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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 F_1 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.

ADDITIONAL KEYWORDS: chromosome pairing – fecundity – host plant adaptation – host shift – hybrid sterility – Lepidoptera – meiosis – speciation – specialization – *Yponomeuta*.

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

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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; Cruickshank & 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-Barrientos *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 (F_1) 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. F_1 sterility; Carpenter *et al.*, 2005). Therefore, we also studied the occurrence of sterility in second-generation (F_2) 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°07′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 cagnagella* females were offered *E. 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 Axioplan 2 Zeiss microscope at ×1000 magnification.

Males: spermatocytes

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 zygotene 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 zygotene 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 F_1 hybrids but performed reciprocal backcrosses only with F_1 *Y. padella* × *Y. cagnagella* [F_1(pc)]. This cross was chosen because preliminary experiments showed that F_1(cp) hybrids suffer slightly more from sterility than the F_1(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 F_1 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 F_1 hybrids

The F_1 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 F_1 hybrids were provided with *E. europaeus* (both reciprocal F_1 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 F_1 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 F_1s (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 F_1 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 F_1 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 (Z_1 and Z_2). The diploid chromosome number in females is therefore $2n = 61$ ($29AA + WZ_1Z_2$), whereas males have 31 chromosome pairs and $2n = 62$ ($29AA + Z_1Z_1Z_2Z_2$) (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 F$_1$ hybrids

Cross type* (female × male)	Number of pairs	Percentage of pairs with offspring	Sign†	Lifetime fecundity [mean ± SD number of eggs (N‡)]	Sign§	Eggs hatched [% (N‡)]	Sign†
A. Crosses with *Yponomeuta cagnagella*							
Cag × Cag (control)	27	70.4	a	–		–	
Cag × F$_1$(cp)	32	71.9	a	107.4 ± 93.1 (17)	ab	93.6 (2)	a
Cag × F$_1$(pc)	29	58.6	a	99.0 ± 60.8 (13)	ab	82.0 (7)	a
F$_1$(cp) × Cag	34	67.7	a	79.3 ± 34.8 (21)	b	79.8 (9)	a
F$_1$(pc) × Cag	39	66.7	a	143.4 ± 65.4 (19)	a	84.0 (10)	a

G-tests for pairs with offspring: G_4 = 1.6136, n.s. over all crosses; G_3 = 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_3 = 1.2908, n.s.

Cross type* (female × male)	Number of pairs	Percentage of pairs with offspring	Sign†	Lifetime fecundity [mean ± SD number of eggs (N‡)]	Sign§	Eggs hatched [% (N‡)]	Sign†
B. Crosses with *Yponomeuta padella*							
Pad × Pad (control)	24	91.7	a	110.5 ± 36.7 (22)	b	90.8 (22)	a
Pad × F$_1$(cp)	29	72.4	ab	113.5 ± 46.4 (19)	b	95.0 (9)	a
Pad × F$_1$(pc)	26	57.7	b	110.5 ± 58.2 (10)	b	81.8 (5)	a
F$_1$(cp) × Pad	27	74.1	ab	126.7 ± 61.7 (18)	ab	95.9 (8)	a
F$_1$(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.

Cross type* (female × male)	Number of pairs	Percentage of pairs with offspring	Sign†	Lifetime fecundity [mean ± SD number of eggs (N‡)]	Sign§	Eggs hatched [% (N‡)]	Sign†
C. F$_1$ hybrid crosses							
F$_1$(cp) × F$_1$(cp)	108	75.9	a	107.1 ± 54.9 (74)	b	89.8 (46)	a
F$_1$(pc) × F$_1$(pc)	105	66.7	a	168.8 ± 94.3 (63)	a	80.6 (41)	a

G-tests for pairs with offspring: G_1 = 0.5996, n.s. ANOVA for lifetime fecundity: F_1 = 22.6, P < 0.001. G-test for percentage of eggs hatched: G_1 = 0.4921, n.s.

