

Ecologically dependent and intrinsic genetic signatures of postzygotic isolation between sympatric host races of the leaf beetle *Lochmaea capreae*

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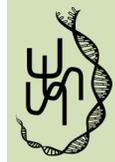
The fitness of hybrids might be compromised as a result of intrinsic isolation and/or because they fall between ecological niches due to their intermediate phenotypes (“extrinsic isolation”). Here, we present data from several crosses (parental crosses, F1, F2, and backcrosses) between the two host races of *Lochmaea capreae* on willow and birch to test for extrinsic isolation, intrinsic isolation, and environmentally dependent genetic incompatibilities. We employed a reciprocal transplant design in which offspring were raised on either host plant and their survival was recorded until adulthood. We also applied joint-scaling analysis to determine the genetic architecture of hybrid inviability. The relative fitness of the backcrosses switched between environments; furthermore, the additive genetic–environment interaction was detected as the strongest effect in our analysis. These results provide strong evidence that divergent natural selection has played a central role in the evolution of hybrid dysfunction between host races. Joint-scaling analysis detected significant negative epistatic effects that are most evident in the poor performance of F2-hybrids on willow, indicating signs of intrinsic isolation. We did not find any evidence that genetic incompatibilities are manifested independently of environmental conditions. Our findings suggest the outcome of natural hybridization between these host races is mainly affected by extrinsic isolation and a weak contribution of intrinsic isolation.

KEY WORDS: Ecological speciation, hybrid fitness, joint-scaling analysis, epistasis, hybrid breakdown, reproductive isolation.

The idea that ecological adaptation is responsible for biological diversification has received substantial renewed interest within the last two decades (Schluter 2001; Weissing et al. 2011). This concept was inherent in the hypothesis that adaptive radiations resulted from access to new ecological niches (Simpson 1955; Funk et al. 2006). Several theoretical models have further shown how the adaptive fixation of alternative alleles in ecologically diverging populations might incidentally lead to the evolution of reproductive isolation, a process now referred to as “ecological speciation” (Dobzhansky 1937; Mayr 1942; Funk et al. 2006). Models also predict that ecological speciation is somewhat independent to the classical modes of speciation by geographical arrangement and can proceed between geographically isolated (allopatric) populations (Schluter 2001), as well as lineages that are not extrinsically prevented from gene exchange (that is in sympatry) (Berlocher and Feder 2002; Rundle and Nosil 2005).

Although examples of ecologically driven reproductive isolation have started to accumulate, the role of ecology in the formation of any particular species still remains elusive (Via 2009). One potential reason is that the multifaceted process of speciation potentially involves a range of reproductive barriers that can arise through both ecological and non-ecological causes (Sobel et al. 2009; Butlin et al. 2012).

The causes of reduced hybrid fitness, which are known as postzygotic isolation, present an important issue in ascertaining the ecological nature of speciation (Coyne and Orr 2004; Peccoud et al. 2014). Postzygotic isolation can be characterized as either *extrinsic* or *intrinsic* (Turelli et al. 2001; Coyne and Orr 2004). Intrinsic postzygotic isolation (hereafter referred to as “intrinsic isolation” for simplicity) is more or less independent of environment and results from constitutive incompatibilities between the parental genomes, or from the



breakup and reassortment of co-adapted gene combinations (Hurst and Pomiankowski 1991). In contrast, extrinsic postzygotic isolation or environmentally dependent postzygotic isolation (hereafter referred to as “extrinsic isolation”) occurs when the fitness of hybrids is compromised because their intermediate phenotypes are maladaptive in their parental habitats (Egan and Funk 2009). In other words, extrinsic isolation arises as populations adapt to alternative environments, thus evolving toward separate adaptive peaks and intermediate phenotypes, as a result, fall into fitness valleys (Schluter and Conte 2009). Although intrinsic isolation could arise during both ecological and non-ecological speciation, direct reduction of hybrid fitness by environment-specific selection pressures provides a unique support for ecological speciation (Schluter 2001). However, despite the importance of extrinsic isolation and support from theoretical models (Thibert-Plante and Hendry 2010), it has received much less empirical attention than any other form of reproductive isolation and many have lamented how little we know about its prevalence, strength, and character in nature (Gavrilets 2003; McBride and Singer 2010).

