Genetic architecture of host specialisation in Yponomeuta

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Limited intrinsic postzygotic reproductive isolation despite chromosomal rearrangements between closely related sympatric species of small ermine moths

K.H. HORA, F. MAREC, P. ROESSINGH & S.B.J. MENKEN

Abstract. 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. Here we studied meiosis and fertility in hybrids of two closely related small ermine moths, *Yponomeuta padellus* and *Y. cagnagellus*, 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. Fertility of reciprocal F1 and surprisingly also of backcrosses with both parent species was not significantly decreased compared to intraspecific offspring. Results indicate that intrinsic postzygotic reproductive isolation between these closely related species is limited. We therefore suggest that the rearrangements observed do not result from an accumulation of postzygotic incompatibilities preventing hybridization but rather reflect genomic changes established during adaptation to a new host plant.

INTRODUCTION

Although an abundance of species definitions has been postulated since the New Synthesis (Coyne & Orr 2004; Matsubayashi et al. 2010), the biological species concept is probably the most widely used [see Nosil (2008) and Sobel et al. (2010) for reviews]. Following this definition, species are groups of individuals, 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, intrinsic postzygotic incompatibility has often not evolved yet (Mallet 2008), but extrinsic prezygotic or postzygotic reproductive isolation mediated by specialisation on the host plant is thought to reduce gene flow to the point at which speciation is complete (Rundle & Whitlock 2001; Matsubayashi et al. 2010). The shift to a new host and subsequent adaptation to that host (i.e., host race formation) has long been viewed as the basic step leading to new species through divergent selection (Bush 1994; Mallet et al. 2009). Studies modeling the process of host specialisation in an initially randomly mating population 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 such systems, is unlikely (Felsenstein 1981; Matsubayashi et al. 2010). It is now generally accepted that decreased hybrid fitness between species can be caused by Dobzansky/Muller-type between-locus incompatibilities (Dobzhansky 1937; Muller & Pontecorvo 1940; Barton & de Cara 2009; Fierst & Hansen 2010).

Between-locus incompatibilities cause intrinsic hybrid sterility through the inability of parental genes to cooperate in regulating the development of gonads. Alternatively, hybrid sterility can be caused by disturbance of meiosis, in particular irregularities during gametogenesis, leading to the production of genetically unbalanced gametes. Such irregularities are likely to occur when there are structural differences between the chromosomes of the two hybridizing taxa, known as Robertsonian variation (White 1978; Oliver 1979; King 1993). The contribution of chromosomal rearrangements and in particular of inversions to processes of speciation are generally seen as important (Kandul et al. 2007; Hoffmann & Rieseberg 2008; Matsubayashi et al. 2010). Such changes are thought to provide a mechanism to reduce recombination (Kulathinal et al. 2009), resulting in the preservation of whole genomic regions, the so-called ‘islands of speciation’ (Turner et al. 2005). These speciation islands could help to maintain genetic divergence between populations in the presence of gene flow. The actual role of reduced recombination due to chromosomal rearrangements in speciation is, however, still a subject of debate (cf. Feder & Nosil 2009; Noor & Bennett 2009; Faria & Navarro 2010) and more research is needed to determine which structural factors and selective forces are fundamental to the process of speciation.

In this paper, we studied the role of chromosomal rearrangements and hybrid sterility in the early stages of reproductive isolation using closely related species of the small ermine moth genus *Yponomeuta* (Lepidoptera, *Yponomeutidae*). The species in this genus are specialised herbivores on one or a few related plants (Menken et al. 1992). Species in the so-called *Y. padellus*-complex are thought to have evolved in sympatry, and their differential host specialisation following a host shift probably played a principal part in the evolution of the group. As the phylogenetic relationships and host associations of the species are well known (Turner et al. 2010), this genus provides an exemplary model for multidisciplinary studies of speciation through host specialisation (Menken et al. 1992; Menken 1996; Gershenson & Ulenberg 1998; Menken & Roessingh 1998; Bakker et al. 2008).

We performed a cytogenetic analysis of hybrid meiosis to determine whether gametogenesis is disturbed as a conse-
quence of structural differences between the parental chromosomes. Parallel to this, sterility of both males and females of reciprocal interspecific hybrids of Y. padellus L. and Y. cagnagellus (Hübner) was assessed. Viability of F1 hybrids will not suffer from chromosomal rearrangements if the combined parental genomes are sufficiently compatible to secure all vital functions. The effect of chromosomal rearrangements should only become apparent in the production of unbalanced gametes in hybrid individuals (hybrid sterility; Carpenter et al. 2005). For this reason, we also studied the occurrence of sterility in second generation hybrids (F2) and in backcrosses. The combination of these data allows for an empirical estimate of the extent of postzygotic reproductive isolation between Y. padellus and Y. cagnagellus and the role of chromosomal rearrangements in the evolution of reproductive isolation between these species of small ermine moths.

