Asymmetric habitat isolation and sexual isolation predicted by the cost of migration and hybridization generate novel signatures of reinforcing selection

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Abstract

Reinforcement is an evolutionary process whereby increased prezygotic reproductive isolation evolves in response to the cost of hybridization. Despite theory predicting that multiple prezygotic barriers can evolve via reinforcement, most empirical studies examine a single barrier. We test novel predictions for the reinforcement of both habitat isolation and sexual isolation between ecologically divergent lineages under asymmetric migration—the lineage that emigrates more should evolve stronger habitat isolation due to the lower fitness of immigrants in the alternative habitat, while the lineage that receives more immigrants should exhibit stronger sexual isolation due to the lower fitness of hybrids. We found both signatures of reinforcement in two sympatric sister species of gall wasps that are host specific to the southern live oaks, *Quercus virginiana* and *Q. geminata*, respectively. Specifically, we observed stronger habitat isolation in the species with higher emigration rates, *Belonocnema treatae*, and stronger sexual isolation in the species facing more immigrants, *B. fossoria*. In contrast, comparisons of both species to a third, allopatric, species showed that *B. kinseyi* exhibited both lower habitat isolation and sexual isolation than the sympatric species, consistent with the classic predictions of reinforcement. Our study provides a rare examination of the interplay of ecology and geography in the evolution of multiple reproductive barriers to gene flow. Given that asymmetric migration between ecologically divergent lineages increasingly appears to be the rule rather than the exception, concomitant asymmetries in the strength of habitat and sexual isolation could be more widespread than currently understood.

Keywords: ecological speciation, gall wasp**,** reinforcement, reproductive isolation

Introduction

Reinforcement describes the evolutionary process by which selection favors increased prezygotic reproductive isolation in response to the costs of hybridization, which include reproductive interference or lower hybrid fitness [1]. Despite theory predicting that multiple prezygotic barriers (e.g., temporal, habitat, and/or sexual isolation) could evolve via reinforcement [2,3], the majority of studies addressing reinforcement focus exclusively on sexual isolation [4–7]. Relatively few studies have tested the response of other forms of prezygotic reproductive isolation to reinforcement (e.g., habitat isolation [8] and gametic isolation [9]). To our knowledge, only one empirical study to date has tested the role of reinforcement on two or more prezygotic barriers simultaneously within a single natural study system [10]. Filling this knowledge gap is important as multiple prezygotic barriers are often observed among closely related sympatric lineages [11–16]. In particular, habitat isolation has been identified as an important and common prezygotic barrier during ecological speciation [11,17–20]. Therefore, empirical tests that assess whether habitat isolation and/or sexual isolation evolve jointly via reinforcement within single study systems are needed.

Few theoretical studies have examined whether habitat isolation or sexual isolation more readily evolves under reinforcement and how these barriers interact when evolving simultaneously [3,21]. Model predictions are mixed, with Yukilevich and True (2006) finding that assortative mating evolves more readily than habitat fidelity under reinforcing selection [3], while Kyogoku and Kokko (2020) documented the opposite pattern [21]. Despite the opposing outcomes, which may lie in differences in model structure and underlying assumptions (e.g., one-allele vs. two-allele models; infinite vs. finite choice), both models predict that the isolating barrier that evolves most readily would not suppress the evolution of the less readily evolving isolating mechanism.

Thus, both models ultimately lead to the prediction that habitat isolation and sexual isolation could evolve simultaneously or sequentially as a result of reinforcement. In particular, under niche-based divergent selection, by which both immigrants and hybrids have lower fitness than residents, both habitat isolation and sexual isolation are likely to evolve [3,10].

Another important aspect of the dynamics of population divergence and speciation, often underappreciated in theoretical studies but increasingly reported by empirical research, is that migration of individuals is commonly found to be asymmetrical between populations and species. This pattern is observed across multiple spatial scales and across taxa including plants [7,22–24], fish [25–27], birds [28], and insects [29]. As well, meta-populations studies within species document general asymmetric migration patterns where the net movement of individuals between any two subpopulations is typically non-zero and where subpopulations vary in size in tandem with rates of immigrants/emigration. For instance, meta-populations in river networks display highly asymmetric patterns of migration that reflect unidirectional water currents [30,31].

Examining the effects of reinforcement on both habitat isolation and sexual isolation is particularly interesting under the scenario of asymmetric migration, as each reproductive barrier may respond differently to any fitness cost associated with migration. Lineages exhibiting stronger dispersal capability (higher emigration rates), but receiving less immigrants, are hypothesized to evolve stronger habitat isolation in response to the cost of migration, especially if divergent selection associated with different habitats results from immigrant inviability [19,32]. Conversely, lineages receiving a higher proportion of immigrants are predicted to evolve stronger sexual isolation in response to the fitness costs of hybridization [33,34]. Hence, under asymmetric migration between diverging lineages in which hybrids suffer reduced fitness, reinforcement theory predicts that the strength of habitat isolation and sexual isolation will also be asymmetric

between lineages but in opposing directions. Here, both selection against hybrids and selection against migrants can drive the evolution of prezygotic isolation. In systems where both ecologically based immigrant and hybrid inviability are present it is nearly impossible to isolate the effects of selection against migrants vs. selection against hybrids on the evolution of habitat isolation. Thus, we refer to the process of increased habitat isolation whether driven by selection against hybrids or by selection against migrants as reinforcement [1,21].

In this study, we test for the signatures of selection arising from reinforcement under asymmetrical migration for both habitat isolation and sexual isolation by use of a closely related group of three host-plant-specific insect herbivores: gall-forming wasps in the genus *Belonocnema* locally adapted to live oaks in the genus *Quercus* (subsection Virentes). Two species of *Belonocnema*, *B. treatae* and *B. fossoria*, co-occur over a portion of their range across the coastal southeastern United States on the southern live oak, *Q virginiana*, (*Qv*) and the sand live oak, *Q. geminata* (*Qg*), respectively (Fig 1) [35,36]. A third species, *B. kinseyi*, is allopatric to both *B. treatae* and *B. fossoria*, where it develops on *Qv* host plants along the Gulf coast east of the Mississippi River and *Q. fusiformis* (*Qf*) in the more western portion of its range [35,36]. Recent population genomic analysis showed that sympatric *B. treatae* and *B. fossoria* are genomically distinctive (average $G_{ST} = 0.371 \pm 0.006$ confidence interval [CI]), whereas allopatric *B. kinseyi* from the western range of *Qv* exhibit stronger divergence from *B. fossoria* ($G_{ST} = 0.708 \pm 0.006$) [35].

The sister species, *B. treatae* and *B. fossoria*, provide an opportunity to investigate the evolution of multiple reproductive barriers given asymmetric gene flow in a natural system in which the biology, ecology, and reproductive isolation between lineages is well characterized [16,20,35–42]. First, the two sympatric gall wasp species exhibit multiple prezygotic reproductive

barriers, including temporal isolation [40], sexual isolation [42], and habitat isolation [20,38]. Second, immigrants suffer both reduced viability and fecundity on alternative host plant species [16,39]. Third, asymmetric migration between these sympatric species is suggested by differences in flight capability [36] and supported by population genomic data [35]. Flight test assays demonstrate that asexual *B. fossoria* exhibit under-developed wings and are flightless, whereas asexual *B. treatae* exhibit fully developed wings and are capable of flight [36]. Based on multi-locus genomic data, a small number of *B. treatae*-like migrants have been detected by population genomic analysis among *B. fossoria* populations on *Qg* (3 migrants out of 172 individuals genotyped at 40,699 shared SNPs; a migration rate of 1.74%), whereas no *B. fossoria*-like individuals were found among *B. treatae* populations on *Qv* (0 migrants out of 313 individuals) [35]. Last, this system exhibits important prerequisites required for reinforcing selection to play an important role in speciation: (a) a cost of habitat switching due to immigrant inviability and decreased immigrant fecundity [16], (b) a cost of hybridization (Fig S1, [41]), and (c) a history of introgression inferred by the shared mtDNA haplotypes among sympatric species [43].

Here, we test the hypothesis that the cost of asymmetric migration arising from selection against maladaptive immigration and/or hybridization predicts the direction and relative strength of prezygotic reproductive isolation between sympatric *B. treatae* and *B. fossoria* (Fig 2). Specifically, we predict that *B. treatae*, which has a higher capacity to migrate, will exhibit stronger habitat isolation due to the cost of immigration, while *B. fossoria*, which exhibits reduced capacity to migrate, will exhibit stronger sexual isolation as a result of receiving increased emigrants. Last, we estimate and compare observed habitat isolation and sexual isolation between each of these sympatric species and the allopatric species *B. kinseyi* to test a critical prediction of

reinforcement: prezygotic reproductive isolation between closely related sympatric species is expected to be higher than between allopatric species [1,5,7].

