Host race formation in the leaf-mining moth
Acrocercops transecta (Lepidoptera: Gracillariidae)

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Received 23 January 2007; accepted for publication 18 April 2007

Genetic differentiation in ecological traits plays an important role in the reproductive isolation of phytophagous insects. The present study aims to elucidate the genetic changes involved during the process of host shifts, by combining analyses for (1) host adaptations, (2) pre- and postmating isolation, and (3) phylogeny among populations, using a leaf-mining moth, Acrocercops transecta. This species is associated with Juglans ailanthifolia and Lyonia ovalifolia. Transplantation of the larvae demonstrated that the Juglans-associated population completely failed to survive on Lyonia, whereas the Lyonia-associated population survived on Juglans as well as on Lyonia. Females of respective host-associated populations oviposited on their natal host plant only. An mtDNA-based phylogeny clearly separated the Lyonia-associated population from the Juglandaceae-associated population, and indicated that the Lyonia-associated population once evolved from the Juglandaceae-associated population. These results indicate that the processes of host shifting from juglandaceous species to Lyonia involved genetic changes both in larval ability to use host plants and in host preference of females. The derived Lyonia-associated population has retained the potential to assimilate the ancestral host, Juglandaceae. Mating between the two host-associated populations was successful for both directions of crossing, and there were no significant differences in egg hatchability between hybrids and control crosses. No adults emerged when the F1 hybrid larvae were maintained on Lyonia; however, on Juglans the F1 hybrid larvae grew to adulthood as well as in the control, suggesting a lack of genomic incompatibilities between the two host-associated populations. In conclusion, the results showed that the two host-associated populations are host races that are partially reproductively isolated, and that the differences in performance and preference function as strong barriers against gene flow between the host races. © 2008 The Linnean Society of London, Biological Journal of the Linnean Society, 2008, 93, 135–145.


INTRODUCTION

Ecological differences between environments cause divergent selection that may lead to reproductive isolation between populations in different environments (Schluter, 2000). When a novel environment emerged, frequent speciation events and adaptive radiation occurred through divergent selection in some animal groups (Grant & Grant, 1982; Schluter, 1993; Seehausen & Bouton, 1997). Most phytophagous insects are specialized to their natal host plant species physiologically and behaviourally, and such specialists predominate among phytophagous insect taxa (Strong, Lawton & Southwood, 1984; Bernays & Chapman, 1994; Schoonhoven, Jermy & Van Loon, 1998). Specialization entails differentiation in ecological traits such as host preference, larval performance, and phenology between insect populations. Differences in ecological traits may contribute to barriers against gene flow between populations when they are symparic, leading to ecological speciation (Rundle & Nosil, 2005). A host shift to a novel host plant, whether it occurs allopatrically or sympatrically, could lead to an incipient stage of reproductive isolation through changes in ecological traits, although the
likelihood of ecological speciation has been disputed (Bush & Butlin, 2004; Coyne & Orr, 2004; Kawecki, 2004).

Phytophagous insect species often consist of subpopulations that are specialized to different species of host plants, that is, host races (Drès & Mallet, 2002). Host races are known to differ in some ecological traits (e.g. host preference, larval performance, or phenology) (Akimoto, 1990; Craig et al., 1993; Feder, 1998; Ikonen et al., 2003; Vanbergen et al., 2003; Forister, 2004; Gross, Fatouros & Hilker, 2004). An increasing number of studies using molecular markers have documented genetic differentiation between host races (Feder, Chilcote & Bush, 1988; Brown, Abrahamson & Way, 1996; Lopez-Vaamonde, Godfray & Cook, 2003; Diegisser, Seitz & Johannesen, 2006). Such genetic differentiation may be a result of reduced adaptations to non-natal hosts (ecological incompatibilities) or reduced viability and fecundity in hybrids (genomic incompatibilities). If there are no genomic incompatibilities between host races, differences in host specificity could be a principle cause of reproductive isolation. For this reason, distinguishing between ecological and genomic incompatibilities is crucial for understanding the mechanisms that create and maintain the remarkable diversity of phytophagous insects (Forister, 2005; Nygren, Nylin & Stefanescu, 2006).