Different types of genes and gene networks may be involved in generating these two broad categories of hybrid dysfunction. The available evidence suggests that negative epistatic interactions—Dobzhansky–Muller incompatibilities—between heterospecific loci inherited from the parental species are the most frequent cause of intrinsic isolation mechanisms, because the alleles contributing to hybrid inviability generally do not cause problems within the genetic background in which they arose (Dobzhansky 1937; Muller 1942; Orr 1995; Presgraves 2007; Ellison and Burton 2008; Seehausen et al. 2014). The main point about intrinsic isolation mechanisms is that deleterious epistatic interactions between parental genomes might occur between alleles that never appeared together in a single lineage. Therefore, hybrid dysfunction does not indicate that “unfit” alleles were fixed within a lineage, or these lineages passed through adaptive valleys (Orr 1995; Coyne et al. 2000; Turelli et al. 2001). Genetic mechanisms of reduced hybrid fitness due to intrinsic incompatibilities are generally independent of ecological context, although environmental effects may affect the specific interactions underlying hybrid breakdown. Unlike intrinsic isolation, only a small number of studies have recently focused on determining the genetic basis of extrinsic isolation (Filchak et al. 2000; Gross et al. 2004; Fuller 2008). One potential reason could be that, theoretically, extrinsic isolation need not invoke epistatic interactions between loci and a purely additive model of gene action would be adequate to explain it, provided that there are no intermediate environments in which hybrids are efficient at exploiting resources (Fuller 2008).

The distinction between the two forms of postzygotic barriers highlights a perhaps underappreciated role of the ecology and environment in determining barrier strength (Harrison 2012). However, as both mechanisms involve hybrid inferiority,

differentiating between extrinsic and intrinsic isolation is challenging. An unambiguous demonstration would be to identify the loci contributing to hybrid inviability and reproductive isolation and determine whether the fitness of allele variants change with the environmental conditions or not (Bordenstein and Drapeau 2001; Willett and Burton 2004; Schluter 2009). Alternatively, extrinsic isolation can be detected rigorously by a quantitative genetic model derived by Rundle and Whitlock (2001). This method compares the relative fitness of both backcrosses in both parental environments while controlling for any intrinsic genetic incompatibilities that may be present. A comparison of the fitness of both backcrosses in both environments evaluates the additive gene effects that act in an environment-dependent manner, independent of contribution of any intrinsic incompatibilities. This is based on the fact that both backcross types exhibit the same degree of hybridity yet are similarly influenced by deleterious epistatic interactions between parental genomes and are not predicted to exhibit considerable differences in environment-specific fitness. If postzygotic isolation is ecologically dependent, each backcross type should display relatively higher environment-specific fitness in the habitat of the parent to which it is more genetically similar. In parallel to the prediction concerning the relative fitness of backcrosses, ecological speciation also requires that hybrid fitness is reduced relative to the native parental species in each environment (i.e., selection must be divergent) (Rundle and Whitlock 2001; Egan and Funk 2009).

Lochmaea capreae is a chrysomelid beetle that mainly feeds on willow (*Salix capreae*, Salicaceae), birch (*Betula pendula*, Betulaceae), and occasionally on poplar (*Populus* sp. Salicaceae). This species exhibits sympatric host races (i.e., “genetically differentiated, sympatric populations of herbivorous insects that feed on different hosts and between which there is appreciable gene flow,” Drès and Mallet 2002) on willow and birch with about 2% gene flow (Kreslavskiy and Mikheyev 1994, for further details about the biology see Soudi et al. 2015). Previous studies have demonstrated that populations are highly sympatric in Western Russia, near Lake Baikal and further east (Kreslavskiy and Mikheyev 1994); yet sympatric populations also seem to extend westward at least to our study populations in the western parts of Germany (K. Reinhold pers. obs.). Reciprocal transplant experiments have previously revealed that each race is locally adapted on each native host plant and has acquired different adaptive sets of preference and performance traits through close association with its own host plant (Soudi et al. 2015). However, it has been suggested that differentiation on different hosts is associated with a monogenic recessive mutation preventing individuals from the willow race from developing on birch (Kreslavskiy and Mikheyev 1994). The pronounced host-associated ecological divergence and local adaptation is accompanied by multiple forms of premating isolation, specifically habitat choice and immigrant inviability

(Soudi et al. 2015). These host races thus provide an informative system for investigating ecological speciation.

