wasps (equally divided by sex) based on two putatively neutrally evolving mtDNA genes, a 416 bp long segment of the cytochrome *b* (*cyt b*) gene and a 593 bp segment of the cytochrome oxidase 1 (*COI*). Sample sizes for the six collecting sites were *S* = 7, *ABS* = 8, *AP* = 7, *HH* = 9, *KSP* = 7 and *GR* = 6 (figure 1a). See electronic supplementary material for sequencing methods. Sequences were deposited in GenBank (accession nos. JQ438777–JQ438824). *Cyt b* and *COI* sequences were concatenated, and mean pairwise sequence divergences among populations were calculated in MEGA v. 5 [14] with a Kimura two-parameter model [15]. We also sequenced a 257 bp nuclear ITS2 region, but found no evidence of variation at this marker, as one might expect for recently diverging lineages (see electronic supplementary material).

(e) **Statistical analysis**

Our study design generated datasets in the form of matrices of pairwise comparisons. To compare elements between matrices, Mantel tests [16] were run in the ‘ecodist’ package in R v. 2.11.1 with 10 000 randomizations and one-tailed hypothesis testing. Additionally, we used logistic regression to examine the effects of male host plant, female host plant and their interaction across all populations on copulation frequency, and then separately for each pairwise comparison. Finally, we estimated the degree of sexual isolation for each pairwise population comparison by using a standard metric of sexual isolation, $I_{PSI}$ (0, random mating, +1, complete assortative mating) [17].

3. RESULTS

Pairwise mtDNA sequence divergence among populations ranged from 1.0 to 5.1 per cent but was not associated with host use, as same-host pairs exhibited similar patterns of divergence as different-host pairs (Mantel $r = 0.06$, $p > 0.25$; figure 1b). Isolation-by-distance was not detected (Mantel $r = 0.18$, $p = 0.32$). However, consistent differences in both body size (Mantel $r = 0.85$, $p < 0.05$; figure 1c) and gall structure (Mantel $r = 0.83$, $p < 0.05$; figure 1d) were associated with differences in host-plant use. Larger adults and more chambers per root gall were evident for *B. treatae* populations on *Q. geminata* (figure 1c,d).

Most importantly, patterns of copulation frequency (no. of copulations/no. of mating trials) revealed strong evidence of host-associated sexual isolation. Overall, copulation was more likely if paired individuals were from the same host plant (logistic regression: male host plant × female host plant interaction, likelihood-ratio test $= 37.84$, d.f. = 1, $p < 0.0001$; figure 2) and this was consistent across host plant origin. Indeed, the mean $I_{PSI}$ value for sexual isolation between pairs of allopatric populations using different host plants ($0.31 \pm 0.06$ s.e.) was significantly greater than the mean value between allopatric pairs of populations using the same host plant ($0.01 \pm 0.04$ s.e.; Mantel $r = 0.76$, $p < 0.05$; table 1).

4. DISCUSSION

We have demonstrated that divergent host-plant use is associated with differences in trait values and prezygotic reproductive isolation between *B. treatae* populations (table 1 and figure 2). Our results are
consistent with partial sexual isolation evolving in this gall wasp species as a by-product of adaptation to different hosts [18].

Reproductive isolation often increases with time, where time is estimated using genetic distance at neutral loci [18]. However, time since divergence among the six allopatric B. treatae populations examined is unlikely to confound the observed associations between host-plant use and reproductive isolation for two reasons. First, allopatric different-host pairs are not more genetically divergent than allopatric same-host pairs. Second, direct comparison of sexual isolation with genetic distance among populations shows that the degree of between-population sexual isolation is not correlated with genetic distance (Mantel test statistic from likelihood-ratio test of male pop. interaction in logistic regression).

Table 1. Sexual isolation among B. treatae populations (population host association: Qv, Q. virginiana; Qg, Q. geminata; pop. label subscripted as in figure 1a. I_psi index of sexual isolation; n, sample size per comparison; χ², test statistic from likelihood-ratio test of male pop. x female pop. interaction in logistic regression).

<table>
<thead>
<tr>
<th>population comparisons</th>
<th>I_psi</th>
<th>n</th>
<th>χ²</th>
<th>p-value</th>
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<tr>
<td>different-host</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>QvGR × QvHH</td>
<td>0.19</td>
<td>39</td>
<td>3.15</td>
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<td>QvGR × QvKSP</td>
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<tr>
<td>QvGR × QvGR</td>
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<td>34</td>
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<td>0.019</td>
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<tr>
<td>QvHH × QvHH</td>
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<td>34</td>
<td>7.69</td>
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<td>0.23</td>
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<td>QvKSP × QvHH</td>
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<td>QvKSP × QvKSP</td>
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<td>1.25</td>
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<tr>
<td>QvAP × QvAP</td>
<td>−0.03</td>
<td>35</td>
<td>1.90</td>
<td>0.168</td>
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<td>0.01</td>
<td>0.993</td>
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<td>QvAP × QvKSP</td>
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<td>1.12</td>
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<td>QvAP × QvAP</td>
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<td>0.39</td>
<td>34</td>
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<td>0.019</td>
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<tr>
<td>QvHH × QvKSP</td>
<td>0.03</td>
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<td>0.17</td>
<td>0.677</td>
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</table>

In conclusion, the same-host/different-host comparisons produced evidence consistent with divergent selection promoting speciation for B. treatae populations on alternative host plants and hints at the possible general role of host plant-driven diversification in cynipid oak systems. Our study provides the first evidence that supports predictions of ecological speciation theory for the species-rich and ecologically diverse Cynipidae.

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