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Limited intrinsic postzygotic reproductive isolation despite chromosomal rearrangements between closely related sympatric species of small ermine moths (Lepidoptera: Yponomeutidae)

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In evolutionarily young species and sympatric host races of phytophagous insects, postzygotic incompatibility is often not yet fully developed, but reduced fitness of hybrids is thought to facilitate further divergence. However, empirical evidence supporting this hypothesis is limited. To assess the role of reduced hybrid fitness, we studied meiosis and fertility in hybrids of two closely related small ermine moths, Yponomeuta padella and Yponomeuta cagnagella, and determined the extent of intrinsic postzygotic reproductive isolation. We found extensive rearrangements between the karyotypes of the two species and irregularities in meiotic chromosome pairing in their hybrids. The fertility of reciprocal F1 and, surprisingly, also of backcrosses with both parental species was not significantly decreased compared with intraspecific offspring. The results indicate that intrinsic postzygotic reproductive isolation between these closely related species is limited. We conclude that the observed chromosomal rearrangements are probably not the result of an accumulation of postzygotic incompatibilities preventing hybridization. Alternative explanations, such as adaptation to new host plants, are discussed.


INTRODUCTION

Although an abundance of species definitions has been postulated since the New Synthesis (Coyne & Orr, 2004; Mallet, 2010), the biological species concept is probably the most widely used (Nosil, 2008; Sobel et al., 2010). Following this definition, species are groups of individuals that are reproductively isolated from each other by intrinsic and genetically determined barriers (Dobzhansky, 1937; Mayr, 1940). In sympatric host races and evolutionarily young species of phytophagous insects, postzygotic incompatibility has often not yet evolved (Mallet, 2008), but prezygotic or extrinsic postzygotic reproductive isolation mediated by specialization on the host plant is thought to form a barrier to gene flow to the point at which speciation is complete. The shift to a new host and subsequent adaptation to that host (i.e. host race formation) has long been viewed as a basic step leading to new species through divergent selection (Bush, 1994; Mallet, 2008; Powell et al., 2013). Studies modelling the process of host specialization in an initial randomly mating population (or at secondary contact after a period of geographical isolation) often assume selection against hybrids between the diverging taxa. Without this lower fitness of intermediate genotypes, the evolution of prezygotic isolation in the form of assortative mating, the key event of speciation in the presence of...
gene flow, is unlikely (Felsenstein, 1981; Matsubayashi et al., 2010). Although recent work indicates that reproductive isolation might evolve by coupling of all types of barriers to gene flow (Barton & de Cara, 2009; Butlin & Smadja, 2018), decreased fitness of hybrids is still viewed as a key mechanism.

It is generally accepted that decreased hybrid fitness is caused by Bateson–Dobzhansky–Muller-type between-locus incompatibilities (DBMI; Dobzhansky, 1937; Muller & Pontecorvo, 1940; Barton & de Cara, 2009; Fierst & Hansen, 2010). Between-locus incompatibilities can cause hybrid sterility or reduced reproduction through the inability of parental genes to collaborate in regulating the development of gonads. In addition, hybrid sterility can be caused by disturbance of meiosis, especially by irregularities during gametogenesis, leading to the production of genetically unbalanced gametes. Such irregularities, known as Robertsonian variation, are likely to occur when there are structural differences between the chromosomes of the two hybridizing taxa (White, 1978; Oliver, 1979; King, 1993). The contributions of such chromosomal rearrangements and, in particular, of chromosomal inversions to processes of speciation (Noor et al., 2001; Rieseberg, 2001) have been (and are) broadly discussed (Noor & Bennett, 2009; Faria & Navarro, 2010; Jackson, 2011; Blankers et al., 2018; Faria et al., 2019). It has been suggested that a single inversion could generate most of the genetic barriers needed for speciation (Ayala et al., 2013). Consensus is emerging that chromosomal inversions and structural variation play a crucial role and should receive more attention in the study of adaptation and speciation (Pennisi, 2017; Wellenreuther & Bernatchez, 2018; Campbell et al., 2018; Faria et al., 2019).

In general, differences in chromosome structure between two populations can play a role in reproductive isolation and speciation by affecting the chances of evolution of reproductive isolation between the populations both directly and indirectly. Direct effects of structural alteration of chromosomes can cause intrinsic postzygotic effects that lower hybrid fitness (Orr & Turelli, 2001; Presgraves, 2002, 2010a, b; Ayala et al., 2013; Crucickshank & Hahn, 2014), favouring selection against genotypes mating outside the population. In addition, two types of indirect effects have been suggested. Inversions can prevent perfect bivalent formation during meiosis and, in this way, oppose the break-up of favourable gene combinations by recombination (Rieseberg, 2001; Trickett & Butlin, 1994; Pegueroles et al., 2010; Ortiz-Barrios et al., 2016). More recently, it was suggested that structural changes, such as chromosome fusions, might bring favourable combinations of genes into close proximity (Guerrero & Kirkpatrick, 2014) and, exactly like inversions, reduce recombination. Structural changes, herefore, can facilitate the build-up of linkage disequilibrium (Yeaman, 2013; Flaxman et al., 2014; Thompson & Jiggins, 2014). Hypotheses about such indirect effects gained ground in recent years (Flaxman et al., 2014; Charlesworth, 2016; Britton-Davidian et al., 2017; Branco et al., 2018). However, there is still a lack of empirical data to test these ideas and evaluate the extent of their role in reproductive isolation and the evolution of barriers to gene flow (Feder et al., 2014; Butlin & Smadja, 2018; Faria et al., 2019). In this study, we tested the classical prediction from DBMI theory that reduced hybrid fitness is a strong force in the development of prezygotic barriers and that structural differences between the chromosomes of two hybridizing taxa contribute to a lower hybrid fitness.