In the present study, we follow three main goals; first, we aimed to establish whether extrinsic isolation (additive genetic \times environment effect) exists between the host races of *L. capreae*. Second, to test for intrinsic isolation (overall deleterious epistatic effects) and finally whether the expression of genetic incompatibilities is dependent on the environment (epistatic effects that vary with environmental conditions, see Demuth and Wade 2007a; Fuller 2008) by comparing the survival of different cross-types on both host plants. To achieve these goals, we applied joint-scaling analysis to a series of various crosses between the host races of *L. capreae* that had been raised on either host plant. The aim of line cross-analysis is to determine the (1) additive genetic effects, (2) dominance effects, (3) epistatic effects, (4) environmental effects, and (5) the extent to which the genetic effect may vary with the environment. By examining genetic architecture of postzygotic isolation during population differentiation it may be possible to determine the factors that account for reduced hybrid fitness, and as such, potentially offer insight into the evolutionary forces that lead to the formation of reproductive barriers.

Methods

EXPERIMENTAL CROSSES AND LARVAL PERFORMANCE OR FITNESS ASSAYS

We collected overwintered adults of *L. capreae* on birch and willow on two locations in Kottenforst (50.715°N, 7.002°E; 50.671°N, 7.009°E) near Bonn, Germany. Eggs were collected from these field collected females and larvae from these eggs were reared to adulthood on leaves from their native host plants. We refer to these adults as Generation 1. From these adults, F1-hybrids and pure parental crosses were generated; we refer to them as Generation 2. F1-hybrids were obtained only from the matings between willow females and birch males, whereas the reciprocal cross between birch females and willow males was not possible due to an inability of the willow males to transfer sperm to the birch females during copulation and therefore a prezygotic isolation mechanism (Soudi et al. in prep.). F1-hybrids from Generation 2 were subsequently crossed with other F1-hybrids and backcrossed with individuals of the parental lines (in both male–female combinations) to generate Generation 3. In total we thus created eight different crosses; two pure parental crosses ($WW = WW_{\text{♀}} \times WW_{\text{♂}}$ and $BB = BB_{\text{♀}} \times BB_{\text{♂}}$), one F1 ($WB = WW_{\text{♀}} \times BB_{\text{♂}}$), one F2 ($WB_{\text{♀}} \times WB_{\text{♂}}$), two willow-like backcrosses ($BcW1 = WW_{\text{♀}} \times WB_{\text{♂}}$ and $BcW2 = WB_{\text{♀}} \times WW_{\text{♂}}$) and two birch-like backcrosses ($BcB1 = BB_{\text{♀}} \times WB_{\text{♂}}$ and $BcB2 = WB_{\text{♀}} \times BB_{\text{♂}}$).

All test families from Generations 2 and 3 were derived from individual male/female matings, and no beetles were used more

than once. Mated females were subsequently kept in single 9-cm petri dishes and provided with fresh leaves of its native host plant, thus providing the next generation of test offspring. We reared half of the F1-hybrids on willow and the other half on birch. However, since all of those F1-hybrids reared on birch died during overwintering phase, we could continue experiments only with those that survived successfully on willow during winter. Parental hosts can have an effect on offspring fitness (Agrawal 2001), but we could not test it here. Moreover, as all surviving F1s were raised on the same host plant, this possible influence cannot explain our results in the current study. Eggs from mated females were collected from the petri dishes every second day and maintained in 5-cm petri dishes lined with dry filter paper. Abiotic conditions were maintained at 23–25°C, 18L: 6D light cycle, and 70% relative humidity. Upon hatching, the larvae were collected randomly from each family type and split into two groups (20–25 individuals per family). The first group was assigned to feed on birch and the second group to feed on willow. Each test larva was maintained individually in a 5-cm single petri dish lined with moist filter paper and a cutting of its test plant. Mortality of each individual was checked daily until the larva died or reached the adult stage. We focused on survival as the main performance trait because previous reciprocal-transplant experiments revealed that mortality of willow and birch race individuals on the non-native host plant was considerable and likely to present the most important factor determining host environment-dependent fitness (Soudi et al. 2015). In total over 1700 offspring from 130 families were used.