To distinguish ecological incompatibilities from genomic incompatibilities, assessment of the fitness of F₁ hybrids is required (Hatfield & Schluter, 1999; Rundle & Whitlock, 2001). However, most studies on host race formation have used univoltine species, so that difficulties crossing and rearing continuously have hampered the assessment of hybrid fitness. As a result, few studies have distinguished these two incompatibilities (Katakura, Shioi & Kira, 1989; Craig, Horner & Itami, 1997; Forister, 2005; Nygren et al., 2006).

Bush (1975) pointed out that genetic changes in larval performance (host-based fitness) and the preference of ovipositing females are the most important factors for host shifts. These two traits are most likely to be under different genetic control (Sezer & Butlin, 1998a, b; Forister, 2005; Hora, Roessingh & Menken, 2005; Nygren et al., 2006), so that the process of host shifting will involve at least two mutations. Most studies have not determined the relative importance of host preference and larval performance for the evolution of host range. Evolutionary changes in host preference may create selection for increased performance on a new host, or variation in larval performance among plants may exert selection on female preference for favourable plants (Via, 1990; Sezer & Butlin, 1998b). Empirical work suggests that the host ranges of insects are limited by specialization in either performance or preference (Bush & Butlin, 2004). For instance, larvae of a derived host race have the potential to feed on ancestral host plants (Ikonen et al., 2003; Vanbergen et al., 2003; Gross et al., 2004) or exhibit the highest performance on ancestral host plants (Forister, 2004), even though adult females prefer to oviposit on derived hosts. This incongruence between performance and preference in derived host races reflects the genetic process of adaptation to a novel host plant (Stastny et al., 2006). Thus, precise understanding of the direction of host shifts, as well as assessing the extent of genetic differentiation, is important for elucidating the process of ecological speciation via host shifts.

In this paper, I have used a multivoltine micromoth species, Acrocercops transecta Meyrick, 1931 (Gracillariidae: Lepidoptera), which is readily reared in the laboratory over several generations (Ohshima, 2005). This species is associated with several tree species of the family Juglandaceae and with the shrub Lyonia ovalifolia (Wall.) Drude (Ericaceae) (Kumata, Kuroko & Ermolaev, 1988a). In many sites, the Juglandaceae- and Lyonia-associated populations are sympatric in respect that the two populations are within the range of the normal dispersal ability of the adult moth. A. transecta constitutes the Acrocercops leucophaea complex together with Acrocercops leucophaea and Acrocercops defigurata. Within this species complex, larvae of all species feed on juglandaceous plants, whereas A. transecta and A. leucophaea are also associated with L. ovalifolia. A molecular phylogenetic study using mtDNA indicated that the Juglandaceae association is ancestral for this species complex, and that the two host-associated populations in A. transecta are molecularly distinct (Ohshima & Yoshizawa, 2006). However, as the previous study was based on a small number of A. transecta samples, the direction of host shifting within A. transecta has not been determined. The host association of a single species with distantly related plant families is a rare example for leaf-mining insect groups (Van Nieukerken, 1986; Kumata et al., 1988a; Kumata, Kuroko & Ermolaev, 1988b). Thus, this unique host association, coupled with the information on genetic differentiation at the molecular level, suggests that there should be ecological differentiation and some mechanisms restricting gene flow between the two populations.

The present study first aims to assess the larval performance and oviposition preference of the two host-associated populations. Second, mating compatibility between the two host-associated populations is examined in order to check for premating isolation. Third, this study evaluates the hatchability and viability of F₁ hybrids when they are reared on Juglans or on Lyonia to test whether reduced hybrid
fitness results from genomic or ecological incompatibilities. Finally, this study determines the direction of the host shift by inferring a molecular phylogeny. Thus, this study combines analyses for host adaptations and reproductive isolation and phylogenetic information of A. transecta to elucidate the genetic changes during initial stages of host shifts.

