Genetic mechanisms preventing the fusion of ecotypes even in the face of gene flow

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Understanding the genetics behind adaptation and reproductive isolation contributes to our knowledge about how biodiversity is created and maintained. Host races of phytophagous insects are host-associated ecotypes and have been considered as candidates for ecological speciation, but very little is known about the genetic backgrounds of host adaptations. A leaf-mining moth, Acrocercops transecta, consists of Juglans- and Lyonia-associated host races. This study assesses the genetic bases of oviposition preference and larval performance using F1, F2 and backcross hybrids between the two host races. Segregation patterns in the hybrid generations revealed that larval performance on Juglans is dominant, but oviposition preference for Lyonia is dominant. This result indicates that genetic components introgressed from the Lyonia race are removed from the Juglans race even though hybrid larvae are viable on Juglans. Thus, simple genetic controls with contrasting dominance directions in host-adaptation traits function as barriers to prevent a fusion of host races.

A adaptation to a novel environment often requires the evolution of multiple traits, and hybridization between ecologically divergent taxa could produce maladaptive phenotype combinations, resulting in isolation barriers1–4. In phytophagous insects, a precise combination of preference (e.g., ovipositing female preference) and performance (e.g., larval tolerance to secondary compounds) for particular host plants is crucial because a new host plant can be incorporated into an insect’s diet only if adults accept it for oviposition and if the larvae are able to complete their development on it5,6. In most cases, preferences and performances in phytophagous insects are under genetic control8. Thus, differences in the mode of inheritance between these two traits may result in isolating barriers between host races.

To address the genetic mechanisms preventing the fusion of host races, I studied a leaf-mining moth, Acrocercops transecta (Gracillariidae), which consists of Juglans (Juglandaceae)- and Lyonia (Ericaceae)-associated host races (see Supplementary Background Text for details). The two host races clearly differ in the host preferences of ovipositing females and larval performances on host plants but mate readily in the laboratory, producing fertile hybrids7. Because the resistance to Lyonia is completely recessive to resistance to Juglans, the F1 hybrid larvae can survive only when they feed on Juglans, indicating that gene flow should be directed from the Lyonia race to the Juglans race. However, because F1 larvae exhibit high viability on Juglans7, gene flow from the Juglans race to the Lyonia race is also possible if eclosed F1 hybrid males mate with females of the Lyonia race. The Juglans and Lyonia races are often sympatric in the wild, and there is no phenological or host-associated premating isolation between them10,11. Indeed, gene flow has occurred in both directions between the two host races in the wild12,13. Thus, there should be postmating genetic mechanisms that maintain the differences between the two host races even in the face of gene flow.

Maladaptive alleles introgressed through hybridization are likely to be eliminated from respective host races, but the strength and extent of purifying selection against the alleles depend on the mode of inheritance of each locus. Although a previous study has revealed the direction of dominance of larval performance5, the segregation patterns in F2- and backcross-hybrid larvae are still unknown. Further, the oviposition preferences in F1-, F2- and backcross-hybrid females are also still unknown. Thus, detailed observations of phenotypes in both oviposition preference and larval performance in hybrid generations are crucial to infer the genetic bases of host adaptation and to evaluate their contribution to an isolating barrier between the two host races of A. transecta.
To investigate the genetic bases of oviposition preference and larval performance, two sympatric and five allopatric host-associated populations (Juglans race: Sendai, Sapporo and Yamagata; Lyonia race: Sendai, Okazaki, Kyoto and Kirishima; see Supplementary Fig. S1 online) in Japan were used for the experiments. First, F₁, F₂ and all combinations of backcrosses were established using the sympatric populations (Sendai) (see Supplementary Fig. S2A-H online). Next, I further assessed the segregation patterns of oviposition preference using backcrosses between Juglans females and F₁ hybrid males (J x JL backcross) that were established from Sapporo (Juglans race) and Kyoto (Lyonia race) populations (see Supplementary Fig. S2I online). Finally, segregation patterns of larval performance were further assessed using backcrosses between F₁ hybrid females and Lyonia males and vice versa (JL x L<JL and L<JL x JL backcrosses, respectively). I used Yamagata (Juglans race) and Okazaki and Kirishima (Lyonia race) populations for establishing the backcrosses (see Supplementary Fig. S2J, K online).