Results

1. Asymmetry in habitat isolation and sexual isolation between sympatric species

The strength of habitat isolation and sexual isolation differed in magnitude and direction between sympatric populations of *B. treatae* and *B. fossoria*. The species more capable of emigration, *B. treatae*, displayed higher host fidelity than *B. fossoria* (GLMM: $Z = -2.430$, $P = 0.015$; least square means ± standard errors (SE), *B. treatae*: 0.755 ± 0.039, n = 80; *B. fossoria*: 0.615 ± 0.036, n = 109; Fig 3; Table S3). In contrast, the species more likely to interact with emigrants, *B. fossoria*, exhibited higher probability of assortative mating than *B. treatae* (Fig 4), for which a higher proportion of *B. fossoria* males engaged in courtship displays (i.e., wing buzzing) when paired with conspecific females (0.358 ± 0.036; n = 216) compared with heterospecific females (0.134 ± 0.036; n = 96; Tukey's test: $t = 3.676$, $P = 0.0015$; Fig 4A). In comparison, *B. treatae* males engaged in courtship displays to conspecific (0.248 ± 0.066) and heterospecific females (0.316 ± 0.060) similarly ($t = -0.769$, $P = 0.868$). The asymmetry in the strength of mate preference of male *B. treatae* compared with *B. fossoria* is also supported by the significant male species × species pair interaction from the GLMM ($Z = 2.98$, $P = 0.0029$; Table S4). Moreover, the observed pattern of asymmetry in mate preference was robust to the possible influence of unbalanced sample sizes between species (*B. treatae*, n = 118; *B. fossoria*, n = 312). Of the 1000 permutation models in which we randomly drew 118 of the 312 *B. fossoria* and tested the hypothesis of no difference in mate preference between males of each species, 82% of the models found significant asymmetry ($\alpha = 0.05$) in the probability of assortative mating for *B. treatae* and *B. fossoria* (probability of randomly getting 820 significant results: $P < 10^{-16}$). Thus, our original

GLMM and our resampling approach both support the observation of stronger assortative mating in males of *B. fossoria* compared with *B. treatae*.

We did not detect statistically significant evidence of mate preference expressed by female *B. treatae* (probability of conspecific mating: 0.781 ± 0.121, n = 11; heterospecific mating: 0.464 ± 0.136, n = 17; t = 1.650, P = 0.355) or female *B. fossoria* (conspecific mating: 0.570 ± 0.065, n = 72; heterospecific mating: 0.377 ± 0.112, n = 23; t = 1.489, P = 0.556), although females of each species exhibited trends of preferring conspecific males (Fig 4B). Similarly, the strength of assortative mating did not differ between female *B. treatae* and female *B. fossoria* as evidenced by the female species × species pair term in the GLMM model (Z = −0.633, P = 0.526; Table S5).

2. Asymmetric habitat isolation and sexual isolation are consistent with reinforcement

If asymmetric habitat isolation among sympatric species is the result of selection arising from the fitness cost of switching habitats, in this case host plants, we would expect that the strength of habitat isolation exhibited by *B. kinseyi*, whose host plant *Qv* is allopatric with host plant *Qg* (Fig 1), to be lower than the strength of habitat isolation exhibited *B. treatae*, whose host plant *Qv* is sympatric with the alternative host plant *Qg*. Consistent with this prediction, we found significantly lower host plant fidelity in allopatric populations of *B. kinseyi* (0.647 ± 0.023, n = 237) compared with sympatric populations of *B. treatae* (0.772 ± 0.036, n = 237, GLMM: Z = -2.712, P = 0.007, Fig 5, Table S3).

Reinforcement also predicts that sympatric *B. fossoria* males will exhibit stronger sexual isolation than allopatric males of *B. kinseyi*. Consistent with the prediction of reinforcement (Fig 6, Table S6), males from sympatric *B. fossoria* displayed stronger sexual isolation than allopatric *B. kinseyi* males, as indicated by the comparison of male mate preference for females of *B. fossoria* and *B. kinseyi* (t = 4.076, P < 0.001). Alternatively, males of *B. kinseyi* did not discriminate among

females of *B. fossoria* or *B. kinseyi* ($t = 0.463$, $P = 0.967$). The asymmetry in the strength of sexual isolation of male *B. fossoria* and *B. kinseyi* is also supported by the significant male species × species pair interaction from the GLMM ($Z = 3.531$, $P < 0.001$, Table S4).

Discussion

Reinforcement is a key mechanism promoting the evolution of prezygotic isolation [1]. While studies of reinforcement focus primarily on sexual isolation [44,45], other prezygotic reproductive barriers may simultaneously respond to reinforcing selection and perhaps respond differently depending on the direction of gene flow [19,46,47,48–50]. Rarely, however, have the combined (additive) and interactive (multiplicative) effects of habitat and sexual isolation been examined empirically (but see [8]), even though theory has predicted that their interactions can be important [3, 21]. By examining two prezygotic barriers within the same system, our study is one of a few cases that addresses whether more than one prezygotic isolating mechanism evolves via reinforcement [3,21].

Our study also addresses an important knowledge gap in ecological speciation research. While it is widely acknowledged that divergent selection can initiate partial reproductive isolation at the early stages of speciation [19], less is known about whether and how divergent selection can complete the speciation process, where reinforcement may play a key role [51]. This gap may exist because reinforcement in nature has commonly been assessed without necessarily including the contribution of divergent selection [4,5,7] as reinforcement can complete instances of non-ecological speciation as, for example, when reproductive isolation evolves via neutral mechanisms [47]. In addition, many studies investigate the role of divergent selection in promoting prezygotic reproductive isolation between sympatric populations without specifically isolating the potential role of reinforcement [23,42,52,53]. Our study is one of a few that links these evolutionary

processes and detects a signature of reinforcement under divergent selection, which is hypothesized to reduce gene flow at the later stages of ecological speciation (e.g., [12,54]). Combined with temporal isolation and immigrant inviability in this system, the estimated total prezygotic reproductive isolation between sympatric *Belonocnema* species is near complete (mean = 0.95; [16,40]). Thus, the *Belonocnema*–live oak system adds to a limited but growing number of cases investigating the evolution of strong reproductive isolation under divergent selection [14,23], with reinforcement arising as a result of the ecologically based costs of dispersal and hybridization [16,41].

Last, our study in the *Belonocnema*–live oak system presents an example in which ecological adaptation outweighs neutral genetic differentiation in promoting the evolution of prezygotic reproductive isolation. First, we found that the strength of sexual isolation was stronger between the more closely related sympatric *B. treatae* and *B. fossoria* (G_{st} = 0.36; [35]) that reside on different host plants compared with the strength of sexual isolation between the more distantly related allopatric *B. kinseyi* and *B. treatae* (G_{st} = 0.8; [35]) that utilize the same host plant species. Moreover, in the absence of gene flow from other closely related lineages, allopatric *B. kinseyi* exhibit significant habitat preference for its natal host plant and strong mate preference for individuals from its natal host plant (*Qv*) relative to individuals from the alternative host plant (*Qg*) (Fig S2). These results are consistent with the prediction that ecological adaptation to divergent environments acts to promote prezygotic reproductive isolation [12].

Comparing data with models: Evolution of habitat and sexual isolation via reinforcement

The speciation model by Nosil and Yukilevich [10] predicts that the conditions favoring the rise and fixation of one isolating mechanism would favor the evolution of other isolating mechanisms under divergent selection at an intermediate strength. Essentially, if the strength of

divergent selection is too strong, the evolution of habitat isolation is likely to interfere with the evolution of sexual isolation. Consistent with this theoretical prediction and empirical findings in *Timema* stick insects [11], we observed that both habitat isolation and sexual isolation exhibit patterns consistent with elevated reproductive isolation in sympatry where there is an intermediate strength of selection against migrants among sympatric *Belonocnema* species (~0.36, Fig S1, [16]). This observed strength of selection is within the range of the optimum predicted by Nosil and Yukilevich (0.30–0.50), where increased reproductive isolation in both habitat isolation and sexual isolation in sympatry is favored [10].

Meanwhile, the patterns of prezygotic reproductive isolation found among *Belonocnema* species are also compatible with the two-allele-based model of Kyogoku and Kokko [21]. If habitat isolation evolves first, which is likely if the fitness costs of migration due to divergent selection is present [3], the evolution of sexual isolation could follow [21]. We suspect that the evolution of habitat isolation and sexual isolation via reinforcement is common across organisms for which populations use different habitats, especially among host-specific herbivorous insects. For instance, Funk, Filchak, and Feder [55] detected both habitat isolation and sexual isolation in 70% of the cases included in their review of speciation patterns among herbivorous insects [55]. Despite the fact that none of the studies examined prezygotic reproductive isolation under the context of reinforcement, the coexistence of habitat isolation and sexual isolation among many study organisms highlights the possibility of reinforcement in promoting multiple prezygotic reproductive isolating mechanisms.

Both Nosil and Yukilevich [10] and Kyogoku and Kokko [21] predict habitat isolation and sexual isolation could evolve within a single study system under reinforcing selection; however, neither model examined the effect of asymmetric migration on the evolvability of multiple

prezygotic reproductive isolating mechanisms. Asymmetric migration within and between species is a common phenomenon across different taxa [22–29]. Therefore, we stress the need for both theoretical and empirical studies to test the generality of asymmetric reinforcement in driving the asymmetry in the direction of habitat isolation and sexual isolation.