**MATERIALS AND METHODS**

**Biology, collection, and rearing of insects**

*Yponomeuta padellus* and *Y. cagnagellus* are sympatric, reproductively synchronous, and closely related species (Sperling et al. 1995; Turner et al. 2010). They are univoltine, with gregarious first instars, which hibernate under a hibernaculum formed by their egg capsules. Eggs are laid on stems of host plants in masses of 30-100 eggs. *Yponomeuta padellus* is the only oligophagous species in the genus in Western Europe, and feeds on a restricted number of species of the Rosaceae. In the Netherlands, it is most commonly found on *Crataegus* spp. and on *Prunus spinosa*. *Yponomeuta cagnagellus*, on the other hand, is specialised on *Eunynus europeaus* (Celastraceae). The two species do not hybridize in nature as a result of pre-zygotic reproductive isolation mediated by differential diet breadth and sex-pheromones (Hendrikse 1988; Menken et al. 1992). In other highly specialised herbivores – e.g. *Rhagoletis* spp. (Feder et al. 2003) – populations utilizing different hosts may become separated because mating is strongly correlated to the larval host plant (host fidelity). In *Yponomeuta*, mating usually does not take place on the host plant (Bakker et al. 2008), excluding host fidelity as a means to establish assortative mating. Under laboratory conditions, forced matings between *Y. padellus* and *Y. cagnagellus* produce fully viable F1 hybrids. These in turn are capable of producing viable F2 and backcross offspring (Menken 1980; Roessingh et al. 1999; Hora et al. 2005). When reared under laboratory conditions, mortality and sex ratios in these crosses do not differ from those of the parental species (Chapter 6 in this thesis).

Insects to be used as parents of the interspecific F1 crosses and those used for intraspecific control crosses were collected as fifth instars from their host plants in the field (both species at Meyendel, The Hague, The Netherlands (52°07'55"N, 4°19'59"E) and Flevopark, Amsterdam, The Netherlands (52°21'46"N, 4°56'54"E)). Larvae were fed ad libitum on fresh leaves of their host in big plastic cylinders (45 cm high, 20 cm diameter) or glass jars (20 cm high, 11 cm diameter). The resulting pupae were placed individually in glass tubes (8 cm long, 1.2 cm diameter). *Yponomeuta padellus* reaches sexual maturity somewhat sooner after eclosion as *Y. cagnagellus* (Hendrikse 1979); therefore, pupae of the latter were kept at a higher temperature (24 vs. 18 °C for *Y. padellus*) until eclosion; this synchronized the development of the two species to the extent that sexually ripe individuals were available at the same time.

**Crosses**

After emergence, moths were sexed and one female and one male were put together in glass vials for 1-2 weeks, with a strip of filter paper as a mating substrate (at 18 °C, 16:8 LD). After this period, pairs were transferred to Perspex cylinders (16 cm high, 4 cm diameter) 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 cagnagellus* females were offered *E. europeaus* and *Y. padellus* females *P. spinosa* for oviposition (Hora & Roessingh 1999). In all abbreviations of crosses the mother is given first, i.e., F1(pc) denotes a female *Y. padellus* crossed with a male *Y. cagnagellus*.

Following the same procedure, backcrosses (BC) were produced by crossing interspecific F1 hybrids with *Y. cagnagellus* or *Y. padellus* that were reared from the intraspecific crosses. As small ermine moths do not respond well to manipulation of their univoltine life cycle, an artificial diapause of at least 4 months was introduced by storing egg masses in total darkness at 4 °C, until they were used the next spring.