The goal of this study is to reveal the modes of inheritance of adaptive traits and to uncover the genetic mechanisms causing isolating barriers between ecologically divergent taxa.

Results

Oviposition preferences in F₁ hybrid females. Reciprocal hybrids (JL and LJ) were assessed in this experiment. F₁ hybrid females significantly preferred to oviposit on Lyonia rather than Juglans regardless of the direction of crosses, except for two females from JL crosses that laid more eggs on Juglans than on Lyonia (JL t = 7.471, df = 38, P = 5.727e-9; LJ t = 4.225, df = 4, P = 1.343e-2; paired t-test) (Fig. 1A–D). Because all F₁ hybrids fed on Juglans during their larval stages, the results indicate that the oviposition preference of adult females is determined not by larval experiences but by genetic factors. The results also indicate that the loci determining oviposition preference are located on autosomes and that the preference for Lyonia is dominant over that for Juglans. Thus, I used only JL F₁ hybrids for establishing F₂ and backcross hybrids in the subsequent experiments.

Oviposition preferences in F₂ and backcross hybrid females. Assuming that oviposition preference is determined by a single-locus, two-allele system and that the Lyonia-prefering allele is dominant to the Juglans-prefering allele, the expected segregation ratios are 1:3 (prefer to oviposit on Juglans:Lyonia) in F₂, 0:1 in JL x L and L x JL, and 1:1 in J x JL (JL x JL backcross is lethal, see below). In each of the pooled JL x L, L x JL, and J x JL backcrosses, F₁ hybrid individuals segregated in the expected ratio (Fig. 1G–N; Table 1A). All females preferred to oviposit on Lyonia in the JL x L (Sendai) and L x JL (Sendai) backcrosses (JL x L t = 9.798, P = 1.202e-7, df = 14; L x JL t = 4.757, P = 1.828e-4, df = 17; paired t-test) (Fig. 1G, H, M, N). In the J x JL (Sendai) backcross, 95 females preferred to oviposit on Juglans and 87 preferred Lyonia, although three females laid eggs evenly on Juglans and Lyonia (Fig. 1I). For the 95 females that preferred Juglans, the mean number of eggs deposited on Juglans was significantly larger than that on Lyonia (paired t-test, t = 13.1666, P = 2.2e-16, df = 94), and the 87 females that preferred Lyonia deposited significantly more eggs on Lyonia (paired t-test, t = 10.9945, P = 2.2e-16, df = 86) (Fig. 1K, L). These results are consistent with the hypothesis of Mendelian inheritance, with the dominance of a Lyonia-prefering allele in the preference gene.

However, F₂ (Sendai) and the J x JL backcross (Sapporo x Kyoto) demonstrated biased segregation ratios (Fig. 1F, P; Table 1A). One possible hypothesis to explain the deviation from the expected ratio in F₂ hybrids is that the preference gene is physically linked to performance genes. Because all F₂ hybrid larvae were reared on Lyonia because of the oviposition preference of F₁ hybrid females and because the resistance to Lyonia is completely recessive to resistance to Juglans', approximately three-quarters of F₂ hybrid larvae failed to survive on Lyonia (Fig. 2; Table 1B). Thus, given the linkage between preference and performance loci, all F₂ hybrids possessing a Juglans-prefering allele died on Lyonia. In contrast, the biased segregation pattern in the J x JL backcross (Sapporo x Kyoto) is difficult to explain. One of the possible reasons for this deviation is the experimental condition: a shortage of fresh Lyonia leaves may have inhibited the oviposition of females because the experiments were...
conducted during winter and I used Lyonia plants maintained in a greenhouse. Alternatively, oviposition preference may be governed by several genes or influenced by maternal effects (e.g., symbionts) in the Sapporo population. There were much fewer females assessed in the J x JL backcross (Sapporo x Kyoto) than in the J x JL backcross (Sendai), so additional experiments with a larger sample size of J x JL backcross (Sapporo x Kyoto) females could reveal the factors for this deviation. However, what can be concluded is that Lyonia-preference is dominant to Juglans-preference and that a few loci control oviposition preference.