Alternative mechanisms of elevated prezygotic reproductive isolation in sympatry

While elevated prezygotic reproductive isolation among sympatric populations compared with allopatric populations is a key prediction of reinforcement, alternative processes have been hypothesized that generate similar patterns [56,57]. One notable alternative is the Templeton Effect, also termed differential fusion [58]. This theory posits that isolated populations experiencing strong prezygotic reproductive isolation persist in sympatry whereas weakly isolated populations fuse. Thus, the degree of prezygotic reproductive isolation observed among sympatric species pairs represents a non-random sample of the range of prezygotic reproductive isolation between all species that have come into secondary contact. However, differential fusion is unlikely to be the cause of elevated reproductive isolation in regions of sympatry for the *Belonocnema* system for three reasons. First, differential fusion assumes that sympatric species are in secondary contact with each other, which would predict that sympatric lineages are more closely related to allopatric lineages than they are between sympatric lineages. This assumption is contrary to the observed patterns of genomic divergence among *Belonocnema* lineages: the two sympatric species, *B. treatae* and *B. fossoria*, are more closely related to each other than to the allopatric species, *B. kinseyi* (Fig 2, [35]). Second, the Templeton Effect also predicts elevated postzygotic reproductive isolation among sympatric species, which would prevent fusion of divergent species during secondary contact [56]. To date, we have no support of this pattern among *Belonocnema* species as the degree of hybrid inviability between sympatric *B. fossoria* and allopatric *B. kinseyi* is similar

to the degree of hybrid inviability between sympatric species *B. fossoria* and the other sympatric species, *B. treatae* [41]. Finally, the Templeton Effect predicts that little to no gene exchange occurs between sympatric species. While no current genetic admixture between sympatric *B. treatae* and *B. fossoria* has been documented [35], shared mtDNA haplotypes among sympatric species compared with allopatric species suggests a history of introgression [43].

Another evolutionary process that has been proposed to generate patterns of increased reproductive isolation is that competition among species in sympatry, rather than selection against hybridization, promotes greater divergence in ecological traits such as mating signals [59]. However, competition between sympatric species of *Belonocnema* is unlikely to occur as species suffer significant fitness cost when switching to non-native host plants [16,39], thus making direct competition rare. In this regard, local adaptation to each host environment is also unlikely to be the direct result of avoiding interspecific competition, as allopatric lineages also exhibit a significant fitness cost of switching host plants [39].

Alternative mechanisms of asymmetric prezygotic reproductive isolation

The magnitude of reinforcing selection is a combined effect of the degree of gene flow and the fitness costs associated with dispersal and hybridization [34,48,60]. Thus, the pattern of asymmetric habitat isolation and sexual isolation that we observed in the *Belonocnema* system could be the result of either (a) the asymmetric costs of migration and/or hybridization, (b) asymmetric gene flow, or (c) a combination of the two. In our study, we failed to detect a significant asymmetric cost in habitat switching between sympatric *Belonocnema* species (Fig S1A, [16]), whereas a cost to asymmetric hybridization was found where the offspring produced by *B. fossoria* males mated with *B. treatae* females had a significantly lower fitness than residents (Fig S1B, [41]). The concordant asymmetry between sexual isolation and postzygotic isolation is

partially consistent with the prediction of reinforcement in that the lineage that suffers a higher fitness cost of hybridization should exhibit stronger sexual isolation [33,48,60]. However, if the cost of forming hybrids is the only mechanism promoting asymmetric sexual isolation via reinforcement, we would also expect to see *B. treatae* females displaying significant sexual isolation. However, we did not detect such a pattern in our study. The lack of assortative mating among *B. treatae* females could be due to the limited sample size (n = 28) and/or reflect the combined effect of asymmetric fitness cost of hybridization and unequal dispersal, as *B. treatae* experiences fewer immigrants from sympatric species, reducing the opportunity for reinforcement. In future studies, it will be important to evaluate the combined effect of fitness costs and the degree of migration in driving prezygotic reproductive isolation.

An additional cause of asymmetry in the reproductive isolating barriers of species could be due to differences in the underlying genetic architecture that translates into differences in sensitivity to varying degrees of gene flow [61]. For example, a genetic architecture with loci located in a genomic region characterized by low recombination will be more likely to be preserved in the face of gene flow. If such genetic architecture underlies sexual isolation, but not habitat isolation, a lineage could maintain sexual isolation while also displaying weaker habitat isolation because of the homogenizing effect of the gene flow [62,63]. Studies that simultaneously examine the strength of prezygotic reproductive isolation in relation to the genetic architecture of multiple contributing reproductive barriers could address these issues.

Conclusion

Reinforcement is an important, yet historically controversial, mechanism underlying the evolution of prezygotic reproductive isolation. Our study tested a hitherto unexplored signature of reinforcing selection against migrants: discordant asymmetries in the strength of habitat isolation

and sexual isolation based on the widespread observation of asymmetric migration among populations in nature. Our study presents an example in which the relative roles of ecological divergence and gene flow on the evolution of prezygotic isolation and reinforcement have been examined. Moreover, this research also contributes toward the understanding of how gene flow interacts with ecologically based divergent selection to shape the evolution of prezygotic reproductive isolation, which may promote and/or complete the speciation process.

Methods

1. Study system

The genus *Belonocnema* (Hymenoptera: Cynipini: Cynipidae) comprises three gall wasp species that specialize on American live oaks (genus *Quercus*; subsection Virentes) [36]. As with many cynipids, each species exhibits cyclical parthenogenesis whereby the sexual and asexual generations alternate seasonally to complete an annual life cycle [36,64]. Sexual generation males and females develop within single sex, multi-chambered root galls [64]. In the spring, adults emerge from root galls and immediately mate and oviposit into newly flushed leaves. Oviposition at the onset of new leaf growth is critical for successful gall initiation [39,40]. Successful oviposition and larval establishment results in a single-chambered spherical gall that forms on the underside of new leaves. Each gall houses a single developing asexual generation female [64]. Asexuals emerge from leaf galls in early fall within the canopies of trees and descend to the ground and search for root tissue suitable for gall induction. The study described herein is motivated by the potential movement of asexual generation *B. treatae* and *B. fossoria* among the two sympatric host plant species in their search for suitable root tissue. It is the ability of asexuals to recreate the sexual generation that sets the stage for potential gene flow between *Belonocnema* species during emergence of the ensuing sexual generation.

2. Sample collection

In the spring of 2016 through 2019, we collected maturing root galls containing late-stage pupal wasps of each of the three species from their respective host plants: *B. fossoria* from *Qg* at three sites, *B. treatae* from *Qv* at four sites, and *B. kinseyi* from *Qv* at three sites (Fig 1; Supplemental Table 1). Peak emergence of *B. treatae* can take place earlier than that of *B. fossoria* [40], therefore we placed one-half of the root galls collected from *Qv* at each site into a refrigerator at 4 °C for one week to delay and thus synchronize emergence with *B. fossoria*. Vials were monitored once every two days and, upon emergence, one- to two-day-old adults were used to perform host preference tests and mate choice assays. All assays were conducted under constant greenhouse conditions at Rice University, Houston, Texas, in 2016, 2018, and 2019, and in a screened-in outdoor enclosure at Archbold Biological Station, Venus, Florida, in 2017.

3. Host preference tests

To test the hypothesis of asymmetrical habitat isolation between the two sympatric host-associated lineages in the direction predicted by the greater dispersal capability of *B. treatae*, we compared host plant preference for each of the two sympatric lineages as well as the allopatric lineage *B. kinseyi* using a controlled two-choice experimental approach (Table S1, [20]). Female host choice directly determines the environment in which development proceeds for the next generation, and previous study showed that males display lower host fidelity [20]. We therefore used only female individuals for measurement of host preference. In 2016 and 2017, host preference trials were conducted within sealed 500 mL clear-plastic cups. A single, fresh 15–20 cm stem cutting of each host plant (*Qv* and *Qg* from Florida) with similar numbers of leaves were placed inside a cup to minimize any effect of differences in plant biomass on insect choice. In 2019, trials were conducted within 60 × 15 mm (diameter × height) petri dishes stocked with a

single newly flushed leaf of each host plant positioned at opposing ends of the dish. For all trials, a single unmated female was aspirated into each container and was observed at 2-minute intervals for 30 minutes for a total of 15 observations. For each observation, we recorded the location of each female as either on *Qv*, on *Qg*, or on the surface of the cup/petri dish. Host fidelity (host preference) of each individual was calculated as the number of observations on the individual's native host plant divided by the total number of observations across both natal and non-natal host plants during a trial. In total, 557 preference assays were conducted using 126 *B. treatae* from *Qv*, 130 *B. fossoria* from *Q*g, and 301 allopatric lineage *B. kinseyi* from *Qv*. Only those trials in which individuals were observed on a host plant at least once were retained for analysis (76.5% of trials, Table S1). In total, we made 8355 individual observations over 278.5 testing hours for the assessment of host preference.

4. Mate preference tests

To test asymmetry in sexual isolation between two sympatric species, as predicted by asymmetric reinforcement, we compared mate preference for two sympatric species and subsequently compared the mate preference of sympatric species with allopatric species by conducting no-choice preference trials (Table S2). Trials were conducted in 2016, 2017, and 2018 within 60 × 15 mm petri dishes fitted with moist filter paper at the base. For each trial, one male and one female were aspirated into the petri dish and their behaviors observed at 2-minute intervals for 30 minutes for a total of 15 observations (for pairings and replication see Table S2). At each interval, we recorded one of three courtship and mating-related interactions: (a) whether the male was engaged in wing buzzing (or fanning), an important male courtship display [65], (b) whether the male mounted the female, and (c) whether the pair copulated (defined as abdomen contact for over five seconds). We then evaluated mating preference expressed by each sex separately. Both

male and female mate preference were considered as binary variables (0 or 1). Male preference was estimated by evaluating whether each individual displayed the wing buzz courtship behavior in one or more of the 15 two-minute intervals. Female preference was based on whether or not a female allowed the male to mount or copulate given that male courtship (wing buzzing) occurred during the 30-minute observation period [20]. In total, 1123 preference assays were conducted with 225 females and 153 male *B. treatae* from *Qv*, 455 females and 563 male *B. fossoria* from *Qg*, and 335 females and 515 male *B. kinseyi* from *Qv* (Table S2). In total, we made 16,845 individual observations over 561.5 testing hours for this mate preference assessment.