We examined the occurrence of chromosomal rearrangements and sterility in hybrids between closely related species of the small ermine moth genus Yponomeuta (Lepidoptera: Yponomeutidae). These insects are specialized herbivores on one or a few related plants (Menken et al., 1992), and differential host specialization after a host shift probably played an important role in the evolution of the group. The genus provides an excellent model for studies of speciation through host specialization (Menken et al., 1992; Menken, 1996; Gershenson & Ulenberg, 1998; Menken & Roessingh, 1998; Bakker et al., 2008). We exploit the fact that these species do not appear to hybridize in the field (Arduino & Bullini, 1985; Hendrikse, 1988; Menken et al., 1992) but can still be crossed easily in the laboratory. We performed a cytogenetic analysis of hybrid meiosis to determine whether gametogenesis is disturbed as a consequence of structural differences between the parental chromosomes. Furthermore, the sterility of reciprocal interspecific hybrids of Yponomeuta padella L. and Yponomeuta cagnagella (Hübner) was assessed. It is expected that viability of first-generation (F₁) hybrids will not suffer from chromosomal rearrangements unless the combined parental genomes are not sufficiently compatible to secure vital life functions. However, the effect of chromosomal rearrangements should become apparent in the production of unbalanced gametes in the hybrids (i.e. F₂ sterility; Carpenter et al., 2005). Therefore, we also studied the occurrence of sterility in second-generation (F₂) offspring and in backcrosses. Together, these data allow for an empirical estimate of the extent of postzygotic reproductive isolation between Y. padella and Y. cagnagella. The results of this study will further our understanding of the evolution of reproductive isolation and the role of hybrid fitness in the formation of barriers to gene flow.
MATERIAL AND METHODS

Biology, collection and rearing

_Yponomeuta padella_ and _Y. cagnagella_ are sympatric, reproductively nearly synchronous, and closely related species (Menken _et al._, 1992; Sperling _et al._, 1995; Turner _et al._, 2010). _Yponomeuta cagnagella_ is strictly monophagous on _Euonymus europaeus_ (Celastraceae), whereas _Y. padella_ feeds on a restricted number of Rosaceae (Crataegus _spp._ and _Prunus spinosa_). The two species do not hybridize in nature, but in no-choice laboratory conditions _F_1 hybrids can be produced easily (Roessingh _et al._, 1999; Hora _et al._, 2005).

Insects to be used as parents of the interspecific _F_1 crosses and those used for intraspecific control crosses were collected as fifth instars from their host plants in the field at two locations in The Netherlands: Meyendel, the Hague (52°21′55″N, 4°19′59″E) and Flevopark, Amsterdam (52°21′46″N, 4°56′54″E). Larvae were fed ad libitum on fresh leaves of their host in plastic cylinders (45 cm high, 20 cm in diameter) or glass jars (20 cm × 11 cm). The resulting pupae were placed individually in glass tubes (8 cm × 1.2 cm). _Yponomeuta padella_ reaches sexual maturity somewhat sooner after eclosion than _Y. cagnagella_ (Hendrikse, 1979); therefore, pupae of the latter were kept at a higher temperature (24 °C, vs. 18 °C for _Y. padella_) until eclosion; this synchronized the development of the two species.

Crosses

After emergence, moths were sexed and individual pairs put in glass vials for 1–2 weeks at 18 °C, 16 h–8 h light–dark cycle, with a strip of filter paper as a substrate for mating. After mating, pairs were transferred to Perspex cylinders (16 cm × 4 cm) that were slid over the branches of potted host plants in a greenhouse (at 20–25 °C and natural light) and closed off with cotton-wool plugs. _Yponomeuta padella_ females were offered _Euonymus europaeus_ and _Y. padella_ females _P. spinosa_ for oviposition (Hora & Roessingh, 1999). In the abbreviations of the crosses, the mother is given first, i.e. _F_1(pc) denotes progeny of a _Y. padella_ female crossed with a _Y. cagnagella_ male.

Following the same procedure, backcrosses (BC) were produced by crossing interspecific _F_1 hybrids with the parental species _Y. cagnagella_ and _Y. padella_ that originated from intraspecific laboratory crosses. Given that small ermine moths do not respond well to manipulation of their univoltine life cycle, an artificial diapause of ≥ 4 months was introduced by storing egg masses in total darkness at 4 °C, until they were used the next spring.

In all experiments, first instar larvae (L1) were carefully removed from their hibernaculum, counted, and reared in plastic Petri dishes (2.5 cm × 10 cm) at 18 °C and 16 h–8 h light–dark cycle until pupation. The size of rearing groups was kept at 15–30 first instars per Petri dish and a maximum of 15 L5 larvae per dish. _Euonymus europaeus_ was used as the host for _F_1 interspecific hybrids. For the backcrosses, the host plant of the backcross parent was chosen. Fully mature pupae were taken out of their cocoons on day 4 after the first signs of pupation and weighed to the nearest 0.1 mg on an Ohaus Analytical Standard Balance. They were placed individually in glass vials at 18 °C and 16 h–8 h light–dark cycle until eclosion. After eclosion, adults were sexed and fed with 0.5 cm³ blocks of 1% honey in 1% agar, which were replaced two or three times per week.

Chromosome preparations

Meiotic chromosome pairing was investigated using different methods for males and females.

Females: oocytes

In females, we examined three individuals of _Y. cagnagella_, three _Y. padella_, four _F_1(pc), four _F_1(cp) and one BC(c×pc). We applied pachytene mapping (Traut, 1976). This method allows the identification of chromosome regions by their chromomere pattern and the sex chromosomes by the W chromosome heterochromatin.

Immature ovaries of late fifth instars or young pupae were dissected and fixed in freshly prepared Carnoy’s fluid (ethanol:chloroform:acetic acid, 6:3:1). Cells were dissociated in 60% acetic acid, spread and heat dried onto the slide at 45 °C, then stained and mounted in 2.5% lactic acetic Orcein. Slides were inspected and photographed with phase-contrast optics in an Axiosplan 2 Zeiss microscope at ×1000 magnification.