MATERIAL AND METHODS

STUDY SITES, COLLECTION, AND REARING

To assess larval performance, ovipositing female preference, premating isolation, and F1 hybrid viability, laboratory experiments were performed using the Juglans ailanthifolia Carr. (Juglandaceae)-associated and L. ovalifolia-associated populations found in a locality in Mt Aoba (38°15′N, 140°49′E), Sendai City, northern Honshu (the central main island of Japan) (Fig. 1). In this locality the two host plants grow side by side, and at some sites they are completely intertwined. All moths used in these experiments were collected from the field as larvae, together with the leaves in which they were mining. More than 100 collected from the field as larvae, together with the twined. All moths used in these experiments were obtained by side, and at some sites they are completely inter-twined. In this locality the two host plants grow side

LARVAL PERFORMANCE

Only third instar larvae were used for this experiment, because first and second instar larvae were too small to transplant. Cut leaves were used for all treatments, but cutting does not reduce larval survival (Ohshima, 2005). Larvae sourced from each host plant were carefully extracted from the mines with forceps, and were reciprocally transplanted into the vacated non-natal mines on the cut leaves. As a control, transfers of larvae from their own mines on Juglans/Lyonia to other mines on different Juglans/Lyonia trees were conducted. Whether the larvae could ingest destination plant tissues or not was observed 1 h after transfer. After the transplantation, the larvae were maintained in the laboratory following the methodology described by Ohshima (2005). After all adults emerged, all the mines were examined for larval mortality. Individuals that were parasitized by natural enemies (wasps) were removed from the data because the wasps had already parasitized the larvae before the third stadium. The viability to adulthood was calculated based on 34 Juglans larvae and 36 Lyonia larvae that were transferred into mines on the other host species, and 32 Juglans larvae and 31 Lyonia larvae as controls. This experiment and the experiments on oviposition preference, and on the viability of F1 hybrids, were conducted from early June to late September in 2003 and 2004, and the experiment on mating compatibility was conducted from late July to late October in 2002, and from early June to late August in 2003.

OVIPPOSITION PREFERENCE

A single pair of virgin moths that had emerged within 24 h of each other was transferred with an aspirator to one plastic vial (118-mm long, 28-mm diameter, centrifuge tube) for pairing. Females were crossed with males from the same population. After mating, each female that had started oviposition on a host leaf in a mating tube was individually introduced into a plastic container (100 × 100 × 50 mm). In the container, one pair of fresh young leaves of Juglans and Lyonia were placed. 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. The Juglans and Lyonia leaves used in these
trials were collected from plants that had been transplanted from the study locality to a greenhouse. Forty adult females from the Juglans population and 40 females from the Lyonia population were used.

**Mating compatibility**

Pairs of eclosed moths were crossed separately in plastic vials following the same procedure as in the experiment on oviposition preference. Four mating combinations were tested and the combinations were abbreviated as follows: Jf × Jm, Juglans female × Juglans male; Lf × Lm, Lyonia female × Lyonia male; Jf × Lm, Juglans female × Lyonia male; Lf × Jm, Lyonia female × Juglans male. A total of 74 pairs for the Jf × Jm combination, 47 pairs for Lf × Lm, 42 pairs for Jf × Lm, and 52 pairs for Lf × Jm were prepared. The adults were allowed to mate until one member of the pair died. At the end of the experiment, all the females were dissected and examined for the presence of spermatophora in the bursa copulatrix, which was considered as evidence of copulation.