5. Testing for asymmetric habitat and sexual isolation among sympatric lineages

To test for the predicted asymmetry in habitat isolation between sympatric *B. treatae* and *B. fossoria*, we used a generalized linear mixed model (GLMM) with host plant fidelity (i.e., proportion of time each female spent on native host plant out of the total time spent on both native and non-native plants) as the response variable (ranging from 0 to 1) with a beta-binomial distribution. A beta-binomial distribution is often used to control for overdispersion of binomially distributed data, which is the case in our data [66]. The independent variables of "arena type" (cup or petri dish) and "gall wasp species" were included as fixed effects, with "collection site" treated as a random effect. Asymmetry in host fidelity was inferred by a significant gall wasp species term. Similarly, asymmetry in sexual isolation was tested by comparing the strength of mate preference between sympatric *B. treatae* and *B. fossoria* populations via GLMM in which male mate preference and female mate preference were evaluated separately. For each model, mate preference (Bernoulli trials: 0 or 1) was treated as the response variable with a binomial distribution, while the independent variables collection year, gall wasp species, species pair (conspecific or heterospecific), and the interaction term gall wasp species × species pair were

included as fixed effects with collection site treated as a random effect. A significant interaction between species and species pair (conspecific or heterospecific) is interpreted as evidence of asymmetric sexual isolation. To rule out the possibility that any observed asymmetric male mate preference between *B. treatae* and *B. fossoria* was an artifact of unbalanced sample sizes for the two species (*B. treatae*: n = 118; *B. fossoria*: n = 312), we down-sampled mate preference tests from *B. fossoria* to match the number of *B. treatae* tested. We performed this random resampling process 1000 times, each time running the GLMM as described above. We then summarized the number of models runs that returned a significant interaction term supporting the observation of asymmetric mate preference expressed by males.

6. Assessing the signature of reinforcement in habitat and sexual isolation

A core prediction of reinforcement theory is that prezygotic reproductive isolation between closely related sympatric species is expected to be higher than between allopatric species [1,5,7]. We tested this prediction by comparing the strength of both habitat isolation and sexual isolation between sympatric *B. treatae* and *B. fossoria* with that observed between *B. fossoria* and allopatric *B. kinseyi* (Fig 1). One important distinction between our analysis of habitat isolation and sexual isolation is that habitat isolation is a property of the individual interacting with its environment (e.g., host plants), whereas sexual isolation is a property of the pairwise interaction between the male and female in the mating trial. Thus, the statistical models for comparison will be slightly different but address the same general question of elevated reproductive isolation in sympatry.

To test for the signature of reinforcement in habitat isolation, we compared the strengths of habitat isolation of females between sympatric and allopatric species pairs by using GLMM as described in section 5 with the added fixed effect of geography (sympatry or allopatry). The strength of habitat isolation was quantified by the degree of host plant fidelity. A finding of

significantly higher host plant fidelity for female sympatric *B. treatae* compared with the host plant fidelity of allopatric female *B. kinseyi* would be consistent with reinforcing selection.

To test for the signature of reinforcement in sexual isolation, we compared whether the males of the sympatric species (*B. fossoria* [*Bf*]) differed in the strength of sexual isolation compared with the allopatric species (*B. kinseyi* [*Bk*]). The strength of sexual isolation was quantified by comparing the male mate preference toward conspecific vs. heterospecific females. If males of sympatric *B. fossoria* exhibit stronger sexual isolation than males of allopatric *B. kinseyi*, we predict a pattern of asymmetric sexual isolation between *B. fossoria* and *B. kinseyi*, which would be tested by the interaction term between male species and the type of species pair (conspecific or heterospecific). We conducted a GLMM as described above in section 5. A finding of significantly higher sexual isolation for male sympatric *B. fossoria* compared with allopatric male *B. kinseyi* would be consistent with reinforcing selection.

All GLMM models were followed by multiple pairwise comparison among lineages with different geographic and host plant associations by means of a Tukey's post hoc test. The least square means of the response variables in each GLMM were calculated using function *lsmeans* in package emmeans [67] and are presented graphically with corresponding standard errors.

Acknowledgments

We thank the staff and volunteers at Archbold Biological Station, especially Mark Deyrup and Hilary Swain, for accommodating our large field and lab experiments in Florida; Amanda Driscoe, Robert Busbee, and Mattheau Comerford for sample collection; and Zachariah Gompert and Arthur Weis for helpful discussions and comments. In particular, Arthur Weis provides great input on designing Figure 2. The authors acknowledge Lake Lizzie Conservation Area in Osceola

County and South Florida Water Management District at Kissimmee River for access to experimental sites.

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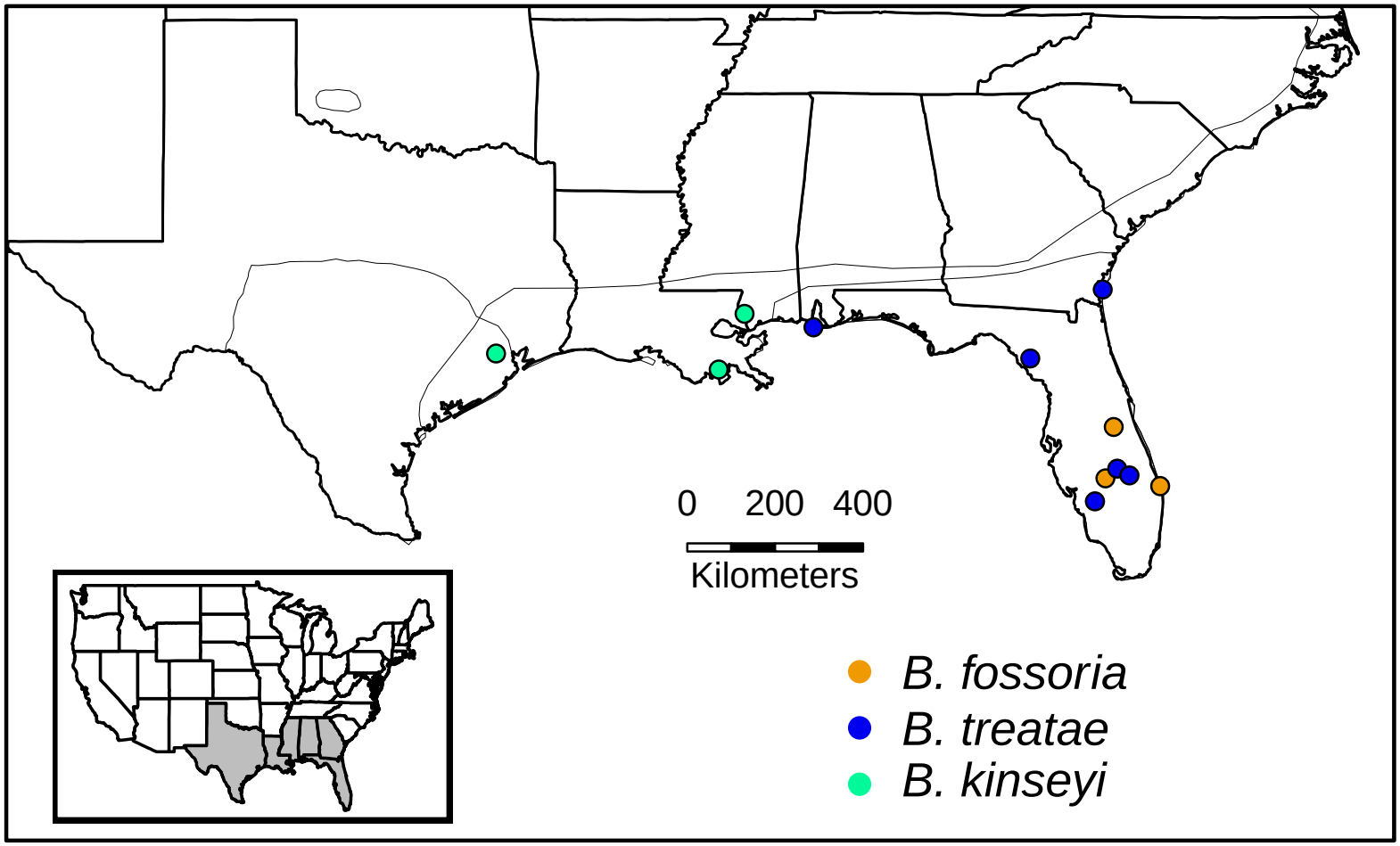

Figure 1. Map of host plant ranges (modified from Driscoe et al. 2019) of *Q. fusiformis* (light grey), *Q. virginiana* (intermediate grey), *Q. geminata* (dark grey), as well as the location of study sites where root galls containing males and females used in habitat isolation and sexual isolation experiments were collected. Green, blue, and orange dots represent collection sites for *B. kinseyi*, *B. treatae*, and *B. fossoria*, respectively. This color scheme is used throughout all figures to represent each *Belonocnema* lineage.