Males: spermatoocytes

In males, we examined two individuals of _Y. cagnagella_, three _Y. padella_, three _F_1(pc), two _F_1(cp) and one BC(c×pc). Meiosis was studied on microspread preparations of late zygote or pachytene nuclei from the testes of early fifth instars. The microspreading technique was performed following the procedure described for _Ephestia kuehniella_ (Marec & Traut, 1993). Preparations were examined and micrographs taken in a Jeol 1010 transmission electron microscope operated at 80 kV. This technique enabled a detailed investigation of chromosome pairing during the zygote and pachytene stages; it visualizes the synaptonemal complexes (SCs) involved in the matching of homologous chromosomes during the formation of bivalents (for a review, see Marec, 1996).
HYBRID STERILITY

Sterility is a complex, multifactorial trait. We tested the following components: percentage of hybrids producing offspring; average lifetime fecundity; and percentage of hatched eggs.

We used males and females of both reciprocal F₁ hybrids but performed reciprocal backcrosses only with F₁, Y. padella × Y. cagnagella (F₁(pc)). This cross was chosen because preliminary experiments showed that F₁(pc) hybrids suffer slightly more from sterility than the F₁(cp) reciprocal hybrids (see Table 1A). This is expected to lead to more pronounced effects on fitness in the second hybrid generation. For both BC and F₁ progeny, male and female hybrid sterility was assessed by again backcrossing them with Y. cagnagella or Y. padella. As a control, we used intraspecific crosses of both parental species. All experiments were carried out in three complete blocks to ensure that all comparisons were made with insects reared on food of the same quality at the same time of year. The resulting total sample sizes are listed in Tables 1 and 2.

CROSSES WITH F₁ HYBRIDS

The F₁ hybrids were combined with Y. cagnagella or Y. padella of the opposite sex and placed as single pairs on plants grown in a greenhouse as described above. The host plant was chosen according to the female parent of the cross: Y. cagnagella and F₁ hybrids were provided with E. europaeus (both reciprocal F₁ hybrids prefer E. europaeus for oviposition; Hora et al., 2005), and Y. padella was provided with P. spinosa. A pair was counted as producing offspring when at least one egg mass was produced. Given that unmated female Yponomeuta hardly lay any eggs (Parker et al., 2013) egg masses reflect successful fertilization, and indeed, complete egg masses do not hatch only very rarely. Lifetime fecundity was determined by counting the number of individual eggs in all egg masses produced by one female under a stereomicroscope (at ×40 magnification). For a limited number of pairs (Table 1), the percentage of hatched eggs was determined by counting the numbers of emerged L₁ in an egg mass. This number was limited because we needed the F₁ hybrids for the production of BC hybrids, and disturbance caused by counting freshly hatched larvae severely reduces their survival.

CROSSES WITH BC HYBRIDS

BC2 was produced by crossing the parental species with backcrossed F₁s (BC1). After eclosion, moths were sexed and moved to a climate room set at 21 °C and 16 h–8 h light–dark cycle, with lights on at 14.00 h, shifting the daylight period forward in time. This allowed convenient observation of mating behaviour. In contrast to the experiment with F₁ hybrids, mating was observed to distinguish between pairs not producing offspring because they had not mated and pairs not producing offspring because of post-mating prezygotic barriers, such as incomplete sperm transfer, no sperm transfer or sterile sperm. Pairs were assigned at the start of the experiment but kept in separate vials. Two hours before the start of the photophase, mating pairs were put together. For a period of 4 h, pairs in copula were scored every hour. Hourly intervals were sufficient, because pairs stay in physical contact for a number of hours. After this period, unmated moths were returned to their separate vials. This procedure was repeated the next dark phase until the moths had mated or one of the pair had died. The percentage of mated pairs was recorded for all cross types. Mated females were allowed to oviposit individually in cages in the same climate room on cut twigs of E. europaeus and P. spinosa (Hora & Roessingh, 1999) for the rest of their life span in order to establish lifetime fecundity. Further assessment of BC hybrid sterility proceeded as described for F₁ hybrids.

STATISTICAL ANALYSIS

Percentages of pairs producing offspring and hatched L1 were analysed using G-statistics with William’s adjustment for low cell numbers (Sokal & Rohlf, 1995). If significant differences were found, the simultaneous test procedure (Sokal & Rohlf, 1995) was applied to determine maximally non-significant sets. Differences in average lifetime fecundity were analysed using one-way ANOVA with Tukey’s HSD multiple comparison post hoc test. All statistics were done with the R v.2.9.1 program (R Development Core Team, 2014).

RESULTS

CHROMOSOMAL REARRANGEMENTS

Oocytes

Females of Yponomeuta species have 29 pairs of autosomes (A) and three sex chromosomes, which in meiosis form a trivalent, consisting of one W chromosome and two Z chromosomes (Z₁ and Z₂). The diploid chromosome number in females is therefore 2n = 61 (29AA + WZ₁Z₂), whereas males have 31 chromosome pairs and 2n = 62 (29AA + Z₁Z₁Z₂Z₂) (Nilsson et al., 1988). With the pachytene mapping technique, the W chromosome can be recognized in many Lepidoptera by its partial or complete heterochromatinization, allowing the identification of sex chromosome bivalents (or trivalents in the case of Yponomeuta species under study), whereas...
Table 1. Reproductive characteristics of intraspecific and interspecific crosses in: A, *Yponomeuta cagnagella* (Cag); B, *Yponomeuta padella* (Pad); and C, their F₁ hybrids