**Viability of F1 hybrids**

To assess the extent of genomic incompatibilities, hybrid eggs were prepared by crossing Juglans moths and Lyonia moths. The following four cross combinations were prepared: Jf × Jm, Lf × Lm, Jf × Lm, and Lf × Jm. To obtain F1 eggs, three or four leaves of a host plant were placed in a plastic container (100 × 100 × 50 mm). Where F1 eggs were laid on the female’s natal host plant, ten broods (one brood per female) were prepared for each combination, with each female producing 15–36 eggs. However, females did not normally oviposit on non-natal host plants, and it was impossible to transfer eggs to other leaves. Similarly, transferring hatched larvae was impossible because early instar larvae were too small to transfer, and because they always died on nonsuitable hosts (see Results). Thus, I tried to induce the females to misoviposit on non-natal host plants by placing a single non-natal host leaf over a natal host leaf in the container. Where misoviposition occurred on non-natal host leaves, a small number of replications (between two and four broods) were available with a few eggs (11.8 eggs on average) per replication. By this approach, I obtained three F1 broods from the Jf × Jm combination for rearing on Lyonia, two F1 broods from Lf × Lm for rearing on Juglans, two F1 broods from Jf × Lm for rearing on Lyonia, and four F1 broods from Lf × Jm for rearing on Juglans. Rearing in the laboratory was conducted as described in Ohshima (2005). Egg hatchability was assessed 4 days after the eggs were laid. The proportion of individuals that developed successfully into adulthood was calculated as an index of viability.

**Statistical analyses**

The percentage of larvae that developed successfully into adulthood in the performance experiment and the percentage of females that copulated in the mating experiment were analysed with goodness-of-fit tests (G-tests) (Sokal & Rohlf, 1995). The sequential Bonferroni correction (Rice, 1989) was applied to the P-values to keep the significance level at 0.05 throughout the tests. The corrected P-values were indicated as P(adj). The pair total index (PTI) coefficients (see Rolán-Alvarez & Caballero, 2000) were also calculated to measure the deviation from the hypothesis of equal mating frequency using the data set of mating compatibility. The PTI coefficients vary from zero to infinity, with 1 representing random mating. The significance of the PTI coefficients was calculated by resampling the observed frequencies of mating pairs 10 000 times using the program JMATING version 1.0.7 (http://webs.uvigo.es/genxb2; see Carvajal-Rodríguez & Rolán-Alvarez, 2006). The egg hatchability and viability per brood of F1 generations were transformed to an arcsine square-root in order to satisfy the requirement of normality, and the transformed values were analysed using ANOVA. Although a small number of replications were available for oviposition on non-natal host leaves, individual broods were not pooled for the analysis. The results of the oviposition preference experiments were analysed using paired Student’s t-tests, in which the numbers of eggs laid on the two leaves by a female were paired. Statistical tests were carried out using the StatView package version 5.0 (SAS Institute, 1998).

**Molecular phylogenetic analyses**

In order to construct a phylogenetic tree for populations of A. transecta, I added seven new sequences from the Juglandaceae-associated moths (five were collected from J. ailanthifolia and two were from Platycarya strobilacea Siebold et Zucc.) and eight from the Lyonia-associated moths to the published mtDNA data (partial sequences of COI and ND5) (Ohshima & Yoshizawa, 2006) (Fig. 1, Appendix). The present samples included three sets of sympatric populations (Sendai, voucher number IO-001, IO-005-7, IO-037, IO-084-5; Susami, IO-050-053; Niimi-sym, IO-054, IO-055, IO-078, IO-079) (Fig. 1, Appendix). In these localities, larvae were collected both from a Juglans tree and from a Lyonia tree that were intertwined with each other. Mined leaves containing larvae were collected from the host plants,
and the larvae were reared in the cut leaves in the laboratory. Eclosed adults were used for the analysis. A. leucophaea and A. defigurata were selected as outgroups (Appendix).