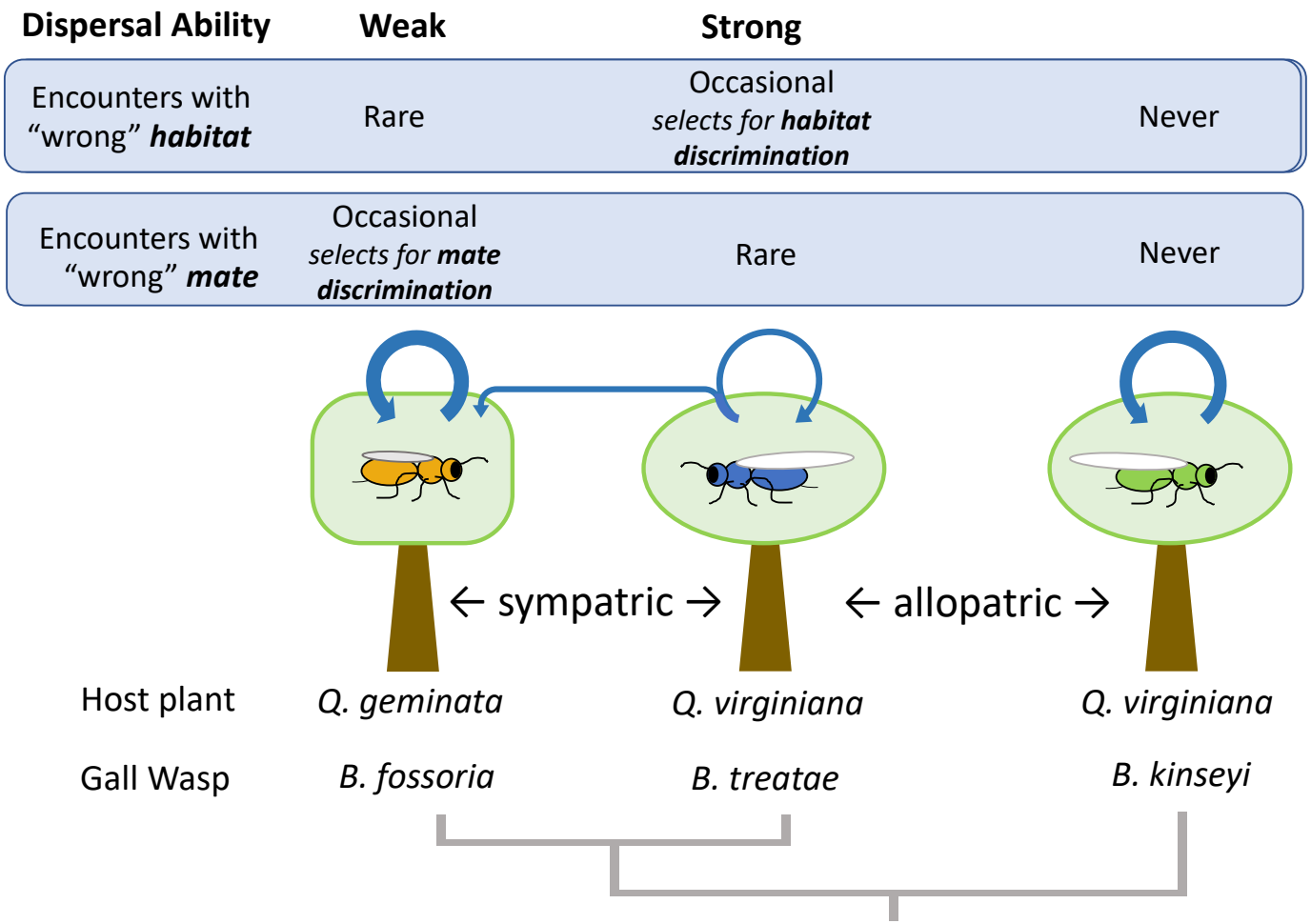

Figure 2. Illustration of the hypotheses tested in this study. *B. treatae* with stronger dispersal ability encounter the wrong habitat more often, therefore selection would favor the evolution of strengthened habitat preference. In contrast, *B. fossoria* with weaker dispersal ability likely encounter the wrong mate more often, therefore selection would favor the evolution of strengthened mate preference.

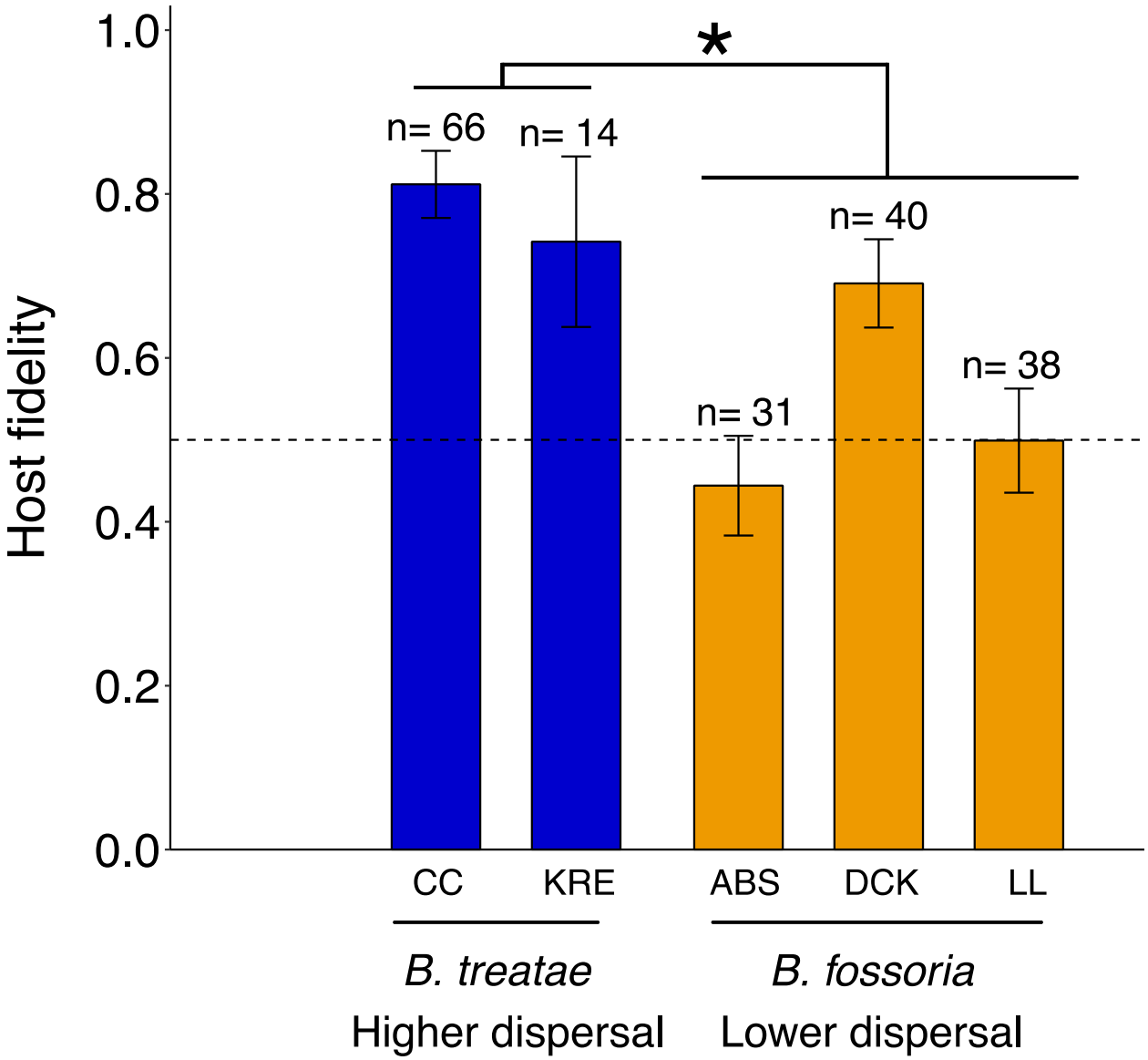

Figure 3. Strength of host fidelity (mean ± SE) evaluated for sympatric *B. treatae* and *B. fossoria* collected from five populations (abbreviated below bars; see Table S2 for population details). The strength of habitat isolation is quantified by the strength of the host fidelity (defined as the percent of time females spent on the native host plant when given the choice of their native and novel host plant). The horizontal dashed line at 0.5 represents the null hypothesis of no host fidelity, where wasps spent an equal amount of time on both hosts. Asterisk indicates statistical difference in host fidelity of females between *B. treatae* and *B. fossoria* ($P < 0.05$).