<table>
<thead>
<tr>
<th>Cross type* (female × male)</th>
<th>Number of pairs</th>
<th>Percentage of pairs with offspring</th>
<th>Sign†</th>
<th>Lifetime fecundity [mean ± SD number of eggs (N‡)]</th>
<th>Sign§</th>
<th>Eggs hatched [% (N§)]</th>
<th>Sign†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Crosses with <em>Yponomeuta cagnagella</em></td>
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<tr>
<td>Cag × Cag (control)</td>
<td>27</td>
<td>70.4</td>
<td>a</td>
<td>–</td>
<td>–</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Cag × F₁(cp)</td>
<td>32</td>
<td>71.9</td>
<td>a</td>
<td>107.4 ± 93.1 (17)</td>
<td>ab</td>
<td>93.6 (2)</td>
<td>a</td>
</tr>
<tr>
<td>Cag × F₁(pc)</td>
<td>29</td>
<td>58.6</td>
<td>a</td>
<td>99.0 ± 60.8 (13)</td>
<td>ab</td>
<td>82.0 (7)</td>
<td>a</td>
</tr>
<tr>
<td>F₁(cp) × Cag</td>
<td>34</td>
<td>67.7</td>
<td>a</td>
<td>79.3 ± 34.8 (21)</td>
<td>b</td>
<td>79.8 (9)</td>
<td>a</td>
</tr>
<tr>
<td>F₁(pc) × Cag</td>
<td>39</td>
<td>66.7</td>
<td>a</td>
<td>143.4 ± 65.4 (19)</td>
<td>a</td>
<td>84.0 (10)</td>
<td>a</td>
</tr>
<tr>
<td>G-tests for pairs with offspring: (G_4 = 1.6136, n.s.) over all crosses; (G_4 = 1.4063, n.s.) over the BC crosses. ANOVA for lifetime fecundity: (F_3 = 3.257, P = 0.027). G-test for percentage of eggs hatched: (G_1 = 1.2908, n.s.)</td>
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</table>

| B. Crosses with *Yponomeuta padella* |                |                                    |       |                                                  |       |                        |       |
| Pad × Pad (control)           | 24              | 91.7                               | a     | 110.5 ± 36.7 (22)                                 | b     | 90.8 (22)              | a     |
| Pad × F₁(cp)                  | 29              | 72.4                               | ab    | 113.5 ± 46.4 (19)                                 | b     | 95.0 (9)               | a     |
| Pad × F₁(pc)                  | 26              | 57.7                               | b     | 110.5 ± 58.2 (10)                                 | b     | 81.8 (5)               | a     |
| F₁(cp) × Pad                  | 27              | 74.1                               | ab    | 126.7 ± 61.7 (18)                                 | ab    | 95.9 (8)               | a     |
| F₁(pc) × Pad                  | 36              | 61.1                               | ab    | 182.3 ± 94.7 (19)                                 | a     | 91.7 (11)              | a     |
| G-tests for pairs with offspring: \(G_4 = 9.7383, P = 0.045; G_4 = 6.286, n.s.\) over all crosses indicated with b. ANOVA for lifetime fecundity: \(F_4 = 4.4495, P = 0.0026.\) G-test for percentage of eggs hatched: \(G_4 = 1.4137, n.s.\) |

| C. F₁ hybrid crosses          |                |                                    |       |                                                  |       |                        |       |
| F₁(cp) × F₁(cp)               | 108             | 75.9                               | a     | 107.1 ± 54.9 (74)                                 | b     | 89.8 (46)              | a     |
| F₁(pc) × F₁(pc)               | 105             | 66.7                               | a     | 168.8 ± 94.3 (63)                                 | a     | 80.6 (41)              | a     |
| G-tests for pairs with offspring: \(G_4 = 0.5996, n.s.\) ANOVA for lifetime fecundity: \(F_4 = 22.6, P < 0.001.\) G-test for percentage of eggs hatched: \(G_1 = 0.4921, n.s.\) |

*Significance: identical letters indicate maximal non-significant sets.
†The number of analysed pairs.
§Post hoc comparisons using Tukey’s HSD; different letters indicate significant differences between values at the 0.05 level.

*Cag, *Y. cagnagella*; Pad, *Y. padella*; F₁, interspecific cross with, in parentheses, the direction of the F₁ (c for *Y. cagnagella* and p for *Y. padella*, with females mentioned first).
Table 2. Reproductive characteristics of intraspecific and interspecific crosses and second backcrosses in *Yponomeuta cagnagella* (A) and *Yponomeuta padella* (B)