Phylogenetic hypotheses inferred from mitochondrial genes sometimes provide inaccurate estimates of species phylogeny resulting from mitochondrial introgression (e.g. Shaw, 2002) or from more rapid evolution than nuclear genes (for a review Ballard & Rand, 2005). However, as mtDNA is inherited maternally, mitochondrial gene phylogeny directly reflects the evolutionary history of female host preference, and is useful for estimating the history of host shifts (Diegiser et al., 2004). The procedures for DNA extracting, PCR, and sequencing were the same as described in Ohshima & Yoshizawa (2006). NEXUS files of the aligned sequences are available from http://insect3.agr.hokudai.ac.jp/~issei/data/index.html or by request to the author. For inferring phylogeny, maximum likelihood and parsimony analyses were conducted with PAUP* 4.0b10 PPC (Swofford, 2002). Parameters for maximum likelihood analysis were chosen on the basis of hierarchical likelihood ratio tests using Modeltest ver. 3.06 (Posada & Crandall, 1998), and the HKY + I + G model was selected (unequal base frequencies: A = 0.4331, C = 0.1218, G = 0.1251, and T = 0.3200; Nst = 2; TRatio = 4.1744; G distribution shape parameter = 0.7172; proportion of invariable sites = 0.7781). Other methods for inferring phylogeny were described in Ohshima & Yoshizawa (2006). Ancestral host use and host shifts were reconstructed using MacClade 4.03 (Maddison & Maddison, 2001).

RESULTS

LARVAL PERFORMANCE

After being transferred into mines made by other larvae, all larvae fed on the destination plant tissues, regardless of the direction of transfer. However, none of the 34 Juglans larvae (0%) transferred to Lyonia reached adulthood, whereas 30 of 36 Lyonia larvae (83.3%) transferred to Juglans grew successfully to adulthood. Thirty-one of 32 Juglans larvae (96.9%) and 29 of 31 Lyonia larvae (93.6%) transferred into mines on a different plant of their own host species successfully developed into adulthood. When the viability was compared pairwise between the transfer from Juglans to Lyonia (JL transfer) and the other three types of transfer, a significant difference was found in each pairwise test [G-tests, for JJ, G = 58.282, d.f. = 1, P > P(adjusted); for LL, G = 53.761, d.f. = 1, P < P(adjusted); for LJ, G = 46.239, d.f. = 1, P < P(adjusted)]. However, there were no significant differences between the JJ, LL, and LJ transfers [G-tests, for JJ vs. LL, G = 0.001, d.f. = 1, P > P(adjusted); for JJ vs. LJ, G = 2.058, d.f. = 1, P > P(adjusted); for LL vs. LJ, G = 0.835, d.f. = 1, P > P(adjusted)]. These results indicated that the inviability of Juglans larvae on Lyonia could be caused by plant secondary chemistry, if the compounds had cumulative toxic effects or hindered digestion, rather than by simply deterring feeding.

OVIPOSITION PREFERENCE

Ovipositing females of both populations had consistently clear preferences for their natal host-plant species. Almost all females from each host-associated population oviposited only on their own host plant (Fig. 2A, B). Of 40 Juglans females, only three (7.5%), and 40 Lyonia females, only one (2.5%) oviposited both on the non-natal plant and on the natal plant. These females oviposited much fewer eggs on the non-natal plant than on the natal plant. Both Juglans and Lyonia females significantly preferred to oviposit on their respective natal host plants (paired Student’s t-test, for Juglans females, t = 10.6, d.f. = 39, P < 0.0001; for Lyonia females, t = 11.0, d.f. = 39, P < 0.0001) (Fig. 2A, B).