First instars (L1) were carefully removed from under their hibernaculum, counted, and reared in plastic Petri dishes (2.5 cm high, 10 cm diameter) at 18 °C and 16:8 LD until pupation in all experiments, unless otherwise stated. The size of rearing groups was kept at 15-30 first instars per Petri dish. When larvae reached their last instar (L5), rearing groups were split in two with a maximum of 15 L5 larvae per Petri dish. Larvae were fed ad libitum with fresh leaves of their optimal host (Hora et al. 2005), which varied between the different crosses, i.e., *E. europeaus* for F1 interspecific hybrids, and the host plant of the backcross parent for backcrosses. Fully mature pupae were taken out of their cocoons on day 4 after the onset of pupation and weighed to the nearest 0.1 mg on an OHAUS Analytical Standard Balance (d = 5x10−5). They were placed in all experiments, unless otherwise stated. The size of rearing groups was kept at 15-30 first instars per Petri dish. When larvae reached their last instar (L5), rearing groups were split in two with a maximum of 15 L5 larvae per Petri dish. Larvae were fed ad libitum with fresh leaves of their optimal host (Hora et al. 2005), which varied between the different crosses, i.e., *E. europeaus* for F1 interspecific hybrids, and the host plant of the backcross parent for backcrosses. Fully mature pupae were taken out of their cocoons on day 4 after the onset of pupation and weighed to the nearest 0.1 mg on an OHAUS Analytical Standard Balance (d = 5x10−5). They were placed individually in glass vials at 18 °C and 16:8 LD until eclosion in all experiments, unless otherwise stated. After eclosion, adults were sexed and fed with 0.5 cm3 blocks of 1% honey in 1% agar, which were replaced 2-3 times per week.

**Chromosome preparations**

Meiotic chromosome pairing in *Y. cagnagellus*, *Y. padellus*, their F1 hybrids, and BC *Y. cagnagellus × F1(pc)* hybrids was investigated using different methods for males and females. In males, we examined two individuals of *Y. cagnagellus*, three of *Y. padellus*, three F1(pc), two F1(cp), and one BC(c×pc). Meiosis was studied on microspread preparations of late zygotene or pachytene nuclei from the testes of early fifth instars. The microspreading technique was performed following the procedure described for *Ephesia kuehniella* (Marec & Traut 1993). Briefly, dissected gonads were disrupted in a hypotonic solution (83 mM KCl and 17 mM NaCl), spread in 0.02% Joy detergent, and centrifuged onto a carbon coated grid through an 0.1 M sucrose cushion containing 1% formaldehdye, fixed with the same solution, and stained in 1% Ethanolic phosphotungstic acid. Preparations were examined and micrographs taken in a Jeol 1010 transmission electron microscope.
Hybrid sterility

Sterility is a complex, multifactorial trait. We tested the following components of hybrid sterility: percentage of hybrids producing offspring, average lifetime fecundity, and percentage of hatched eggs. The method of testing varied slightly between F1 hybrids and backcrosses, and will be described in separate paragraphs below.

We used males and females of both reciprocal F1 hybrids, but performed only reciprocal BC crosses with F1 *Y. padellus × Y. cagnagellus* [F1(pc)]. This cross was chosen because preliminary experiments showed that F1(pc) hybrids suffer slightly more from sterility (see Table 1A) than the F1(cp) reciprocal hybrids. This is expected to lead to more pronounced fitness effects in the second hybrid generation. For both BC and F1 progeny male and female hybrid sterility was assessed by mating them with *Y. cagnagellus* or *Y. padellus* of the opposite sex. 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. Resulting total sample sizes are listed in Tables 1 and 2.

**F1 hybrids**

F1 hybrids were combined with *Y. cagnagellus* or *Y. padellus* of the opposite sex and placed in groups in Perspex cylinders (16 cm high, 4 cm diameter) slid over the branches of potted plants and closed off with cotton wool plugs. Plants were grown in a greenhouse at 20-25 °C and natural light (July-September). The host plant was chosen according to the female parent of the cross: *Y. cagnagellus* and F1 hybrids were provided with *E. europaeus* (both reciprocal F1 hybrids prefer *E. europaeus* for oviposition; Hora et al. 2005), and *Y. padellus* was provided with *P. spinosa*. Cages were inspected every working day, and the male was removed after the first egg mass had been recorded.

A pair was counted as producing offspring when at least one egg mass was produced; unmated female *Yponomeuta* do not lay eggs (Karalius & Budda 1995). Females were kept in their cages until death, and lifetime fecundity was determined by counting the number of individual eggs in all egg masses produced by one female under a stereomicroscope (at 40×). 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. We only did this with a limited number of pairs because F1 hybrids were also used for the production of BC hybrids, and disturbance caused by counting freshly hatched larvae severely reduces their survival (K.H. Hora, unpublished results).