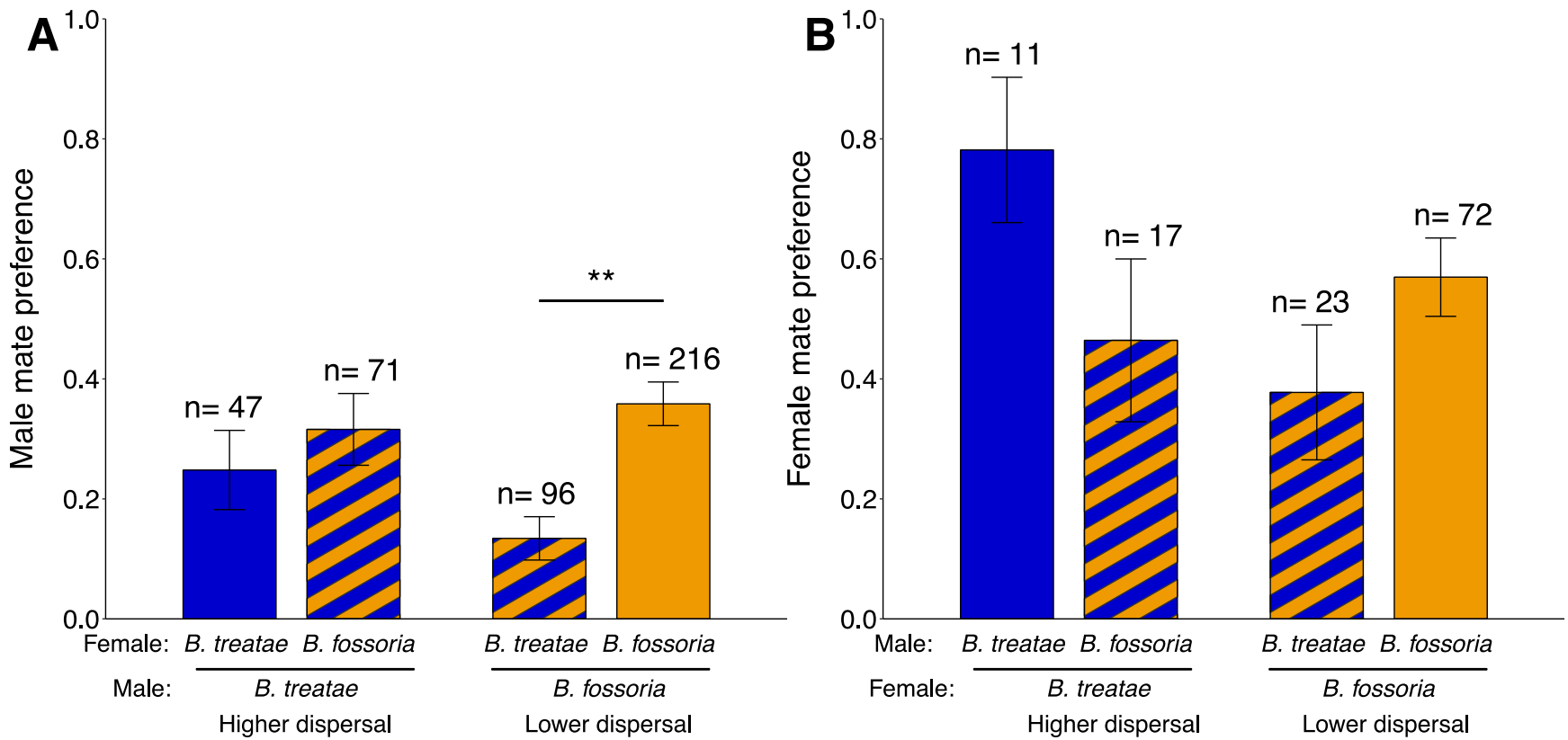

Figure 4. Strength of sexual isolation (mean ± SE) of males (A) and females (B) between sympatric lineages of *B. treatae* and *B. fossoria*. Solid-colored bars represent conspecific matings, and cross-hatched bars represent heterospecific matings. Asterisks indicate significant statistical difference of male mate preference between same and different species pairs ($P < 0.01$). Asymmetrical male mate preference between *B. treatae* and *B. fossoria* is indicated by the significant male species × species pair interaction term in the GLMM ($Z = 2.975$, $P = 0.0029$).

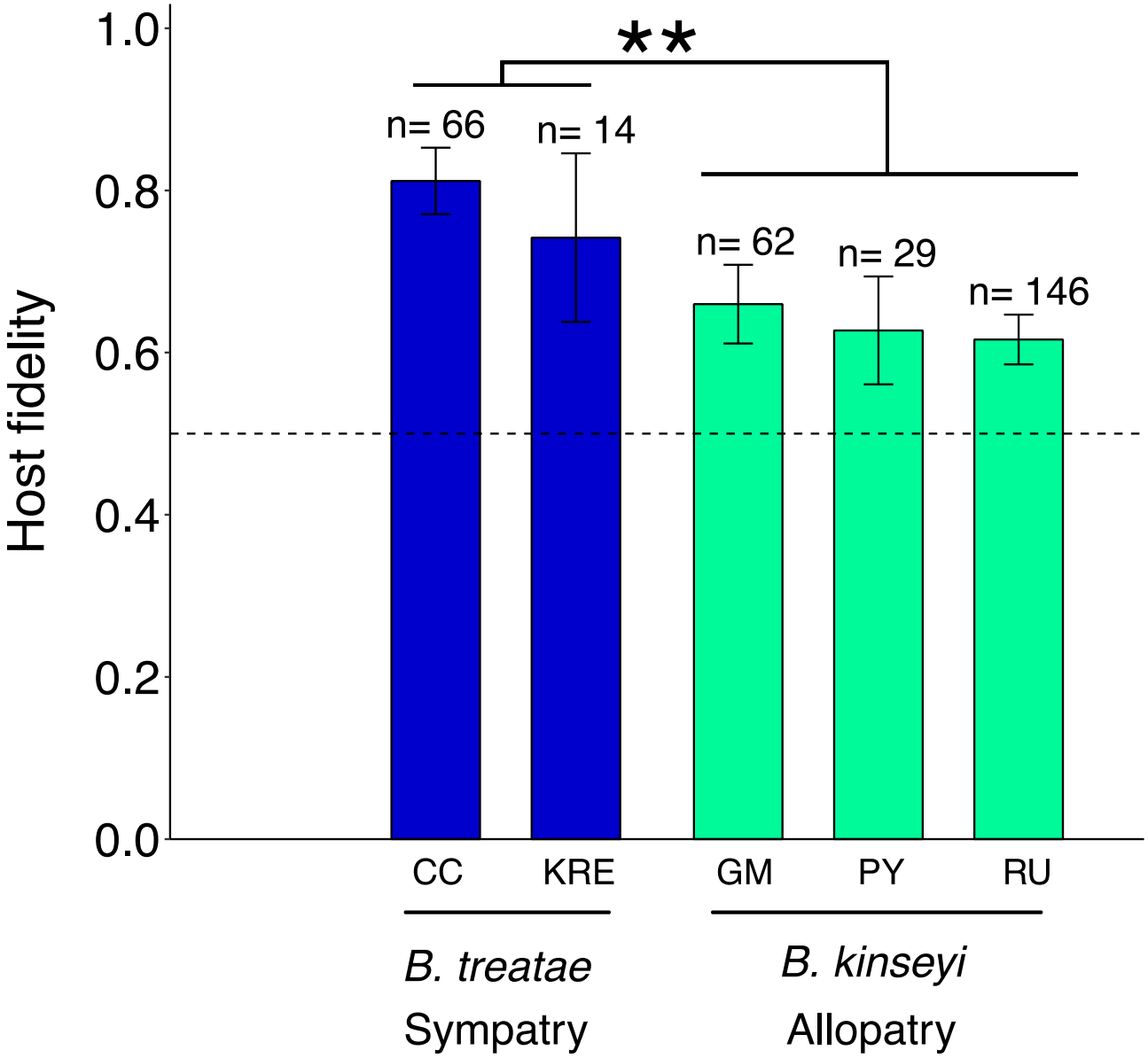

Figure 5. Strength of host fidelity (mean ± SE) evaluated for sympatric *B. treatae* and allopatric *B. kinseyi* populations (see Table S2 for population details). Host fidelity was defined as the percent of time females spent on the native host plant when given the choice of their native and novel host plant. The horizontal dashed line at 0.5 represents the null hypothesis of no host fidelity, where wasps spent an equal amount of time on both hosts. Asterisks indicate significant difference in host fidelity between *B. treatae* and *B. kinseyi* ($P < 0.01$).