<table>
<thead>
<tr>
<th>Cross type*</th>
<th>Number of pairs</th>
<th>Percentage of pairs with offspring</th>
<th>Sign†</th>
<th>Percentage of mated pairs</th>
<th>Sign§</th>
<th>Percentage of mated pairs with offspring</th>
<th>Sign†</th>
<th>Lifetime fecundity [mean ± SD number of eggs (N‡)]</th>
<th>Sign§</th>
<th>Egg hatch [% (N§)]</th>
<th>Sign†</th>
</tr>
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<tbody>
<tr>
<td><strong>A. Crosses with <em>Yponomeuta cagnagella</em></strong></td>
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</tr>
<tr>
<td>Cag × Cag (control)</td>
<td>30</td>
<td>100.0</td>
<td>a</td>
<td>100.0</td>
<td>a</td>
<td>263.6 ± 136.0 (30)</td>
<td>a</td>
<td>93.3 (29)</td>
<td>a</td>
<td></td>
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</tr>
<tr>
<td>Cag × BC(c×pc)</td>
<td>32</td>
<td>78.1</td>
<td>a</td>
<td>78.1</td>
<td>b</td>
<td>234.8 ± 139.4 (25)</td>
<td>ab</td>
<td>88.0 (22)</td>
<td>a</td>
<td></td>
<td></td>
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<tr>
<td>BC(c×pc) × Cag</td>
<td>30</td>
<td>73.3</td>
<td>a</td>
<td>83.3</td>
<td>ab</td>
<td>242.5 ± 114.3 (22)</td>
<td>ab</td>
<td>86.4 (20)</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC(pc×c) × Cag</td>
<td>30</td>
<td>90.0</td>
<td>a</td>
<td>93.3</td>
<td>ab</td>
<td>246.7 ± 96.2 (27)</td>
<td>ab</td>
<td>93.3 (26)</td>
<td>a</td>
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<tr>
<td><strong>G¬tests for crosses with offspring:</strong></td>
<td>G = 6.971, d.f. = 4, n.s.</td>
<td>G-tests for percentage of mated pairs: G = 3.532, d.f. = 4, n.s.</td>
<td>G-tests for percentage of mated pairs with offspring: G = 4.782, d.f. = 4, n.s.</td>
<td>ANOVA for lifetime fecundity: F = 2.806, d.f. = 4, P = 0.028.</td>
<td>G-test for percentage of eggs hatched: G = 0.715, d.f. = 4, n.s.</td>
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<td><strong>B. Crosses with <em>Yponomeuta padella</em></strong></td>
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<tr>
<td>Pad × Pad (control)</td>
<td>30</td>
<td>66.7%</td>
<td>ab</td>
<td>100.0</td>
<td>a</td>
<td>88.0 ± 34.6 (20)</td>
<td>a</td>
<td>95.0 (19)</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pad × BC(p×pc)</td>
<td>32</td>
<td>71.9%</td>
<td>ab</td>
<td>90.6</td>
<td>a</td>
<td>82.7 ± 39.1 (23)</td>
<td>a</td>
<td>95.7 (22)</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pad × BC(pc×p)</td>
<td>6</td>
<td>83.3%</td>
<td>a</td>
<td>100.0</td>
<td>a</td>
<td>119.6 ± 15.4 (5)</td>
<td>a</td>
<td>100 (5)</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC(pc×p) × Pad</td>
<td>17</td>
<td>82.3%</td>
<td>ab</td>
<td>88.2</td>
<td>a</td>
<td>81.6 ± 46.9 (14)</td>
<td>a</td>
<td>85.7 (12)</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC(pc×p) × Pad</td>
<td>12</td>
<td>50.0%</td>
<td>b</td>
<td>91.7</td>
<td>a</td>
<td>92.0 ± 46.6 (6)</td>
<td>b</td>
<td>66.7 (5)</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G¬tests for crosses with offspring:</strong></td>
<td>G = 10.936, P = 0.0273 over all crosses; G1 = 2.620, n.s. over all crosses indicated with a; G2 = 8.301, n.s. over all crosses indicated with b.</td>
<td>G-tests for percentage of mated pairs: G1 = 1.289, n.s.</td>
<td>G-tests for percentage of mated pairs with offspring: G2 = 12.377, P = 0.014 over all crosses; G3 = 4.575, n.s. over all crosses indicated with a; G4 = 7.361, n.s. over all crosses indicated with b.</td>
<td>ANOVA for lifetime fecundity: F1 = 1.025, n.s.</td>
<td>G-test for percentage of eggs hatched: G4 = 8.425, n.s.</td>
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</tbody>
</table>

*Cag, *Y. cagnagella*; Pad, *Y. padella*; BC = backcross (c for *Y. cagnagella* and p for *Y. padella*; females mentioned first).
†Identical letters indicate maximal non-significant sets.
‡The number of analysed pairs.
§Post hoc comparisons using Tukey’s HSD; different letters indicate significant differences between figures at the 0.05 level.
autosomal bivalents show a homologous chromomere–interchromomere pattern, i.e. deeply stained pairs of chromatin beads separated by weakly stained regions (Traut & Marec, 1997). Our study confirmed the existence of the WZ, Z trivalent. In both species of Yponomeuta, the W chromosome appeared to be fully heterochromatic, and the sex chromosomes regularly formed a well-paired trivalent (Fig. 1A, B). The pairing configurations of the sex chromosome trivalent in hybrids, however, indicated that the Z chromosomes of Y. cagnagella and Y. padella might be structurally diverged. This was apparent in F₁ and BC hybrid females, in which the WZ₁Z₂ trivalents were frequently not well synapsed, and Z₁ seemed shortened to adjust for synapsis (Fig. 1D, E; the W chromosome is the evenly dark chromosome).

In all F₁ hybrids, and even more so in BC hybrids, autosomal bivalents displayed a number of irregularities in synapsis (Fig. 1F–H). In contrast, autosomal bivalents in the pure species were fully paired and showed similar chromomere patterns (Fig. 1C). In the parental species, two bivalents carried the nucleolar organizer region (NOR), each associated with the nucleolus, which appeared as a large, weakly stained spot at one end of the bivalents. The observed irregularities in F₁ and backcrosses frequently involved chromosomes associated with the nucleolus (Fig. 1F); one of the chromosomes could be recognized by a conspicuous chromomere at one end and was often found to be involved in multivalents (Fig. 1F, G) or else it was incompletely synapsed or formed a univalent. We repeatedly found an autosomal multivalent, possibly a quadrivalent, in F₁(pc) and BC hybrid females (Fig. 1F, H). Occasionally, dissimilar chromomere patterns in bivalents in F₁ and backcrosses were observed, but this result is hard to interpret, because the chromomere patterns in pure Y. cagnagella and Y. padella were not always identical either, probably as a result of the spreading procedure itself.

**Spermatocytes**

Pachytene bivalents in Y. cagnagella and Y. padella males were well synapsed and formed regular synaptonemal complexes (SCs) (Fig. 2A). In F₁ hybrid males, a number of bivalents also showed complete synapsis (Fig. 2C), but various pairing irregularities were observed in other bivalents (Fig. 2B, D). These irregularities included loops between homologous chromosomes, possibly indicating an inversion, late pairing of some bivalents, lack of synapsis at the distal ends, or peculiar structures associated with lateral elements of the SC resembling polycomplexes (PCs; see the bivalent in Fig. 2D) (Marec, 1996).