**BC hybrids**

After eclosion, moths were sexed and moved to a climate room set at 21 °C, 16:8 LD, with lights on at 14:00 hours, shifting the daylight period forward in time. This allowed convenient observation of mating behaviour, as *Yponomeuta* are sexually active at the end of the scotophase (Hendrikse 1979). In contrast to the experiment with F1 hybrids, mating was checked carefully to distinguish between pairs not producing offspring because they had not mated and pairs not producing offspring because of post-mating barriers, e.g., incomplete or no sperm transfer, or sterile sperm. Pairs of males and females were assigned at the start of the experiment, but were kept in two separate vials. Two hours before the start of the photophase, a mating pair was put together. For a period of 4 hours, pairs *in copula* were scored every hour. Hourly intervals were sufficient, as pairs stay in copula for many hours. After this period, unmated moths were returned to their separate vials. This procedure was repeated the next ‘night’ until the moths had mated or the male or the female 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 2.9.1 program (R Development Core Team 2009).

**RESULTS**

**Chromosomal rearrangements: oocytes**

Females of *Yponomeuta* species have 29 pairs of autosomes (A) in addition to three sex chromosomes, which in meiosis form a trivalent, consisting of one W chromosome and two Z chromosomes (*Z1* and *Z2*). The chromosome number in females is therefore 2n = 29A + WZ1Z2, whereas males have...
31 chromosome pairs and 2n = 29AA+Z1Z2Z2 (Nilsson et al. 1988). With the pachytene mapping technique, the W chromosome can be recognized in many Lepidoptera by its partial or complete heterochromatization allowing thus the identification of sex chromosome bivalents (or trivalents in the case of the *Yponomeuta* species under study), while 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 WZ2Z2 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. cagnagellus* and *Y. padellus* may not be completely homologous anymore. This was apparent in F1 and BC hybrid females, in which the WZ1Z2 trivalents were frequently not well synapsed, and Z2 seemed shortened to adjust for synapsis (Fig. 1d,e; the W chromosome is the evenly dark chromosome).

In 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 pure species, two bivalents carried the nucleolar organizer region (NOR), which appeared as a large, weakly stained

**Figure 1.** Phase-contrast micrographs of LM preparations of pachytene oocytes in *Yponomeuta cagnagellus*, *Y. padellus*, and their F1 and backcrosses stained with lactic acetic orcein. W, female-specific sex chromosome (heterochromatic); Z1 (long) and Z2 (short) sex chromosomes showing chromomere pattern. Bar = 2 µm (a,b,d,e) or 10 µm (c,f,g,h). (a) Detail of the WZ1Z2 trivalent from a *Y. cagnagellus* female. (b) Detail of the WZ1Z2 trivalent from a *Y. padellus* female. (c) Pachytene nucleus from a *Y. cagnagellus* female; arrows indicate examples of pairs of homologous chromomeres in autosomal bivalents. (d) Detail of the WZ1Z2 trivalent from an F1 hybrid (*Y. padellus* × *Y. cagnagellus*) female: the trivalent is not synapsed and shows only partial pairing of the W chromosome from the *Y. padellus* mother with the two paternal *Y. cagnagellus* Z chromosomes. (e) Detail of the WZ1Z2 trivalent in a BC hybrid *Y. cagnagellus* × F1(*Y. padellus* × *Y. cagnagellus*) female; the Z2 chromosome is well paired with the W, the Z1 shows only partial pairing at one distal segment. (f) Pachytene complement in a BC hybrid *Y. cagnagellus* × F1(*Y. padellus* × *Y. cagnagellus*) female showing a quadrivalent (Q) and a multivalent (M) composed of six chromosomes; see (k) for schematic interpretation of Q and M; N, nucleous. (g) Pachytene complement in a BC hybrid *Y. cagnagellus* × F1(*Y. padellus* × *Y. cagnagellus*) female showing one multivalent (M) composed of five chromosomes as illustrated in (i) and a number of pairing irregularities (arrows) such as loops and weakly paired segments. (h) Pachytene complement in a BC hybrid *Y. cagnagellus* × F1(*Y. padellus* × *Y. cagnagellus*) female showing the unusual sex chromosome trivalent (see detail in Fig. 1e), possibly a quadrivalent (Q) indicating a translocation between two autosomes as illustrated in (j) and at least two small loops (arrows) indicating inversions and/or deletions.
spot associated with one end of the bivalents. The observed irregularities in F1 and backcrosses frequently involved chromosomes associated with the NOR (Fig. 1f,g): one of the chromosomes could be recognized by a conspicuous chromomere at one end, and was often found to be involved in multivalents, 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,g). Sometimes non-homologous chromomere patterns in bivalents in F1 and backcrosses were observed, but this result is hard to interpret, as the chromomere patterns in pure *Y. cagnagellus* and *Y. padellus* were not always completely homologous either, probably as a result of the spreading procedure itself.