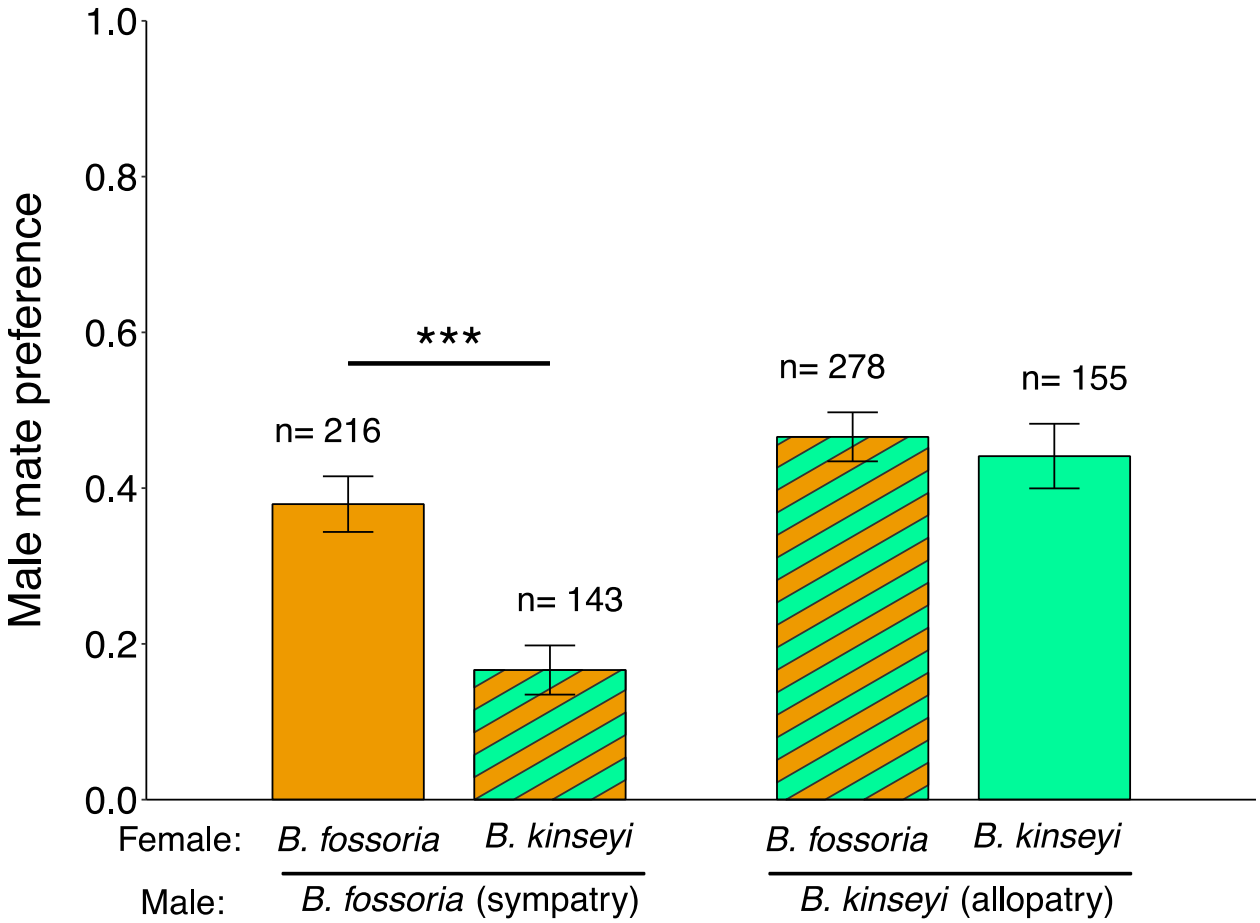

Figure 6. Strength of sexual isolation (mean ± SE) of males compared between sympatric *B. fossoria* and allopatric *B. kinseyi*. Asterisks indicate significant statistical difference of male mate preference between same and different species pairs ($P < 0.001$). Asymmetrical male mate preference between *B. fossoria* and *B. kinseyi* is indicated by the significant interaction term male species × species pair in GLMM ($Z = 3.531$, $P < 0.001$).

Supporting information

Table S1. Details of all collection sites for animals used in this study: site names and abbreviations used in Table S3 and all Figures, host plant (*Qg* – *Quercus geminata*; *Qv* – *Quercus virginiana*); geographic context (sympatry – geographic overlap of host plants, allopatry – no geographic overlap, see map Figure 1); and latitude and longitude.

Species	Site	Host plant	Geography	Latitude	Longitude
B. fossoria	Archbold Biological Station (ABS)	*Qg*	sympatry	27.1846	-81.3521
B. fossoria	Dickinson State Park (DCK)	*Qg*	sympatry	27.0261	-80.1091
B. fossoria	Lake Lizzie (LL)	*Qg*	sympatry	28.2276	-81.1788
B. treatae	Alva (Alva)	*Qv*	sympatry	26.7092	-81.6154
B. treatae	Cross City (CC)	*Qv*	sympatry	29.6192	-83.0988
B. treatae	Dauphin Island (DI)	*Qv*	sympatry	30.2516	-88.0920
B. treatae	Jekyll Island (JI)	*Qv*	sympatry	31.0175	-81.4297
B. treatae	Kissimmee river (KRE)	*Qv*	sympatry	27.3780	-81.0968
B. treatae	Okeechobee (Okee)	*Qv*	sympatry	27.2440	-80.8168
B. kinseyi	Golden Meadow (GM)	*Qv*	allopatry	29.3939	-90.2729
B. kinseyi	Picayune (PY)	*Qv*	allopatry	30.5255	-89.6795
B. kinseyi	Rice University (Rice)	*Qv*	allopatry	29.7174	-95.4018

Table S2. Number of females tested per site to measure habitat isolation. Only females located on a host plant at least once during the 30 minute observation period were retained in the analysis of preference.

Species	Site	# females tested host preference trials	# females retained host preference analysis
B. fossoria	Archbold Biological Station (ABS)	42	31
B. fossoria	Dickinson State Park (DCK)	46	40
B. fossoria	Lake Lizzie (LL)	42	38
B. treatae	Cross City (CC)	99	66
B. treatae	Kissimmee river (KRE)	27	14
B. kinseyi	Golden Meadow (GM)	82	62
B. kinseyi	Picayune (PY)	36	29
B. kinseyi	Rice University (Rice)	183	146
	Total	557	426

Table S3. Number of males and females tested per site to measure sexual isolation in this study.

		B. fossoria **(♂)**			***B. treatae*** **(♂)**					***B. kinseyi*** **(♂)**			
	Site	**ABS**	**DCK**	**LL**	**Alva**	**DI**	**JI**	**KRE**	**Okee**	**GM**	**PY**	**Rice**	**Total**
B. fossoria	**ABS**	21	20	29	2	3	17	9	0	5	7	31	144
(♀)	**DCK**	24	23	16	2	4	16	10	2	9	10	24	140
	LL	7	7	69	2	1	13	13	2	7	3	47	171
B. treatae	**Alva**	3	13	5	7	0	0	2	3	0	0	10	43
(♀)	**JI**	6	5	5	0	1	0	2	0	3	0	0	22
	KRE	8	9	7	1	1	3	15	0	0	0	19	63
	Okee	1	5	2	2	0	0	0	10	0	0	5	25
B. kinseyi	**GM**	26	22	38	0	0	13	3	0	0	10	8	120
(♀)	**PY**	12	17	21	0	0	14	2	0	5	0	8	79
	Rice	21	34	87	5	0	2	30	13	0	0	124	316
	Total	129	155	279	21	10	78	86	30	29	30	276	

Table S4. Results General Linear Mixed Model (GLMM) analysis of host preference between different pairs of wasp species; (Df = degrees of freedom). Method in the model is the effect of different testing environments (cup vs. petri dish) in which the preference trials were conducted.

	GLMM host preference comparison *B. treatae* and *B. fossoria*			**GLMM host preference comparison *B. treatae* and *B. kinseyi***		
	χ^2	*Df*	*P*-value	χ^2	*Df*	*P*-value
(Intercept)	7.425	1	**0.006**	6.552	1	**0.011**
Method	7.839	1	**0.005**	10.165	1	**0.001**
Wasp species	7.356	1	**0.007**	5.904	1	**0.015**

Table S5. Results of General Linear Mixed Model (GLMM) analysis of male mate preference among sympatric wasp species *B. treatae* and *B. fossoria*. The term "Male species" in the model indicates the species of male in each mating trial (*B. treatae* vs. *B. fossoria*) while the term "Species pair" indicates whether the male and female used in each trial were conspecifics or heterospecifics.

	χ^2	*Df*	***P*-value**
Intercept	5.537	1	**0.019**
Year	8.070	2	**0.018**
Male species	7.957	1	**0.005**
Species pair	0.592	1	0.442
Male species x Species pair	8.852	1	**0.003**

Table S6. Results GLMM for female mate preference among sympatric wasp species *B. treatae* and *B. fossoria*. Female species in the model indicates the species of female in each mating trial (*B. treatae* vs. *B. fossoria*) while "Species pair" indicates whether the male and female used in each trial were conspecifics or heterospecifics.

	χ^2	*Df*	*P*-value
Intercept	0.815	1	0.367
Year	3.625	2	0.163
Female species	0.407	1	0.523
Species pair	0.202	1	0.653
Female species × Species pair	0.002	1	0.960

Table S7. GLMM results of test of male preference among sympatric species *B. fossoria* and allopatric species *B. kinseyi*. The term "Male species' in the model denotes the species of male in each mating trial (*B. treatae* vs. *B. fossoria*) while "Species pair" indicates whether the male and female used in each trial were conspecifics or heterospecifics.