Larval performances in F2 and backcross hybrids. Performance on Juglans is dominant over that on Lyonia. Thus, assuming that larval performance is determined by a single-locus, two-allele system, the expected segregation ratios are 1:3 (survive:die on Lyonia) in F2, 0:1 (survive:die on Lyonia) in JL x J, 1:1 (survive:die on Lyonia) in JL x L and L x JL, and 1:0 (survive:die on Juglans) in J x JL backcrosses. All segregation patterns in F2 and backcross hybrids supported the hypothesis except the J x JL backcross hybrids (Table 1B). However, the viability of the J x JL backcross was not significantly different from that of the control crosses (Fig. 2, see Supplementary Table 1 | Segregation patterns and single-locus goodness-of-fit tests in F1, F2 and backcross hybrids. (A) oviposition preference. (B) larval performance.

| Population        | Cross*   | Total* | Juglans type* | Lyonia type* | Expected* | G  | df | P-value |
|-------------------|----------|--------|---------------|---------------|-----------|----|----|---------|
| Sendai            | JL x JL F2 | 20     | 0             | 20            | 1:3       | 6.667 | 1  | 9.823e-3** |
|                   | JL x L BC | 15     | 0             | 15            | 0:1       | 0   | 1  | 1       |
|                   | J x JL BC | 182    | 95            | 87            | 1:1       | 0.3516 | 1  | 0.5532  |
|                   | L x JL BC | 18     | 0             | 18            | 0:1       | 0   | 1  | 1       |
| Sapporo x Kyoto   | J x JL BC | 43     | 36            | 7             | 1:1       | 19.56 | 1  | 9.758e-6*** |

| Population        | Cross*   | Larval food† | Total* | Alive* | Dead* | Expected* | G  | df | P-value |
|-------------------|----------|--------------|--------|--------|--------|-----------|----|----|---------|
| Sendai            | JL x JL F2 | Lyonia       | 343    | 76     | 267    | 1:3       | 1.4781 | 1  | 0.2241  |
|                   | JL x L BC | Lyonia       | 157    | 0      | 157    | 0:1       | 0   | 1  | 1       |
|                   | J x JL BC | Lyonia       | 240    | 113    | 127    | 1:1       | 0.8167 | 1  | 0.3662  |
|                   | L x JL BC | Lyonia       | 148    | 137    | 11     | 1:0       | Infinite | 1  | <2.2e-16*** |
| Yamagata x        | JL x L BC | Lyonia       | 328    | 155    | 173    | 1:1       | 0.9878 | 1  | 0.3203  |
| Okazaki x         | L x JL BC | Lyonia       | 269    | 132    | 137    | 1:1       | 0.0929 | 1  | 0.7605  |
| Kirishima         | L x JL BC | Lyonia       | 399    | 182    | 217    | 1:1       | 3.0702 | 1  | 0.07974 |

*All broods in each cross were pooled. No significant heterogeneity was observed within a given category of mating (see Materials and Methods).
†Female moths were considered to be a Juglans (or Lyonia) type if they laid more than half of their eggs on Juglans (or Lyonia).
‡Expected indicates the hypothesized segregation ratio for single-gene inheritance in each cross.
§The host plant on which larvae were maintained.
∞Numbers of hatched larvae that survived or did not survive until the second stadium.