MATING COMPATIBILITY

Sixty-nine of 74 pairs (93.2%) mated successfully in the Jf×Jm crosses, and 46 of 47 pairs (97.9%) mated successfully in the Lf×Lm crosses. When different host-associated moths were crossed, mating was observed in 35 of 42 pairs (83.3%) for the Jf×Lm crosses, and in 39 of 52 pairs (75.0%) for the Lf×Jm crosses. No significant differences were found in the proportion between the Jf×Lm crosses and the two control crosses, whereas there were significant differences between the Lf×Jm crosses and the control crosses (G-tests, Table 1). Although
Table 1. Statistical tests for mating compatibility. The pair total index (PTI) coefficients for each cross combination, and G-tests (Sokal & Rohl, 1995) for each pairwise comparison between hybridization and control crosses, are shown. The P-values for G-tests were adjusted with the sequential Bonferroni method to keep the significance level at 0.05

<table>
<thead>
<tr>
<th>Crosses</th>
<th>PTI (SD)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Female × male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juglans × Juglans</td>
<td>1.07 (0.09)</td>
<td>0.4792</td>
</tr>
<tr>
<td>Juglans × Lyonia</td>
<td>0.95 (0.09)</td>
<td>0.6044</td>
</tr>
<tr>
<td>Lyonia × Juglans</td>
<td>0.86 (0.09)</td>
<td>0.1174</td>
</tr>
<tr>
<td>Lyonia × Lyonia</td>
<td>1.12 (0.10)</td>
<td>0.2174</td>
</tr>
</tbody>
</table>

Comparisons | G     | d.f. | P     |
-------------|-------|------|-------|
JL – JJ      | 2.84  | 1    | 0.1716<sup>a</sup> |
JL – LL      | 5.73  | 1    | 0.0427<sup>b</sup> |
LJ – JJ      | 8.30  | 1    | 0.0087<sup>a</sup> |
LJ – LL      | 10.64 | 1    | 0.0029<sup>a</sup> |

<sup>a</sup><sub>P (adjusted)</sub>

G-tests revealed significant differences among the four cross combinations, the PTI coefficients did not reject the null hypothesis of equal mating frequency for all cross combinations (Table 1). G-tests are subject to serious biases due to mating propensities peculiar to populations, whereas the PTI coefficients provide unbiased estimates for the true mating preferences (Rolán-Alvarez & Caballero, 2000; Pérez-Figueroa, Caballero & Rolán-Alvarez, 2005). Thus, the results lead to the conclusion that mating between the two populations occurs randomly under artificial conditions.

HATCHABILITY AND VIABILITY OF F<sub>1</sub> HYBRIDS

The hatchability of the eggs from every cross combination was over 90%, and there were no significant differences in hatchability among the eight combinations (Jf × Jm, Lf × Lm, Jf × Lm, and Lf × Jm on Juglans and Lyonia) (<sup>F</sup><sub>7.44</sub> = 1.200, <i>P</i> > 0.05) (Fig. 3). When F<sub>1</sub> larvae from the Jf × Jm, Jf × Lm, and Lf × Jm crosses were reared on Lyonia, 325 larvae 324 died before the second stadium, and no adults emerged (only one larva from the Lf × Jm combination survived until the third stadium) (Fig. 3). In contrast, 50–64% of the F<sub>1</sub> generation from the four cross combinations emerged successfully on Juglans (Fig. 3). There were significant differences among the eight cross combinations in viability until adulthood (<sup>F</sup><sub>7.44</sub> = 59.624, <i>P</i> < 0.0001). However, no significant differences were found among the five cross combinations where adult moths did emerge (Jf × Jm, Lf × Lm, Jf × Lm, and Lf × Jm on Juglans, Lf × Lm on Lyonia) (<sup>F</sup><sub>5.31</sub> = 0.880, <i>P</i> > 0.05). These results indicated that hybrid breakdown on Lyonia was to the result of genomic incompatibilities, although the sample size of hybrids was limited within each cross combination.