	χ^2	*Df*	***P*-value**
Intercept	30.717	1	**< 0.001**
Year	23.897	2	**< 0.001**
Male species	32.825	1	**< 0.001**
Species pair	16.618	1	**< 0.001**
Male species × Species pair	12.471	2	**< 0.001**

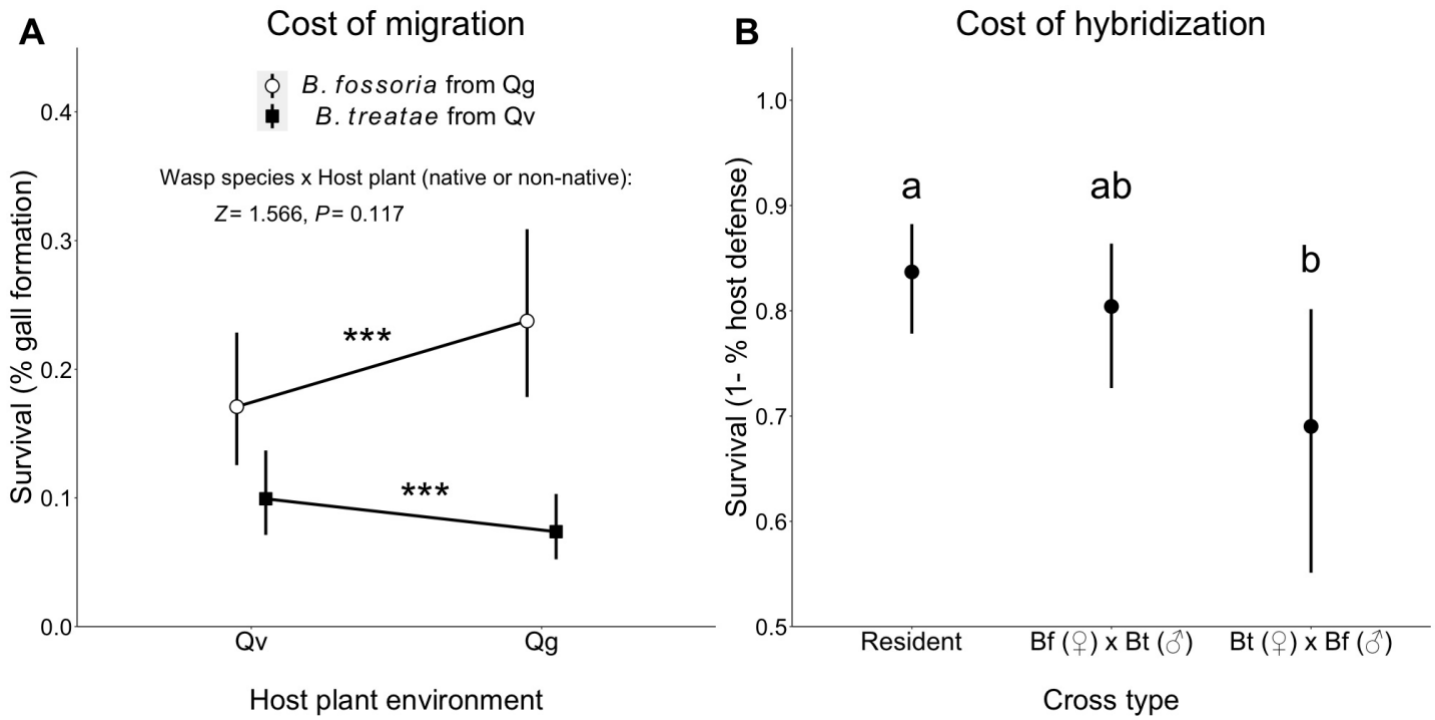

Figure S1. (A) Cost of migration for *B. fossoria* and *B. treatae* to alternative sympatric host plants as measured by the percent of oviposition events that form galls when females oviposit on their natal host plant compared to the alternative host plant (modified from Zhang et al. 2021a). Shown are least square means of % gall formation with 95 % CI. Triple asterisks indicate significant costs of migration for both species ($P < 0.001$). No evidence of asymmetric migration cost between *B. fossoria* and *B. treatae* is indicated by GLMM results for the test of the interaction term: Wasp species × Host plant environment (native/non-native), $Z = 1.566$, $P = 0.117$. (B**)** Cost of hybridization as measured by probability of survival of individual eggs as a function of cross type (modified from Zhang et al. 2021b). Survival is indexed as the ability of developing larvae to evade the host plant's hypersensitive immune response. The resident cross involves matings of conspecific males and females followed by oviposition into the natal host plants. All values compare the least squared mean with 95% CI. Means within a panel labeled with different letters differ ($P < 0.05$).

Methods and Results for Figure S2

1. Test of habitat isolation for *B. kinseyi*

To test whether each population of *B. kinseyi* exhibited significant host fidelity for the native host plant, we conducted one-way t-test by comparing % time spent on the native host plant with the value 0.5, which indicates no preference for either host plant species. All three populations of *B. kinseyi* showed significant host fidelity for their native host plant, *Q. virginiana* (population GM: mean host fidelity = 0.660, t = 3.29, $P < 0.001$; population PY: mean host fidelity = 0.627, t = 1.91, $P = 0.016$; population RU: mean host fidelity = 0.616, t = 3.79, $P < 0.001$).

2. Comparison of the strength of sexual isolation between *B. kinseyi* and *B. fossoria* with the strength of sexual isolation between *B. kinseyi* and *B. treatae*

Species *B. kinseyi* is both allopatric with *B. fossoria* and feeds on a different host plant species. *B. kinseyi* is also allopatric with *B. treatae* but both species share the same host plant species. The genetic divergence between *B. kinseyi* and *B. fossoria* and the genetic divergence between *B. kinseyi* and *B. treatae* are similar (Driscoe et al. 2019). Therefore, the finding of higher sexual isolation between *B. kinseyi* and *B. fossoria that adapt to different host plant species* compared to sexual isolation between *B. kinseyi* and *B. treatae* which share the same host plant species would suggest that ecological adaptation plays an important role in promoting the evolution of sexual isolation.

To test this hypothesis we estimated sexual isolation (SI) using the method described by Sobel and Chen (2014): SI = 1 – 2*[heterospecific mate frequency / (heterospecific mate frequency + homospecific mate frequency)]. We obtained 10,000 bootstrap values of SI for each of the two mating combinations being compared: *B. kinseyi* × *B. fossoria*, and *B. kinseyi* × *B. treatae* by randomly resampling mated and unmated pairs from original data and recalculating SI. Sexual

isolation between *B. kinseyi* and *B. fossoria* was significant with a mean value of 0.138 and 95% bootstrap values ranging from 0.009-0.260 (Figure S2). In contrast, SI between *B. kinseyi* and *B. treatae* was not significantly different from zero with a mean value of 0.078 and with 95% bootstrap values overlapping zero (-0.135 - 0.305). The strength of SI between *B. kinseyi* and *B. fossoria* is higher than that between *B. kinseyi* and *B. treatae* ($t = 43.93$, $P < 1*e^{-16}$).

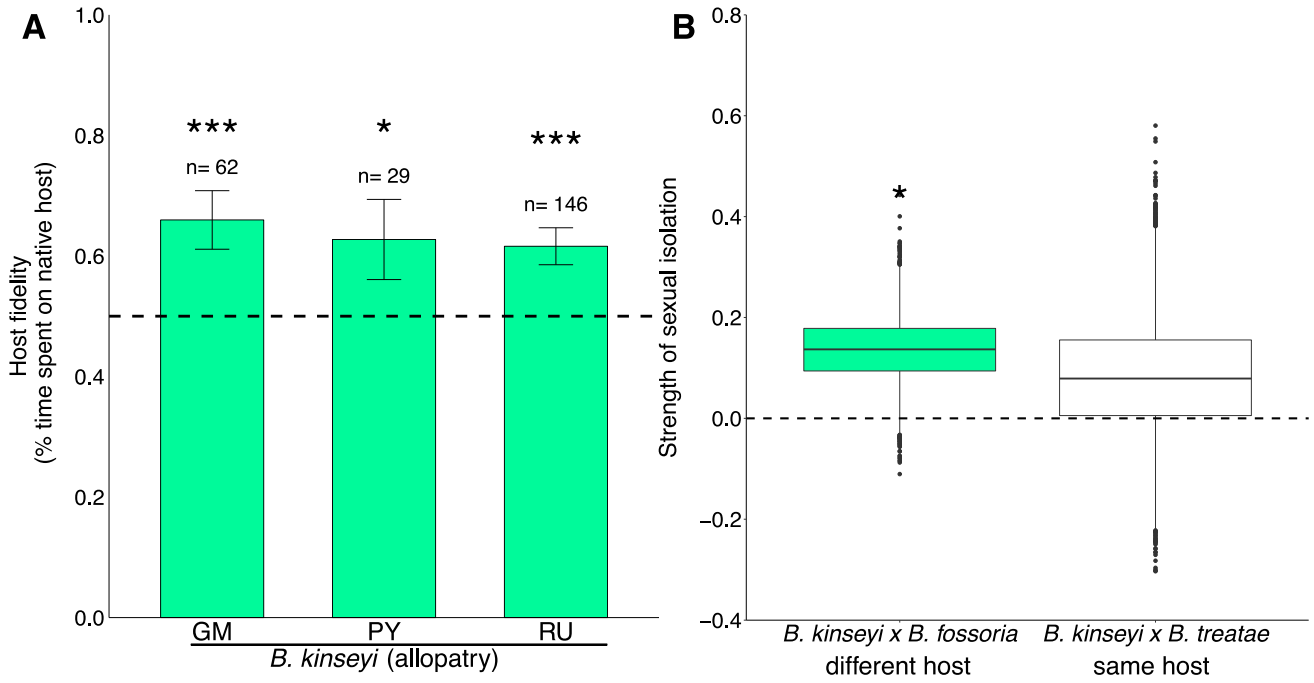

Figure S2. Test of the role of ecological adaptation in both habitat isolation and sexual isolation. (A) Host preference of three allopatric populations of *B. kinseyi*. Each bar represents the average host preference per population with standard error. The bold dashed line identifies the null hypothesis of no host preference. Asterisks denote statistically significant preference: * P < 0.05, ** P < 0.01, *** P < 0.001. (B) Boxplot of bootstrap values of sexual isolation (SI) for each of the two mating combinations: *B. kinseyi* × *B. fossoria* (each species feeds on a different host plant in sympatry) and *B. kinseyi* × *B. treatae* (both species feed on the same host plant in allopatry).The bold dashed line identifies the null hypothesis of no sexual isolation. One asterisk denotes that the 95% CI of bootstrapped SI values does not overlap zero.