STATISTICAL ANALYSIS

The binomially distributed response variable survival was analyzed with generalized linear mixed effects models (GLMMs) using logit as a link factor. Cross-type, diet (i.e., the host on which individuals reared until adulthood), and the interactions between them were considered as fixed effects. Family and family-by-environment, both nested within cross-type, were included as random effects. Subsequently, we performed posthoc tests to examine differences among cross-types within each environment. All post hoc treatments of mean comparisons were conducted using a Tukey HSD test accounting for multiple comparisons.

Joint-scaling analysis—Joint-scaling is a quantitative genetic method by which the net additive and nonadditive genetic effects can be inferred from phenotypic differences among lines of known pedigree (Demuth and Wade 2007a, b). We followed the method described by Rundle and Whitlock (2001), which extends Lynch and Walsh's (1998) model to include two populations in different environments. Briefly, we optimized the linear model describing the composite genetic effects contributing to our different cross-type line means. We used the expected phenotype of F2-hybrid offspring as the point of reference; hence, its

mean phenotype defines the intercept parameter. The composite genetic effects represent the mean and the effect of environment (E), additive genetic effect (a), dominance (d), the epistatic terms (aa), (ad), (dd) describing additive-additive, additive-dominance, and dominance-dominance epistatic interactions, respectively and gene–environment interactions: (a × E), (d × E), (aa × E), (ad × E), (dd × E). These $g \times E$ interactions essentially accounts for mean differences of the same genetic crosses reared on different host plants. The analysis involved assigning coefficients of determination to each cross-type that describe the expected contribution from each genetic effect. The coefficients of determination are listed in Table S1 (Lynch 1991; Lynch and Walsh 1998; Rundle and Whitlock 2001).

With 11 parameters, there are $2^{11} = 2048$ possible models that could potentially be fitted to the data. Following the suggestion of Bieri and Kawecki (2003), we used an AIC-approach to select the model that contained the minimum number of parameters necessary to explain the observed pattern, and this model is referred to as the most parsimonious model. However since statistical inference focusing on the most parsimonious model alone can potentially be misleading (Burnham et al. 2011), we additionally report multimodel inference statistics providing quantities of strength of evidence of different candidate models. Nevertheless, to decrease the risk of over fitting due to a massive number of candidate models, we treated the three epistasis parameter terms (aa, ad, and dd) as one group (e), and the three epistasis environment interaction terms (aa × E, ad × E, and dd × E) as another (e × E) as suggested by Bieri and Kawecki (2003). These terms were thus either all included or excluded from the model. In this way, the number of candidate models was reduced to $2^7 = 128$. The significance of each parameter included in the most parsimonious model was tested with a likelihood-ratio test. All statistical analyses were conducted using the statistical software R 3.0.3 (R Development Core Team 2007). The generalized linear models were performed using *lmer* in the *lme4* package (Bates et al. 2013), and the AIC model selection was done with *dredge* provided in the *MuMIn*-package (Barton 2014). All tests were two-tailed and the null hypotheses were rejected at $P < 0.05$.

Results

PERFORMANCE OF PURE AND HYBRID CROSS TYPES ON BOTH HOST PLANTS

Survival was affected by cross-type, host environment, and the interaction between cross-type and environment (Table 1). Since survival of the two willow-like backcrosses was very similar (see Fig. 1), we pooled them to simplify analyses and refer to them as willow backcrosses. Similarly, the two birch-like backcrosses were pooled as birch backcrosses. The fitness of the willow and birch backcrosses was ecologically dependent and

Table 1. Mixed effect model analysis to compare variation in survival in willow and birch host races of *L. capreae* and hybrids between them reared on different host environments (willow or birch).