The number of pairing irregularities differed among nuclei, but at least one abnormality was found in all F₁ and BC hybrid females, in which the WZ₁Z₂ trivalents were frequently not well synapsed, and Z₁ seemed shortened to adjust for synapsis (Fig. 1D, E; the W chromosome is the evenly dark chromosome).
per nucleus. Meiotic disturbances were even more pronounced in BC hybrid males. We repeatedly found a multivalent consisting of four to six chromosomes. This multivalent, indicating multiple translocations, was found in different configurations in several nuclei (Fig. 3A–C). In some nuclei, one or two bivalents were not synapsed, and their lateral elements were decorated with PCs (Fig. 3D, E).

Figure 3. Electron microscopic microspread preparations of pachytene spermatocytes from backcross (BC) hybrid males Y. cagnagella × F₁ (Y. padella × Y. cagnagella). A, trivalent (T) supposedly associated with a bivalent (B); see panel D for schematic interpretation. B, multivalent configuration that can be interpreted as a trivalent [lateral elements (LEs) 1, 2 and 3] associated with a bivalent (4 and 5) and a univalent (6) or, alternatively, as a hexavalent (1–6); see panel E for schematic interpretation. C, hexavalent (LEs numbered 1–6); see panel F for schematic interpretation. G, bivalent with polycomplex (PC)-like structures associated with lateral elements. H, detail of PCs; note parallel central elements of the PC (PCE) and lateral elements of the PC (PLE) perpendicular to the leading lateral element (LLE) of the chromosome.

Table 1. Offspring production in backcrosses

We found no significant differences in the percentage of pairs that produced offspring among the crosses.
between parental species and F₁ hybrids (G₁ = 1.40, 
\( P = 0.70 \) for crosses with Y. cagnagella, and \( G₂ = 6.29, 
\( P = 0.178 \) for crosses with Y. padella; Table 1A, B). 
There was also no difference between the reciprocal F₁ 
hybrid crosses (\( G₁ = 0.60 \), n.s.; Table 1C). Intraspecific 
Y. cagnagella crosses resulted in similar percentages 
of pairs with offspring as backcrosses, ranging from 
66.7 to 71.9% (\( G₁ = 1.61 \), n.s.). For F₁ hybrids crossed 
with Y. padella, only one of the four hybrid crosses, 
Y. padella \( \times \) F₁(pc), differed from the intraspecific cross 
(57.7 vs. 91.7%, \( G₁ = 9.738, P = 0.045; \) Table 1B); crosses 
with F₁(pc) as a parent consistently showed a lower 
percentage of pairs that produced offspring than those 
involving the reciprocal F₁(cp) hybrid (Table 1A–C).

**Lifetime fecundity**

With Y. padella as the backcross species, significant 
differences in lifetime fecundity were found (\( P = 4.45, 
\( P = 0.0026 \)). However, three out of four possible crosses 
with F₁ hybrids did not differ significantly from the 
intraspecific cross. The difference was caused by F₁(pc) 
\( \times \) Y. padella, and this cross had a significantly higher 
lifetime fecundity than the Y. padella intraspecific cross 
(hybrid vigour). A similar result was found with 
Y. cagnagella as the backcross species (\( P = 3.26, 
\( P = 0.027 \)), and again only F₁(pc) \( \times \) Y. cagnagella 
differed from the intraspecific cross (\( P = 3.257, 
\( P = 0.027 \)), with lifetime fecundity being higher in this 
cross. Unfortunately, lifetime fecundity and egg hatch 
could not be compared with the intraspecific cross of 
Y. cagnagella, because we did not manage to produce 
sufficient numbers of Y. cagnagella to realize this cross 
next to the F₁ hybrids \( \times \) Y. cagnagella crosses.

When comparing all crosses in which F₁ hybrids 
were involved, the F₁(pc) females in backcrosses with 
Y. cagnagella, Y. padella and F₁(pc) males produced 
significantly more eggs (hybrid vigour; 143.4 ± 65.4, 
182.3 ± 94.7 and 168.8 ± 94.3, respectively) than the 
reciprocal F₁(cp) females (79.3 ± 34.8, 126.7 ± 61.7 
and 107.1 ± 54.9, respectively; Table 1A–C). All other 
crosses produced similar numbers of eggs.

**Egg hatch**
The percentage of eggs hatched was 79.8% or higher 
for any cross, and no significant differences were 
observed (Table 1A–C).

**Sterility in backcrosses (Table 2)**

**Production of offspring in crosses of backcrossed 
F₁ hybrids**

All combinations of BC crossed with Y. cagnagella 
resulted in a lower percentage of pairs producing 
offspring (71.4–90.0%) than the pure Y. cagnagella 
cross (100%; Table 2A), but only Y. cagnagella \( \times \) 
BC(c×pc) differed significantly from the intraspecific 
cross in the percentage of mating pairs (78.1 vs. 100%). 
No significant differences were found within the 
interspecific crosses.

All Y. cagnagella females that had mated with a 
conspecific male or a BC(c×pc) male laid eggs, but 
females in other crosses did not always oviposit 
(\( c \times BC(p×pc) = 88\%; BC(c×pc) \times c = 75.6\%; and 
BC(p×pc) \times c = 96.4\% \) ovipositing females), even though 
they had mated (Table 2). In the intraspecific cross, 
females that mated successfully always produced eggs.