**Chromosomal rearrangements: spermatocytes**

Pachytene bivalents in *Y. cagnagellus* and *Y. padellus* males were synapsed well 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 per nucleus. Meiotic disturbances were even more pronounced in BC hybrid males. We repeatedly found a multivalent consisting of 4-6 chromosomes. This multivalent was found in different configurations in several nuclei of the same individual (Fig. 3a-c) and indicated a multiple translocation. In some nuclei, 1-2 bivalents were not synapsed and their lateral elements were decorated with PCs (Fig. 3d,e).

**Figure 2.** EM microspread preparations of pachytene spermatocytes of *Y. cagnagellus*, *Y. padellus*, and F1 hybrid males. SC, synaptonemal complex; CE, central element of the SC; LE, lateral element of the SC; RN, recombination nodule; PC, polycomplex. (a) Part of pachytene nucleus from *Y. padellus* male showing well formed SCs (even CEs are seen); note a bivalent with a well visible RN located between two LEs. (b) Late zygotene nucleus from F1 hybrid (*Y. padellus × Y. cagnagellus*) male showing, besides regular SCs, many pairing abnormalities such as loops (L) indicating inversions, lack of synapsis at distal ends of the SC (arrows), and PCs associated with LEs. (c) Detail of one well-formed SC from the above nucleus showing even a clear CE (one distal end is not fully synapsed probably due to an NOR that prevents synapsis; cf. Marec & Traut 1993). (d) Detail of one partly paired bivalent from the above nucleus showing PCs associated with the LEs of the unpaired segment.
Sterility of F1 hybrids (Table 1)

**Pairs with offspring**

We found no significant differences in the percentage of pairs that produced offspring among the crosses involving pure species and F1 hybrids (G = 1.40, df = 3, p = 0.70 for crosses with *Y. cagnagellus*, and G = 6.29, df = 4, p = 0.18 for crosses with *Y. padellus*; Table 1A,B). There was also no difference in the crosses of F1 hybrids producing second generation F2 (G = 0.60, df = 1, n.s.; Table 1C). Intraspecific *Y. cagnagellus* crosses resulted in similar percentages of pairs with offspring as crosses in which one of the partners was an F1 hybrid, ranging from 66.7 to 71.9% (G = 1.61, df = 4, n.s.). For F1 hybrids crossed with *Y. padellus*, only one of the four hybrid crosses, viz., Pad × F1(pc), differed from the intraspecific cross (57.7 vs. 91.7%; G = 9.738, df = 4, p = 0.045; Table 1B). Crosses with F1(pc) as parent consistently showed a lower percentage of pairs that produced offspring than those involving the reciprocal F1(cp) hybrid (Table 1A,B,C).

**Lifetime fecundity**

With *Y. padellus* as the backcross-species, significant differences in lifetime fecundity were found (F = 4.45, df = 4, 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) × Pad, and this cross had a significantly higher lifetime fecundity than Pad × Pad. A similar result was found with *Y. cagnagellus* as the backcross species (F = 3.26, df = 3, p = 0.027), but again only F1(pc) × Cag differed from the intraspecific cross (F = 3.257, df = 4, p = 0.027), and lifetime fecundity was higher in this cross. Results for the intraspecific cross of *Y. cagnagellus* are missing, because we did not manage to produce sufficient numbers of *Y. cagnagellus* to realise this cross together with the F1 hybrids × Cag crosses.

When comparing all crosses in which F1 hybrids were involved, the F1(pc) females produced in backcrosses with *Y. cagnagellus*, *Y. padellus*, and F1(pc) males significantly more eggs (143.4 ± 65.4, 182.3 ± 94.7, and 168.8 ± 94.3, respectively) than F1(cp) females (79.3 ± 34.8, 126.7 ± 61.7, and...
### Table 1. Reproductive characteristics of intraspecific and interspecific crosses in (A) *Yponomeuta cagnagellus* (Cag), (B) *Y. padellus* (Pad), and (C) their F1 hybrids