Source of variation	df	χ^2	P
Survival			
Cross-type	5	43.7	< 0.0001
Host environment (diet)	1	62.3	< 0.0001
Cross-type by host environment	5	192.6	< 0.0001
Family	5	107.2	< 0.0001
Family-by-environment	5	86.9	< 0.0001

switched across the two host plants. Birch backcrosses exhibited significantly higher survival on birch than on willow ($\chi^2 = 6.07$, $P = 0.01$, Fig. 1). Those individuals from the willow backcrosses that were assigned to be reared on willow showed remarkably higher survival than those on birch ($\chi^2 = 79.41$, $P < 0.001$, Fig. 1). F1- and F2-hybrids also exhibited higher survival on willow than on birch, although the difference was not significant in F2-hybrids (F1: $\chi^2 = 8.52$, $P = 0.003$; F2: $\chi^2 = 3.22$, $P = 0.07$). These differences were further confirmed by a significant interaction between cross-type and environment indicating that the effect of host plant on survival is dependent on the cross-type. The main effect of host environment was also significant, indicating that the mean survival of individuals from different cross-types was higher on willow than on birch (Table 1).

On willow, the differences between cross-types were smaller. Similar to birch, survival was significantly affected by cross-type ($\chi^2 = 32.79$, $P < 0.0001$, Fig. 1a). Individuals from the pure willow cross-type exhibited the highest survival, followed closely by F1, and willow backcrosses (Tukey HSD test, $P > 0.05$). F2, birch backcrosses and the pure birch cross-type exhibited lower survival rates (Fig. 1a).

The largest differences among cross-types occurred on birch and survival of individuals was significantly affected by cross-type on birch ($\chi^2 = 104.1$, $P < 0.0001$, Fig. 1b). Individuals from the pure birch cross-type showed the highest survival rate on their native host plant, followed very closely by birch backcrosses (Tukey HSD test, $P = 0.6$). However, the survival of birch backcrosses was nearly more than twice the survival rate of willow backcrosses (Tukey HSD test, $P < 0.001$). The survival rate of the F1-hybrid did not differ significantly from the pure birch cross-type (Tukey HSD test, $P = 0.06$) and birch backcrosses, but outperformed the F2 hybrid remarkably (Tukey HSD test, $P = 0.03$). All individuals from the conspecific willow cross-type failed to start feeding on birch and died on this host plant (Fig. 1b).

The patterns described above were in full accordance with the predictions of ecological speciation. Specifically, the relative fitness of each cross-type on a given host plant corresponded to its