MTDNA PHYLOGENY

The phylogenetic tree obtained with the maximum likelihood analysis supported monophyly of the Lyonia-associated population, and placed one sample from <i>P. strobilacea</i> (the Juglandaceae-associated population, IO-057) outside the clade comprising the other <i>A. transecta</i> samples (Fig. 4). The parsimony analysis yielded 2418 equally parsimonious trees (not shown), which differed only in the branching within each of the Lyonia- and Juglandaceae-associated populations. Although none of the maximum parsimony (MP) trees were identical with the maximum likelihood (ML) tree, all MP trees also supported monophyly of the Lyonia-associated populations, and separated one sample from the Juglandaceae-associated population (IO-057) from the <i>A. transecta</i> clade. The sample IO-057 composed a monophyletic group together with two samples of <i>A. leucophaea</i> (IO-068, IO-071), and the monophyly of this clade was fairly well supported (bootstrap support MP 70%, ML 70%). Samples from three sympatric populations (Sendai, Susami, and Niimi-sym) were clearly divided into the two host-associated populations in every population. Most parsimonious reconstruction of the host association on the maximum likelihood tree

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showed that the association with Juglandaceae is ancestral within *A. transecta*, and that the Lyonia association evolved once from the Juglandaceae association (Fig. 4).

**DISCUSSION**

The present study tested the hypothesis of ecological speciation between the Juglandaceae- and Lyonia-associated population.
associated populations of A. transecta by assessing the differences in ecological traits, and the degree of pre- and postzygotic reproductive isolation. Hatfield & Schluter (1999) and Forister (2005) demonstrated that when the two parental environments were provided for the F₁ hybrids, the hybrid fitness reduced only in one environment. They concluded that the fitness reduction in the hybrids was not the result of genomic incompatibilities, but was instead caused by ecological incompatibilities. The results they presented agreed with those of the present study. Both Juglans- and Lyonia-associated populations, and hybrid larvae, were viable on the ancestral environment (Juglans), whereas Juglans-associated larvae and hybrids were unable to survive on the novel host environment (Lyonia).

There are three possibilities for evolutionary relationships between host-associated populations: (1) a single polyphagous or oligophagous species, (2) a complex of two host races that are genetically differentiated, or (3) a complex of two morphologically indistinguishable species with complete reproductive isolation. Distinct host specificity of the two host-associated populations and the potential to interbreed without genomic incompatibilities reject the first and third hypotheses. The results of mating compatibility and viability of F₁ hybrids suggest that there is no substantial pre- or postzygotic isolation. This incomplete isolation may permit a level of gene exchange between the two host-associated populations. Therefore, A. transecta can be considered as a complex of two host races.

In his review of postzygotic isolation in Lepidoptera, Presgraves (2002) concluded that genomic incompatibilities among species or populations tend to develop gradually, and are positively correlated with genetic distance. Genomic incompatibilities result from a number of mechanisms, including natural selection, drift, and population bottlenecks, but accrue as a by-product of local adaptations (Turelli, Barton & Coyne, 2001). In contrast, host adaptations result directly from natural selection acting on populations with different host associations. For leaf miners and gall formers with limited larval dispersal, such a strong selection to host adaptation could accelerate the evolution of host specialization, and promote host race formation.

The lack of genomic incompatibilities despite the genetic differences emphasizes the role of ecological isolation between the two host races in A. transecta. In the examination of F₁ viability, when deprived of their own host plant, ovipositing females hardly ever selected the non-natal host plant, suggesting strict host selection in females. This result indicates that even though the larvae of the Lyonia race can develop on Juglans, their host range is limited to Lyonia alone.

Although these observations were based on one population alone (Sendai), the present phylogenetic study strongly implies that oviposition preference is also differentiated between the two host races in other populations. Specialization to respective host plants often leads to phenological isolation between populations with different host association (Craig et al., 1993; Feder, 1998). However, a similar phenology between host plants of the two host races (I. Ohshima, pers. observ.) and multivoltinism in A. transecta (Ohshima, 2005) negate the possibility of phenological isolation between the two host races. Therefore, these results suggest that the two host races are mainly isolated by host preferences of the females.