<table>
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<th>Cross type*</th>
<th>No. pairs</th>
<th>% pairs with offspring</th>
<th>Sign.†</th>
<th>Average lifetime fecundity</th>
<th>Sign.‡</th>
<th>% egg hatch (n§)</th>
<th>Sign.†</th>
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<td>99.0 ± 60.8 (13) ab</td>
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<tr>
<td>F1(cp) × F1(cp)</td>
<td>108</td>
<td>75.9 a</td>
<td>107.1 ± 54.9 (74) b</td>
<td>89.8 (46) a</td>
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<tr>
<td>F1(pc) × F1(pc)</td>
<td>105</td>
<td>66.7 a</td>
<td>168.8 ± 94.3 (63) a</td>
<td>80.6 (41) a</td>
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</table>

G-tests for pairs with offspring: G = 9.7383, df = 4, P = 0.045; G = 6.286, df = 4, n.s. over all crosses. ANOVA for lifetime fecundity: F = 2.806, df = 4, P = 0.028. G-tests for % hatched eggs: G = 0.715, df = 4, n.s.

### Table 2. Reproductive characteristics of intraspecific and interspecific crosses and second backcrosses in (A) *Yponomeuta cagnagellus* and (B) *Y. padellus*

<table>
<thead>
<tr>
<th>Cross type*</th>
<th>No. pairs</th>
<th>% pairs with offspring</th>
<th>Sign.†</th>
<th>% mated pairs</th>
<th>Sign.‡</th>
<th>% mated pairs with offspring</th>
<th>Sign.‡</th>
<th>Average lifetime fecundity</th>
<th>Sign.‡</th>
<th>% egg hatch (n§)</th>
<th>Sign.†</th>
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<tr>
<td><strong>A</strong></td>
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<tr>
<td>Cag × Cag (contr.)</td>
<td>30</td>
<td>100 a</td>
<td>a</td>
<td>100 a</td>
<td>263.6 ± 136.0 (30) a</td>
<td>93.3 (29) a</td>
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<tr>
<td>Cag × BC(c×pc)</td>
<td>32</td>
<td>78.1 a</td>
<td>100 b</td>
<td>234.8 ± 139.4 (25) ab</td>
<td>88.0 (22) a</td>
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<tr>
<td>Cag × BC(pc×c)</td>
<td>30</td>
<td>83.3 a</td>
<td>88.0 ab</td>
<td>242.5 ± 114.3 (22) ab</td>
<td>86.4 (20) a</td>
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<tr>
<td>BC(c×pc) × Cag</td>
<td>35</td>
<td>94.3 ab</td>
<td>75.6 a</td>
<td>163.7 ± 101.8 (26) b</td>
<td>92.3 (24) a</td>
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<tr>
<td>BC(pc×c) × Cag</td>
<td>30</td>
<td>93.3 a</td>
<td>96.4 a</td>
<td>246.7 ± 96.2 (27) ab</td>
<td>93.3 (26) a</td>
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<td>Pad × Pad (contr.)</td>
<td>30</td>
<td>100 a</td>
<td>ab</td>
<td>100 a</td>
<td>88.0 ± 34.6 (20) a</td>
<td>95.0 (19) a</td>
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<tr>
<td>Pad × BC(p×pc)</td>
<td>32</td>
<td>71.9 ab</td>
<td>79.3 ab</td>
<td>82.7 ± 39.1 (23) a</td>
<td>95.7 (22) a</td>
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<tr>
<td>Pad × BC(pc×p)</td>
<td>6</td>
<td>83.3 a</td>
<td>83.3 a</td>
<td>119.6 ± 15.4 (5) a</td>
<td>100 (5) a</td>
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<tr>
<td>BC(p×pc) × Pad</td>
<td>17</td>
<td>88.2 ab</td>
<td>93.3 a</td>
<td>81.6 ± 46.9 (14) a</td>
<td>85.7 (12) a</td>
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<tr>
<td>BC(pc×p) × Pad</td>
<td>12</td>
<td>90.0 b</td>
<td>91.7 a</td>
<td>92.0 ± 46.6 (6) a</td>
<td>66.7 (5) a</td>
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</table>