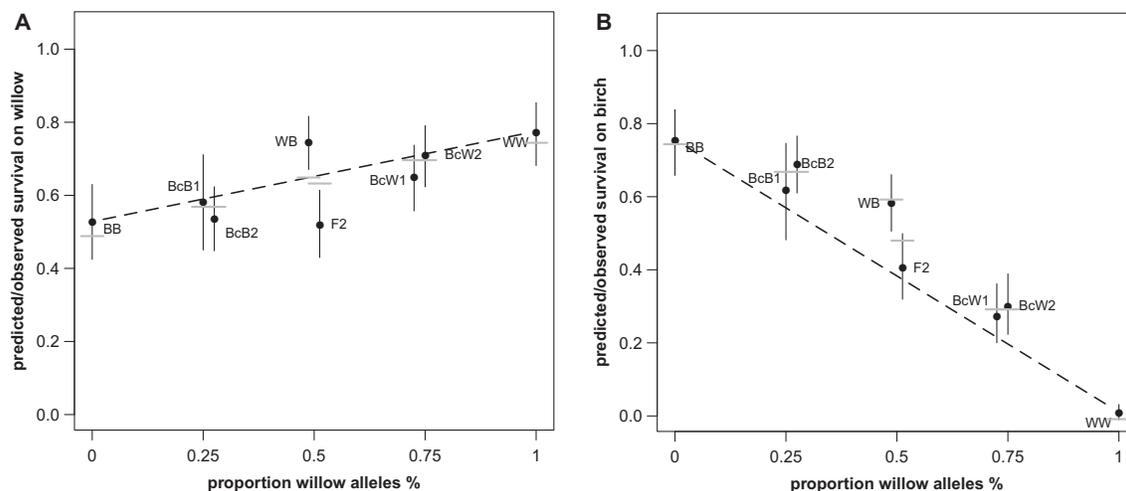


Figure 1. Mean survival rate of each cross-type fed on willow (A) and birch (B). The line indicates the expected hybrid means if inheritance of population differences in survival were purely additive. Gray lines show predicted means without epistatic effects, yet including additive and dominance effects. BB represents pure birch parental type, WW represents pure willow parental type, BcW1 and BcW2 represent two willow-like backcrosses, and BcB1 and BcB2 represent two birch-like backcrosses. Points and bars indicate mean \pm SE.

Table 2. Statistical significance of estimated composite genetic parameters contributing to postzygotic isolation (survival) between the host races of *L. capreae* on different host environments (willow or birch).

Source of variation	χ^2	d.f.	P
<i>Included terms</i>			
Host environment (diet)	28.8	1	< 0.0001
Additive	8.6	1	0.003
Dominance	7.9	1	0.005
Epistasis	8.0	3	0.047
Additive \times environment	105	1	< 0.0001
Dominance \times environment	5.3	1	0.021
<i>excluded terms</i>			
Epistasis \times environment	1.7	3	0.64

Included and excluded terms refer to their presence in the AIC-criteria based most parsimonious model.

genetic similarity to the pure parental type originally associated with that plant (Rundle and Whitlock 2001). With an increasing proportion of environment-specific genes adapted to a given host plant, individuals had higher performance on that host plant. Most importantly the relative fitness of reciprocal backcrosses switched across environments.

JOINT-SCALING ANALYSIS

The best-fit model for the joint-scaling analysis is shown in Table 2; parameters for the six best candidate models estimated by AIC criterion are shown in Table 3. The analysis showed that additive, dominance genetic effects, and the interaction between additive and dominance with environment are the genetic

factors contributing most to the observed patterns of hybrid survival and population differentiation between the host races of *L. capreae* (Fig. 1). The interaction between additive genetic and environmental effects was several times greater than each of the other effects. The most parsimonious model also included statistically significant epistatic effects (Table 2), which were consistent across environments (i.e., no $e \times E$ interaction). Nevertheless, by inspecting the likelihood of the candidate models signifying different genetic effects (a model including epistatic effects is merely 2.5 times more likely than a model excluding them, bottom panel in Table 3), it is also apparent that the strength of evidence in favor of negative epistatic effects is rather moderate.

Discussion

For the ecological speciation hypothesis, it is critical to demonstrate that the relative fitness of backcross types switch between the parental environments. This means that each backcross type should show relatively higher fitness in the environment of the parent to which it is most genetically similar (Rundle and Whitlock 2001). Our results were highly consistent with this prediction of ecological speciation. Survival of birch backcrosses (having a pure birch-associated parent) was significantly higher than willow backcrosses (having pure willow-associated parent) on birch, while on willow the pattern was reversed and willow backcrosses outperformed birch backcrosses. Hence, crosses that carry a higher proportion of willow genes exhibited the highest performance on willow and those that possess a higher proportion of birch genes had the highest performance on birch (Fig. 1). Such a reversal pattern of relative performance of these backcrosses

Table 3. Six candidate models that made the best compromise between the amount of variance explained and the number of parameters (most parsimonious models) selected by Akaike's information criterion (AIC).