Figure 2 | Hatchability and viability until the second stadium in F1, F2, backcross hybrids, and pure Juglans or Lyonia races. For viability, a significant difference was found among crosses ($F_{8, 41} = 82.09, P < 2.2e-16$, one-way ANOVA). Thus, post hoc Tukey-Kramer HSD pairwise comparisons were performed between crosses. Different letters indicate significant differences ($P < 0.05$, Tukey-Kramer HSD test).
Table S1 online for detailed statistical results), suggesting that the slightly reduced viability was not due to the lack of the resistance to Juglans but due to accidental mortality. Thus, the present results indicate that larval performance is governed by a single-locus, two-allele system with complete dominance of resistance to Juglans. In addition, the hatchability of the eggs from every cross combination was higher than 90%. There were no significant differences in hatchability between pure races and hybrids (\(F_{0.10} = 0.7583, P = 0.6406\)) (Fig. 2), indicating that there was not intrinsic reproductive isolation between the two host races.

**Discussion**

The present results provide evidence that the difference in both ovipositing female preference and larval performance between host races of A. transecta is each mainly determined by a single-locus, two-allele system with dominance, respectively. However, a few females showed an intermediate oviposition preference and a biased segregation ratio in the J x JL backcross (Sapporo x Kyoto), implying the existence of modifier genes or maternal effects in host preference. The directions of dominance for female preference and larval performance were opposite, indicating that preference and performance are under different genetic controls.

The present findings have implications for the mechanism that prevent the fusion of the two host races even when gene flow occurs. The allele for resistance to Juglans is dominant. Thus, F₁ larvae could survive if Juglans females mate with Lyonia males, resulting in asymmetrical gene flow from the Lyonia race to the Juglans race (Fig. 3). However, eclosed F₁ females avoid ovipositing on Juglans because of the expression of the Lyonia-prefering allele. This result indicates that genetic components that introgressed from the Lyonia race were removed from the Juglans race (Fig. 3). Thus, the differences in the direction of dominance between preference and performance loci themselves function as a barrier to prevent the fusion of the two host races. A physical linkage between the preference and performance

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**Figure 3** | Summary of the genetic mechanisms that prevent fusion of the two host races in A. transecta. (a) F₁ hybrid eggs from crosses between Lyonia females and Juglans males are deposited on Lyonia. However, F₁ hybrid larvae cannot survive on Lyonia because of an expression of the dominant Juglans-resistance allele. Thus, alleles for Juglans resistance and Juglans preference are eliminated from the Lyonia race immediately. (b) F₁ hybrid larvae from crosses between Juglans females and Lyonia males can develop to adulthood on Juglans, but no eclosed adult females prefer to oviposit on Juglans because of an expression of the dominant Lyonia-preference allele. Even if F₁ males mate with Juglans females, approximately half of the resulting female backcross offspring will avoid ovipositing on Juglans as adults. Therefore, alleles for Lyonia preference are sequentially removed from the Juglans race.
loci would make removal of the genomic components responsible for the resistance to Lyonia from the *Juglans* race more easily, and this possibility should be assessed in future mapping studies. The present study also indicates that the genetics of host adaptation in *A. transecta* contributes to the reproductive isolation of the two host races. If the females of *F1* hybrids mate with males of the *Juglans* race, the females oviposit all eggs on *Lyonia*, but no backcross larvae can survive on *Lyonia* (Fig. 3). Similarly, even if the *F1* hybrids mate with *Lyonia* females or males, only half of the backcross hybrids would express the recessive trait, resulting in reduced viability on *Lyonia* (Fig. 3). Therefore, hybrids suffer from incongruent phenotypes for preference and performance because of the opposite directions of dominance. This incongruent dominance could be a prime barrier against gene flow between the two host races of *A. transecta*.