G-tests for crosses with offspring: G = 6.971, df = 4, n.s. G-tests for % mated pairs: G = 3.532, df = 4, n.s. ANOVA for lifetime fecundity: F = 3.257, df = 4, P = 0.028. G-tests for % hatched eggs: G = 1.4137, df = 4, n.s.
We did not find any of the crosses between BC hybrids and *Y. padellus* to be significantly different from intraspecific *Y. padellus* crosses in terms of the number of pairs with offspring, and there was even a trend for second backcrosses to outperform the control cross (three of four crosses, all non-significant; Table 2B). However, we were not able to rear sufficient numbers of all reciprocal BC hybrids with *Y. padellus*, and accordingly numbers of crosses involving BC(p×pc) females and BC(pc×p) males and females are rather low (Table 2B). Interpretation of these results is further complicated by the fact that only two-thirds of mated females oviposited in the *Y. padellus* intraspecific crosses; such variation is unexplained but not unusual in *Y. padellus* oviposition tests (Geerts et al. 2000). In the cross BC(pc×p) × Pad, the number of pairs producing offspring is low (50%); as 91.7% of all females had been observed in copula, the low figure is caused by the 54.6% females that did mate but did not produce eggs (Table 2B).

**Lifetime fecundity**

Average lifetime fecundity of offspring of BC hybrid crosses was lower than that of intraspecific crosses but only significantly so in BC(c×pc) × *Y. cagnagellus* (Tables 2A,B), a difference that is probably caused by the lower pupal weight of the back cross (Chapter 6 of this thesis).

**Egg hatch**

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

**DISCUSSION**

Chromosomal rearrangements and gene incompatibilities are the two evolutionary mechanisms that have generally been thought to mediate intrinsic postzygotic reproductive isolation. Here, we provided evidence for the lack of synapsis during meiotic prophase I, non-homologous chromomere patterns, and loops between paired homologs that reflect inversions, deletions, and translocations between the closely related ermine moth species *Y. padellus* and *Y. cagnagellus*. 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 in the interspecific hybrids, we found only weak indications of reduced fertility, and virtually the only evidence of hybrid sterility was the significantly lower percentage of pairs that produced offspring in second backcrosses with *Y. cagnagellus*. A very limited number of crosses – Pad × F1 (pc), BC(c×pc) × Cag and BC(pc×p) × pad – showed any other measurable negative effects (see the isolated b’s in Table 2).

**Expected effects of incomplete genomic information in backcrosses not found**

F1 hybrid males possess a full set of genes from both parents. F1 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, F1 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; Rajjmann et al. 1997) and multivalent structures at zygote will be corrected to produce normal bivalents in later stages of meiosis (Rasmussen 1977; Marec et al. 2001). However, in F1 males, unbalanced gametes may be formed through recombination of Z chromosomes or autosomes. Finally, in both sexes distorted segregation of sex chromosomes and autosomes may also lead to unbalanced gametes in the F1. This would suggest that second backcrosses with a hybrid father should display higher sterility or lower fecundity than second backcrosses with a hybrid mother; we did not, however, find such effects in our data.

**Limited effect of inversions**

We found extensive evidence of looping in paired homologous chromosomes in early meiotic prophase I, which might be caused by inversions. F1 hybrids need not suffer in viability from inversions, but they may have an impact on their 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 & Meyers 1993; Tothóvá & Marec 2001). Our data provide no clear evidence in favor of the idea that inversions have important fitness effects.

**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 meioses 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, as well as a lower fecundity and egg hatch, especially in F1 × F1 crosses. Surprisingly, we find little evidence for this: normal numbers of eggs were produced by F1 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 F1 hybrid sterility, the chromosomal rearrangements could cause an indirect decrease in hybrid fitness, which cannot easily be measured in the laboratory, but could lead to selection against hybrids in nature in two ways. First, an impact on viability of F2 hybrids might be expected from chromosomal rearrangements involving the NOR regions. We found evidence of non-homologous NOR associations in *Y. cagnagellus* and *Y. padellus*, and consequently NOR functions in heterozygotes might be impaired. In this case, second generation hybrids would not only suffer from an incomplete genome as a result of unbalanced gametes of their parents, but also from disruption of the integration of growth and development regulated by the NOR (Oliver 1979; Sirri et al. 2008). Non-homology of chromosomes associated
with the NOR has been previously described for subspecies of the grasshopper *Chorthippus parallelus*, resulting in sterile males (Gosálvez et al. 1988). 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 meiosis. The attachment of the polycomplex 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, polycomplexes are frequently found in late prophase I of both oogenesis and spermatogenesis. Although they have been observed in various insect taxa, including *Bombyx mori* females and Trichoptera males, there are no reports on their presence in spermatogenesis of Lepidoptera (Marce 1996). Although we did not find much evidence for hybrid sterility, it cannot be excluded that distortion of spermatogenesis has more subtle effects that eventually reduce male fitness.