Model parameters																
<i>m</i>	<i>E</i>	<i>a</i>	<i>d</i>	<i>aa</i>	<i>ad</i>	<i>dd</i>	<i>a</i> × <i>E</i>	<i>d</i> × <i>E</i>	<i>aa</i> × <i>E</i>	<i>ad</i> × <i>E</i>	<i>dd</i> × <i>E</i>	d.f.	log(L)	AICc	Δ_i	<i>w_i</i>
0.464	0.077	-0.126	0.255	0.358	-0.003	-0.052	0.252	-0.048	—	—	—	11	46.5	-69.7	0	0.472
0.556	0.077	-0.124	0.064	—	—	—	0.252	-0.048	—	—	—	8	42.5	-68.3	1.34	0.242
0.463	0.083	-0.124	0.257	0.362	-0.002	-0.053	0.253	—	—	—	—	10	43.8	-66.6	3.09	0.101
0.464	0.047	-0.126	0.254	0.357	-0.003	-0.052	0.252	—	0.077	-8e-04	0.013	13	47.2	-66.5	3.13	0.099
0.556	0.083	-0.124	0.064	—	—	—	0.252	—	—	—	—	7	39.8	-65.1	4.60	0.047
0.464	0.063	-0.126	0.254	0.357	-0.003	-0.052	0.253	-0.045	-0.008	9e-04	0.038	14	47.4	-64.5	5.14	0.036
fix	~1	~1	~1	~1	0.71	~1	~1	0.75	~1	0.13	~1	Σw_i				
fix	> 10,000	> 10,000	> 10,000	> 10,000	2.5	> 10,000	> 10,000	4.0	0.15	0.15	0.15	Evidence ratio				

Model parameters including *E* represents the effect of environment of *a*, *d* represent additive and dominance genetic effects, respectively. *aa*, *ad*, *dd* represent additive-additive, additive-dominance, and dominance-dominance epistasis effects, respectively. *a* × *E*, *d* × *E* represent interactions between environment and additive and dominance effects, respectively. The terms *aa* × *E*, *ad* × *E*, *dd* × *E* represent the interaction between environment and different epistasis effects. The parameter *m* is the intercept (representing estimated mean survival of F2-hybrids). These six models are the only ones with a $\Delta_i < 10$, and together comprise a summed relative likelihood (Σw_i) larger than 0.995. The bottom panel gives the summed relative likelihood of each model and the evidence ratio of models containing against models lacking the specific term.

on two host plants can only be the product of divergent natural selection and provides strong evidence that ecological mechanisms have contributed to the evolution of postzygotic isolation between these host races. Explicit demonstration of ecological selection against hybrids is still rare and only very few studies have attempted to evaluate the role of ecological mechanisms in arising hybrid dysfunction via rigorous approaches like backcross studies (Rundle 2002; Fuller 2008; Egan and Funk 2009; Kuwajima et al. 2010). Our results are consistent with the previous studies in herbivorous insects by Egan and Funk (2009) that provided strong evidence for ecologically dependent postmating isolation between sympatric host forms of *Neochlamisus bebbianae* leaf beetles on willow and maple, and also sympatric ladybird species on thistle and blue cohosh (Kuwajima et al. 2010). The observed pattern described above is an expected consequence of significant interaction between additive gene effects and environment effects, which was detected as the strongest component in the joint-scaling analysis. This means that additive genetic effects that contribute to hybrid inviability act in an environment-dependent manner.

The evidence for extrinsic isolation in our study might reflect the nature of the alternative host plants evaluated here. For specialized herbivorous insects, like *L. capreae*, the host plant is often the place where most of the life activities occur. Therefore an alternative habitat offers an environment that can be distinctly different, as opposed to a continuously varying environment. In particular, this is true when these alternative host plants belong to disparate plant families that differ in several aspects like phytochemistry and morphology, as in our system here. Hence not only are intermediate habitats suitable for hybrids nonexistent under such conditions, but host-related selection pressures are also expected to be markedly specific, strong, and divergent between host-associated populations. In fact, this may accelerate adaptive divergence and the reproductive isolation predicted to accompany it under assumptions of ecological speciation (Rundle and Nosil 2005). This is in contrast with some other examples of hybridizing taxa such as sticklebacks that inhabit more continuous and less distinct environments (Hatfield and Schluter 1999).