We did not find any of the crosses between BC 
hybrids and Y. padella to be significantly different 
from intraspecific Y. padella crosses in terms of the 
number of pairs with offspring (Table 2B), and there 
was even a trend for BC2 to outperform the control 
cross (three of four crosses: \( p \times (p×pc) \), \( p \times (pc×p) \) and 
(\( p×pc \) \( \times p \), all non-significant). However, we were not 
able to rear sufficient numbers of reciprocal BC hybrids 
with Y. padella, and accordingly, numbers of crosses 
involved BC(p×pc) females and BC(p×pc) males and 
females are rather low (Table 2B). Interpretation of 
the results is complicated further by the fact that only 
two-thirds of mated females oviposited in the Y. padella 
intraspecific crosses; such variation is unexplained 
but not unusual in Y. padella oviposition tests (Geerts 
et al., 2000). In the cross BC(p×pc) \( \times Y. \) padella, the 
number of pairs producing offspring was low (50%). 
Given that 91.7% of all females had been observed in 
copula, the low figure was caused by the 54.6% females 
that did mate but did not produce eggs (Table 2B).

**Lifetime fecundity**
The average lifetime fecundity of offspring of BC hybrid 
crosses was lower than that of intraspecific crosses but only 
significantly so in BC(c×pc) \( \times Y. \) cagnagella (Table 2A, B).

**Egg hatch**
We did not find a significant decrease in egg hatch in 
any of the backcrosses compared with the intraspecific 
crosses (Table 2). More than 84.6% of the eggs resulting 
from BC backcrosses with Y. cagnagella hatched. Eggs from the cross BC(p×pc) \( \times Y. \) padella had a low 
hatching rate of 66.7%, but the difference was not 
significant. However, it should be noted that sample 
sizes in the BC(p×pc) females were low, reducing the 
statistical power of the tests.

**Discussion**

Chromosomal rearrangements and gene 
incompatibilities are the two evolutionary mechanisms

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that have generally been thought to mediate postzygotic reproductive isolation.

We measured postzygotic reproductive isolation by assessing viability and sterility in interspecific crosses of two closely related species of *Yponomeuta*, combined with a cytogenetic study to evaluate the presence of chromosomal rearrangements. The extensive chromosomal reorganization we documented does not appear to contribute to reduced hybrid fitness. The driving force behind the occurrence of the rearrangements is unknown, but we hypothesize that it might be related to the building of coadapted gene complexes.

We provided evidence for a lack of synopsis during meiotic prophase I, dissimilar chromomere patterns, and loops between paired homologs that reflect inversions, deletions, and translocations between *Y. padella* and *Y. cagnagella*. Experimental hybridization of *Yponomeuta* in the laboratory enabled us to evaluate the effects of these chromosomal differences on fitness (as measured by fertility and lifetime fecundity). In spite of the observed rearrangements, we found only weak indications of reduced fertility, and the only evidence of hybrid sterility was the significantly lower percentage of pairs that produced offspring in second backcrosses with a hybrid mother; however, we suggest that second backcrosses with a hybrid father should display higher sterility or lower fecundity than first backcrosses with a hybrid mother; however, we did not find such effects in our crosses.

**LIMITED EFFECTS OF INCOMPLETE GENOMIC INFORMATION IN BACKCROSSES**

The *F*₁ hybrid males possess a full set of genes from both parents. The *F*₁ females also receive a complete genome from their father, but lack the information located on the Z chromosome of the mother. Provided there is no incompatibility between the loci of the two parental species, hybrid viability should not be affected. Also, *F*₁ females do not need to be afflicted in terms of reduced fertility by translocations of autosomes or Z chromosomes between the two parental species. Female Lepidoptera completely lack meiotic recombination (Marec, 1996; Rajmann et al., 1997), and multivalent structures at zygotene will be corrected to produce normal bivalents in later stages of meiosis (Rasmussen, 1977; Marec et al., 2001). However, in *F*₁ males unbalanced gametes may be formed through recombination of Z chromosomes or autosomes. Moreover, in both sexes distorted segregation of sex chromosomes and autosomes may also lead to unbalanced gametes in the *F*₂. This would suggest that second backcrosses with a hybrid father should display higher sterility or lower fecundity than second backcrosses with a hybrid mother; however, we did not find such effects in our crosses.

**LIMITED DIRECT EFFECTS OF INVERSIONS**

In all 26 examined preparations involving interspecific crosses, we found extensive evidence of loops in paired homologous chromosomes in early meiotic prophase I, which can be caused by inversions. Viability of *F*₁ hybrids does not have to suffer from inversions, but they may have an impact on sterility through disturbed meiosis leading to unbalanced gametes. However, inversion is disputed as a factor causing heterozygote disadvantage (King, 1987), and there is indeed empirical evidence that it does not necessarily lead to the formation of unbalanced gametes (e.g. Coyne et al., 1993; Tothová & Marec, 2001). Our data provide no clear evidence in favour of the idea that inversions have important direct effects on fitness.

**EXPECTED EFFECTS OF TRANSLocations AND TANDEM FUSIONS NOT FOUND**

As a rule, translocations or tandem fusions produce genetically unbalanced gametes in animal species, and Lepidoptera are no exception to this (King, 1987). The multivalent structures found in *Yponomeuta* BC hybrid meiosis indicate the existence of reciprocal translocations in the karyotypic differentiation of the two parental genomes (Fig. 3); we would therefore expect a decrease in the ability to produce offspring and a lower fecundity and egg hatch, especially in *F*₁ × *F*₂ crosses. Surprisingly, we found little evidence for this; normal amounts of eggs were produced by *F*₁ and backcrosses, and these eggs hatched to the same extent as those produced in the intraspecific control crosses (Table 1).

Although we found no direct effect on *F*₁ hybrid sterility, the chromosomal rearrangements could cause an indirect decrease in hybrid fitness, which cannot be measured easily in the laboratory but could lead to selection against hybrids in nature in two ways. First, rearrangements involving the NOR regions might affect the viability of *F*₁ hybrids. We observed non-homologous NOR associations in *Y. cagnagella* and *Y. padella*. Consequently, NOR functions in heterozygotes might be impaired, and second-generation hybrids would suffer from disruption of the integration of growth and development regulated by the NOR (Oliver, 1979; Sirri et al., 2008). However, in Lepidoptera, NORs seem to be often located in subtelomeric regions. This enhances their mobility, possibly reducing deleterious effects of chromosomal rearrangements (Nguyen et al., 2010).