It should be noted that Lepidoptera have holokinetic chromosomes: the primary constriction (centromere) typical of monocentric chromosomes 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). As a result, Lepidoptera exhibit some cytogenetic peculiarities, which may mitigate the negative effects on gamete formation of chromosomal rearrangements in hybrids. Lepidoptera are highly resistant to ionizing radiation (Bakri et al. 2005) and it has been shown that irradiated individuals and their progeny may exhibit a considerable amount of chromosomal rearrangement in their genome (fissions, fusions, deletions, and translocations resulting from chromosome breaks) without a large impact on viability and fertility (Tothová & Marce 2001).

**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 in a population when they cause only a small fitness reduction of heterozygotes, but such rearrangements are not likely to much aid the evolution of postzygotic reproductive isolation (Sites & Moritz 1987; Faria & Navarro 2010; Jackson 2011). Even weak selection against heterozygotes may be sufficient to prevent the establishment of a rearrangement in a random mating population unless the population is so small that genetic drift becomes a predominant factor. Therefore, to become established, rearrangements must be associated with traits that increase fitness, e.g., traits for host plant adaptation (Spirito 1998; Faria & Navarro 2010). What benefits could be associated with the considerable chromosomal rearrangements in the two closely related *Yponomeuta* species? One possibility might be that rearrangements facilitate/conserve linkage groups of co-adaptive genes.

In the fly *Rhagoletis pomonella* there is evidence that genes that are involved in diapause reside in complexes of rearranged genes (Feder et al. 2003). By suppressing recombination, chromosomal rearrangements could reduce gene flow over much larger chromosomal regions than would otherwise be possible (Rieseberg 2001; Hoffmann & Rieseberg 2008; Feder & Nosil 2009). Chromosomal rearrangements might thus facilitate as well as conserve linkage disequilibria between loci for assortative mating and those that are under disruptive selection. Mathematical models show that this linkage disequilibrium is a strong requirement for ecological speciation (Felsenstein 1981; Feder & Nosil 2009). Linking these loci in chromosomal rearrangements would favour host shifts which constitute the basic step in ecological speciation (Schluter 2001). In the evolution of *Drosophila* species, extensive chromosomal duplications and subsequent interchromosomal transpositions have been documented for ecologically important olfactory receptor genes (Conceicao & Aguaade 2008). This also points to a role for such changes in the construction of co-adapted gene complexes in the evolution of host associations.

**A role of multiple sex chromosomes in speciation of Yponomeuta**

The sex chromosomes appear to play a role in disturbed meiosis of *Yponomeuta* hybrids. Incomplete synapsed WZ1Z2 trivalents were frequently observed, and in a *Y. cagnagellus × F1* (pc) female an unusual sex chromosome trivalent was found. In spite of this evidence for extensive rearrangements, we did not observe the pattern of Haldane’s rule in F1 *Yponomeuta* hybrids: there was no difference between F1 males and females in being sterile, which would be expected in the heterogametic sex, i.e., the females. This result might at first seem unexpected, as this pattern is one of the most general principles in evolutionary biology (Koevoets & Beukeboom 2009; Schilthuizen et al. 2011). However, Haldane’s rule obviously applies only when sterility is actually found, and we found very little evidence for hybrid sterility.

Evidence for fusion between autosomes and sex chromosomes has recently been documented in detail for the wild silkmoth, *Samia cynthia* (Yoshido et al. 2011). Repeated autosome–sex chromosome fusions may facilitate the formation of favorable gene combinations for prezygotic isolation via mate choice (cf. Bailey et al. 2011), and building linkage groups for host preference and performance. As these observed chromosomal rearrangements in *Yponomeuta* do not seem to contribute to intrinsic postzygotic isolation, it suggests that the two *Yponomeuta* species are in a very early stage of separation, as expected for sister species that recently evolved in sympathy (Menken et al. 1992; Menken & Roessingh 1998). It also reflects the observation that ecological differences appear to be mainly determined by autosomal instead of sex-linked genes at early stages of population divergence (Qvarnström & Bailey 2009; Merrill et al. 2011).

In summary, we propose that the chromosome rearrangements that we found in this study do not play a primary role in postzygotic reproductive isolation, but rather have contributed to the evolution of host specialisation in *Yponomeuta* by advancing co-adaptation of traits necessary for use of the new host of the incipient species.

**Acknowledgements**

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REFERENCES


INTRINSIC POSTZYGOTIC REPRODUCTIVE ISOLATION BETWEEN CLOSELY RELATED SPECIES