In spite of the evidence for the presence of extrinsic isolation, it may not completely prevent introgression between the host races of *L. capreae*. Ecological speciation requires that the fitness of backcross hybrids be reduced relative to the parental species in each environment (Hatfield and Schluter 1999; Rundle and Whitlock 2001; Schluter 2001). Thus, the rank order of survival on birch is predicted to be: birch > birch backcross > F1/F2-hybrids > willow backcross > willow, and this order should be reversed on willow. We observed this expected order, although differences in survival between pairs of cross-types were not all significant; specifically, neither backcross differed significantly from the parental species from which it was mainly derived, but the two backcrosses differed from each other. The F1-hybrid also

had high fitness, particularly on willow. A similar pattern has been reported from other systems in which the fitness of hybrids equals or exceeds that of pure parental species (Arnold and Emms 1998). Hence in our study system, if individuals from F1-hybrid mate with individuals from the willow race (which is likely to be very abundant on willow), or with an individual of the birch race on birch, then the resulting backcross offspring would have relatively high survival on willow and birch, respectively. This suggests that hybridization may have significant evolutionary consequences, such as the introgression of birch alleles into willow alleles and vice versa. However, it is worth noting that we measured only one, though arguably the most important, component of hybrid fitness. Other viability attributes affecting intermediate crosses may further reduce their fitness, including growth rate, reduced predator/parasite resistance, and fecundity (Rundle 2002).

The most parsimonious model in the joint-scaling analysis included significant epistatic effects (Table 2). The signature of negative epistatic effects can be visible in the F2-hybrids, when the F2 has a fitness lower than the average of the midparent and the F1 (Whitlock et al. 1995; Fenster et al. 1997; Johansen-Morris et al. 2006). The observed reduced fitness of F2 relative to F1-hybrids and the midparental line, which is more pronounced on willow, thus suggest negative epistasis. The estimated mean survival rates of the pure and different hybrid crosses deviate from expectations assuming additive and dominance action alone (indicated by gray lines in Fig. 1) in a manner implying signs of intrinsic isolation. Such a pattern may be attributable to the fact that F1-hybrids are heterozygous at all loci (except of those on the sex chromosomes in males) and possess a complete set of alleles including ancestral and mutant alleles due to adaptation to different environments. With all alleles present, congruous allele interactions are expected to continue, and fitness is not negatively affected. F2-hybrids, on the other hand, will be homozygous at many different loci. Most crucially, some loci will have alleles originating from one parental race whereas the alleles at other gene loci will have descended from the other. As these alleles have evolved in different lineages where selection did not act on their ability to positively interact, some of these alleles may not function appropriately together and decrease fitness in the F2-hybrid background (Turelli et al. 2001; Burton et al. 2006). In our study, fitness loss experienced by F2-hybrids may indicate that the deleterious effects of breaking up parental coadapted gene complexes outweigh the favorable dominance effects that are visible in our observation of F1-hybrids (the estimated F1-hybrid survival is well above the expectations from an additive model signified by the lines connecting the two parental means in Fig. 1).

Our analysis did not detect any significant interaction between epistatic genetic effects and environment. This indicates that the signatures of negative epistasis described above are man-

ifested independently from the environmental conditions. Nevertheless, the putative epistatic effect on birch seems rather weak, and we must also keep in mind that only two-way epistatic interactions (i.e., interactions between two loci) are considered in the joint-scaling analysis in our study. Possibly higher order interactions describing more complex epistatic effects might be present. The exclusion of higher order epistatic effects (and their interactions with the environments) may impede the ability to discover environmentally dependent genetic incompatibilities (Fuller 2008). It is also worth noting that although epistasis is included in the most parsimonious explanation of our data, the strength of evidence is rather moderate (Table 3). Altogether, the data strongly implies that extrinsic mechanisms feature much more dominantly compared to intrinsic isolation mechanisms in this system.

Conclusions

In the present study, we rigorously documented a clear example of extrinsic isolation and in addition weaker signs of intrinsic isolation between the sympatric host races of *L. caprae*. In speciation driven by divergent natural selection, extrinsic isolation is expected to evolve first and intrinsic genetic incompatibilities will often only evolve later in the speciation continuum (Seehausen et al. 2014). This is also indicated by the present study, which demonstrates a hitherto stronger accumulation of effects attributed to extrinsic relative to intrinsic isolation. Yet, the evolutionary rates of these two components of postzygotic isolation are still unclear, and one central task for future work to be investigated.

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Table S1. Regression coefficients of the candidate parameters according to Rundle and Whitlock (2001): a (additive), d (dominance), aa, ad, dd: additive-additive, additive-dominance and dominance–dominance epistasis, respectively.