Because of the limited sample sizes and limited number of genes used (mtDNA only), the extent of gene flow between the two host races could not be evaluated. In small-sized phytophagous insects, mating on or near the host plant is widespread (Bush, 1975; Berlocher & Feder, 2002). For this reason, changes in host use may directly affect the choice of mating sites (Craig et al., 1993; Feder et al., 1994; Via, 1999; Emelianov et al., 2003). If oviposition preferences in A. transecta are closely linked with the choice of mating sites, the differentiated host preferences may act as a barrier against hybridization between the two host races. To test this hypothesis, it is necessary to assess the choices of mating sites in the Juglans and Lyonia races. In addition, future studies based on nuclear genetic markers [e.g. amplified fragment length polymorphisms (AFLPs) or microsatellite markers] are needed to elucidate the extent of gene flow between the two host races.

This study was not primarily intended to test whether larval performance and ovipositing female preference are under genetic control or are influenced by larval experiences. However, the complete mortality of F₁ hybrids from the JJ, JL, and LJ crosses on Lyonia indicates that larval performance is determined by genetic factors. Similarly, the fact that all the F₁ females from the reciprocal crosses exclusively preferred to oviposit on Lyonia (Ohshima, unpubl. data) suggests that oviposition preference is also genetically determined, but under different genetic control from larval performance. These results imply that the processes of host shifting from juglandaceous species to Lyonia involved genetic changes both in larval performance and in the preference of ovipositing females.

The fact that larvae of Juglans moths absolutely failed to assimilate Lyonia, coupled with the information of host shifting from Juglandaceae to Lyonia, suggests that novel mutations (e.g. the ability to utilize hosts with toxic secondary compounds) are a prerequisite for larval survival on Lyonia. If oviposition preference for Lyonia emerged first as a mutation
in the *Juglans*-associated population, then it may have been eliminated immediately from the population because of their inability to assimilate *Lyonia* leaves. Therefore, the present results predict that the genes responsible for larval survival on *Lyonia* should have evolved in the *Juglans* population before the mutational appearance of female preference for *Lyonia*. This inference requires that the larvae capable of assimilating *Lyonia* leaves should also retain the potential to assimilate *Juglans* leaves because such larvae are required to appear in the *Juglans* population. Consistent with this prediction, the derived *Lyonia*-associated population does indeed retain the ability to assimilate the ancestral host.

The present findings also have implications for the maintenance of host races. Complete mortality of *F1* hybrids on *Lyonia*, irrespective of the direction of crosses, indicates that gene flow from the *Juglans* race to the *Lyonia* race may be completely blocked. In contrast, the viability of *F1* hybrids on *Juglans* implies the possibility of gene flow from the *Lyonia* race to the *Juglans* race. The prediction of asymmetric gene flow between the host races should be tested using wild populations. In addition, it is necessary to assess larval viability on the two host plants, ovipositing female preference, and the extent of genomic incompatibilities in *F1* and backcross generations, in order to elucidate the genetic mechanisms for maintaining the host races in *A. transecta*.

ACKNOWLEDGEMENTS

I thank R. Deguchi, T. Fukushima, N. Goto, Y. Hirabuki, K. Mizota, and A. Munakata for allowing me to use their laboratory and the greenhouse at Miyagi University of Education; S. Akimoto, C. Lopez-Vaamonde, and two anonymous reviewers for critically reading through the manuscript; M. Ōhara and Y. Saito for allowing the use of their laboratories; N. Fujiyama, N. Kobayashi, T. Kumata, K. Matsubayashi, and K. Yoshizawa for helpful comments on this study. This study was supported by Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists (DC1, 16009250), and in part by a 21st COE grant by the Japanese Ministry of Education, Culture, Sports, Science and Technology for the ‘Neo-Science of Natural History’ Program at Hokkaido University (Leader: H. Okada).

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**APPENDIX**

Sampling information and GenBank accession numbers.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Host plant</th>
<th>Collection site</th>
<th>Voucher number</th>
<th>GenBank accession no. COI</th>
<th>GenBank accession no. NDS</th>
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