Second, our cytogenetic study points to a disturbed male spermatogenesis. The attachment of the polycroplex structures to regular lateral elements in the SCs only occurred in hybrids and during the formation of SCs; this indicates a disruption of the
temporal regulation of hybrid meiosis. Polycomplexes regularly occur in the gametogenesis of various organisms and are interpreted as self-assembly products of SC fragments discarded from the bivalents beyond the pachytene stage (John, 1990; Verma, 1990). In insects, PCs are frequently found in late prophase I of both oogenesis and spermatogenesis, but there are no reports on their presence in spermatogenesis of Lepidoptera (Marec, 1996). Although we did not find much evidence for hybrid sterility, the possibility that distortion of spermatogenesis has more subtle effects that eventually reduce male fitness cannot be excluded.

**Holokinetic chromosomes might mitigate negative effects of chromosomal rearrangements**

Lepidoptera have holokinetic chromosomes: the centromere is missing, and the spindle microtubules attach to a large kinetochore plate extending over most of the length of the chromosome (Wolf, 1996; Wolf et al., 1997, Marec et al., 2010). This might mitigate the negative effects on gamete formation of chromosomal rearrangements in hybrids and suggest mechanisms favouring balanced segregation of chromosomes in chromosomal hybrids that would rescue their fertility. Such mechanisms could include the formation on modified SCs in achiasmatic meiosis of females and inverted meiosis in males, as recently demonstrated in *Leptidea* wood white butterflies (Lukhtanov et al., 2018).

**Chromosomal rearrangements and speciation in *Yponomeuta***

There is a paradox in assuming a large role for chromosomal rearrangements in speciation: chromosomal polymorphisms will be established sooner when they cause only a small reduction in fitness of heterozygotes, but such rearrangements are not likely to aid the evolution of postzygotic reproductive isolation much (Walsh, 1982; Faria & Navarro, 2010; Jackson, 2011). Even weak selection against heterozygotes might be sufficient to prevent the establishment of a rearrangement in a randomly mating population unless the population is so small that genetic drift becomes the predominant factor. Therefore, to become established, rearrangements must be associated with traits that increase fitness; for instance, traits for host plant adaptation (Spirito, 1998; Faria & Navarro, 2010; Butlin & Smadja, 2018).

The traditional explanation for the observed high frequency of structural alterations between *Y. cagnagella* and *Y. padella* would be that they contribute to a reduction in hybrid fitness and, in this way, facilitate the building of linkage disequilibrium that is needed for speciation in the face of gene flow. However, we found no clear evidence for a reduction in hybrid fitness. An alternative explanation could be suppression of recombination. Chromosomal rearrangements could reduce gene flow over much larger ranges than would otherwise be possible (Rieseberg, 2001; Hoffmann & Rieseberg, 2008; Feder & Nosil, 2009) and might thus conserve linkage disequilibria between loci for assortative mating and those that are under disruptive selection. This linkage disequilibrium is a strong requirement for ecological speciation (Felsenstein, 1981; Barton & de Cara, 2009; Feder & Nosil, 2009; Kirkpatrick, 2010). Linking these loci in chromosomal rearrangements could favour host shifts, which constitute the basic step in ecological speciation (Schluter, 2001; Fordyce, 2010).

In addition to suppression of recombination, rearrangements could also facilitate the construction of coadapted gene complexes (Calvete et al., 2012; Yeaman, 2013; Kirkpatrick & Barret, 2015; Feulner & De-Kyne, 2017; Jay et al., 2019). In the fly *Rhagoletis pomonella*, genes that are involved in diapause reside in complexes of rearranged genes (Feder et al., 2003; Powell et al., 2014), and rearrangements might be a source of gene duplications (Calvete et al., 2012). Additionally, repeated autosome–sex chromosome fusions, such as we may have observed for *Y. cagnagella* and *Y. padella*, may facilitate the formation of favourable gene combinations for prezygotic isolation via mate choice (cf. Bailey et al., 2011; Guerrero & Kirkpatrick, 2014) and building linkage groups for host preference and performance (Nguyen et al., 2013).

In *Yponomeuta*, the ability to survive on a host plant is based on multiple genes (Hora et al., 2014), and the observed chromosomal rearrangements might facilitate construction of such adaptive combinations of loci (Fang et al., 2012; Kirkpatrick & Barret, 2015; Conflitti et al., 2015) and produce extrinsic postzygotic isolation that can drive the evolution of prezygotic isolation (Seehausen et al., 2015; Benda et al., 2017; Butlin & Smadja, 2018; Wellenreuther & Bernatchez, 2018).

**Conclusion**

We did not find evidence for effects on fertility and fecundity of the observed chromosomal rearrangements in hybrids between the two investigated *Yponomeuta* species. Such effects are expected under the classical view of Bateson–Dobzhansky–Muller-type between-locus incompatibilities as drivers of the evolution of prezygotic reproductive isolation. Our findings do not fit this model but do support the emerging consensus for a large role of chromosomal rearrangements in the construction and maintenance of coadapted
gene complexes (Jackson, 2011; Wellenreuther & Bernatchez, 2018). The results also fit the observation that ecological differences appear to be determined mainly by autosomal rather than by sex-linked genes at early stages of population divergence (Qvarnström & Bailey, 2009; Merrill et al., 2011). Taken together, our results strongly suggest that intrinsic reproductive isolation caused by between-locus incompatibilities has not been a major factor in the evolution of the closely related Y. padella and Y. cagnagella. Our results are, however, compatible with the view that chromosomal rearrangements might play an important role in intraspecific divergence and speciation (Faria et al., 2019) and might facilitate the construction and maintenance of coadapted gene complexes.

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