Although the genetics of ecological adaptations have received much attention in the study of speciation and species differences34, the implications of such studies for understanding how ecological speciation occurs are unclear. One reason for this lack of clarity is that empirical data for the genetics of ecological adaptation vary among species (e.g., governed by autosomal loci or sex-linked genes, few or many genes, genes of small or large effect, or genes with dominance, epistatic interactions or no dominance)35. However, the present results and a growing number of studies have demonstrated that phytophagous insects have different genetic bases between preference and performance with different modes of inheritance15,25–28. This implies that hybrids are likely to express different, and often functionally incompatible, phenotypes for preference and performance traits. Indeed, Forister24 and Nygren et al.25 have revealed that the differences in dominance directions and sex-linkage for preference and performance loci break the correlation between the two traits in *F1* hybrids, respectively. The present study further demonstrates that differences in dominance directions between preference and performance loci lead to ecological incompatibilities in subsequent backcross generations (Fig. 3).

In phytophagous insects, the growing larvae often complete their entire development on a single host plant individual. Hence, a set of genes that function well together on one host is crucial and could be an expected evolutionary outcome26,27. Therefore, hybrids with incongruent host-adaptation genes are likely to be under strong disruptive selection as indicated by the Bateson-Dobzhansky-Muller (BDM) model for postzygotic genomic incompatibilities26–28. Therefore, the different modes of inheritance for selected traits may be an important postzygotic isolation mechanism in phytophagous insects. Although these mechanisms have rarely been emphasized, they may prove to be a major factor promoting host-race formation and consequently the high specialization and species diversity observed in phytophagous insects.

**Methods**

**Moth collection and rearing.** I collected mined leaves containing larvae from the host plants, *Juglans ailanthifolia* or *J. regia* and *Lyonia ovalifolia*, and maintained them in the laboratory26. Two sympatric (Sendai, Japan [38°15′N, 140°49′E]) and five allopatric host-associated populations (*Juglans* race: Sapporo [43°07′N, 141°34′E] and Yamagata [38°33′N, 140°49′E]; *Lyonia* race: Okazaki [34°94′N, 137°17′E], Kyoto [35°02′N, 135°79′E] and Kirishima [31°86′N, 130°77′E]; see Supplementary Fig. S1 online) were used for the experiments. All moths used for parental crosses were collected from the field as larvae, together with the leaves on which they were reared. Similar-sized leaves were chosen. In this container, ovipositing females were allowed to select leaves for oviposition for 24 h after transfer, and the number of eggs deposited on each leaf was counted. For this reason, I recorded the numbers of hatched eggs and the viability of larvae that reached the second stadium as an index of larval performance.

There was no observed heterogeneity of segregation patterns among broods within a given category of mating (*G*-test, *J. x JL*; *F2* [Sendai] *G* = 6.995, *P* = 0.01361, df = 4; *JL x JL* backcross [Sendai] *G* = 0, *P* = 1, df = 4; *JL x L* backcross [Sendai] *G* = 5.497, *P* = 0.2599, df = 4; *JL x JL* backcross [Sendai] *G* = 7.624, *P* = 0.01063, df = 4; *JL x JL* backcross [Sendai] *G* = 8.389, *P* = 0.05890, df = 4; *JL x L* backcross [Yamagata x Okazaki x Kirishima] *G* = 8.912, *P* = 0.1126, df = 5; *JL x L* backcross [Yamagata x Okazaki x Kirishima] *G* = 1.529, *P* = 0.9922, df = 8). Therefore, the results are presented for pooled data.

**Assessing oviposition preference in *F1*, *F2* and backcross generations.** I further assessed the segregation patterns of oviposition preference using a *J x JL* backcross that originated from Sapporo (*Juglans* race) and Kyoto (*Lyonia* race) populations. Thirteen broods were established for these crosses (see Supplementary Fig. S1I online) in 2007.
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**Author contributions**

I.O. conceived and designed the study, collected materials, reared moths with the assistance of those mentioned in the Acknowledgements, collected data, analyzed the data and wrote the paper.

**Additional information**

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