*Cag, *Y. cagnagella*; Pad, *Y. padella*; F$_1$, interspecific cross with, in parentheses, the direction of the F$_1$ (c for *Y. cagnagella* and p for *Y. padella*, with females mentioned first).
†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.

Table 2. Reproductive characteristics of intraspecific and interspecific crosses and second backcrosses in *Yponomeuta cagnagella* (A) and *Yponomeuta padella* (B)

Cross type* (female × male)	Number of pairs	Percentage of pairs with offspring	Sign†	Percentage of mated pairs	Sign§	Percentage of mated pairs with offspring	Sign†	Lifetime fecundity [mean ± SD number of eggs (N‡)]	Sign§	Egg hatch [% (N‡)]	Sign†
A. Crosses with *Yponomeuta cagnagella*											
Cag × Cag (control)	30	100.0	a	100.0	a	100.0	a	263.6 ±136.0 (30)	a	93.3 (29)	a
Cag × BC(c×pc)	32	78.1	a	78.1	b	100.0	a	234.8 ±139.4 (25)	ab	88.0 (22)	a
Cag × BC(pc×c)	30	73.3	a	83.3	ab	88.0	a	242.5 ±114.3 (22)	ab	86.4 (20)	a
BC(c×pc) × Cag	s	71.4	a	94.3	ab	75.6	a	163.7 ±101.8 (26)	b	92.3 (24)	a
BC(pc×c) × Cag	30	90.0	a	93.3	ab	96.4	a	246.7 ±96.2 (27)	ab	93.3 (26)	a
B. Crosses with *Yponomeuta padella*											
Pad × Pad (control)	30	66.7%	ab	100.0	a	66.7	ab	88.0 ± 34.6 (20)	a	95.0 (19)	a
Pad × BC(p×pc)	32	71.9%	ab	90.6	a	79.3	ab	82.7 ± 39.1 (23)	a	95.7 (22)	a
Pad × BC(pc×p)	6	83.3%	a	100.0	a	83.3	ab	119.6 ± 15.4 (5)	a	100 (5)	a
BC(p×pc) × Pad	17	82.3%	ab	88.2	a	93.3	a	81.6 ± 46.9 (14)	a	85.7 (12)	a
BC(pc×p) × Pad	12	50.0%	b	91.7	a	54.6	b	92.0 ± 46.6 (6)	a	66.7 (5)	a

G-tests for crosses with offspring: $G = 6.971$, d.f. = 4, n.s. *G*-tests for percentage of mated pairs: $G = 3.532$, d.f. = 4, n.s. *G*-tests for percentage of mated pairs with offspring: $G = 4.782$, d.f. = 4, n.s. ANOVA for lifetime fecundity: $F = 2.806$, d.f. = 4, $P = 0.028$. *G*-test for percentage of eggs hatched: $G = 0.715$, d.f. = 4, n.s.

G-tests for crosses with offspring: $G_4 = 10.936$, $P = 0.0273$ over all crosses; $G_4 = 2.620$, n.s. over all crosses indicated with a; $G_4 = 8.301$, n.s. over all crosses indicated with b. *G*-tests for percentage of mated pairs: $G_4 = 1.289$, n.s. *G*-tests for percentage of mated pairs with offspring: $G_4 = 12.377$, $P = 0.014$ over all crosses; $G_4 = 4.575$, n.s. over all crosses indicated with a; $G_4 = 7.361$, n.s. over all crosses indicated with b. ANOVA for lifetime fecundity: $F_4 = 1.025$, n.s. *G*-test for percentage of eggs hatched: $G_4 = 8.425$, n.s.

*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.

autosome 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_1Z_2 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 F_1 and BC hybrid females, in which the WZ_1Z_2 trivalents were frequently not well synapsed, and Z_2 seemed shortened to adjust for synapsis (Fig. 1D, E; the W chromosome is the evenly dark chromosome).

In all F_1 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 F_1 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 F_1(pc) and BC hybrid females (Fig. 1F, H). Occasionally, dissimilar chromomere patterns in bivalents in F_1 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 F_1 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
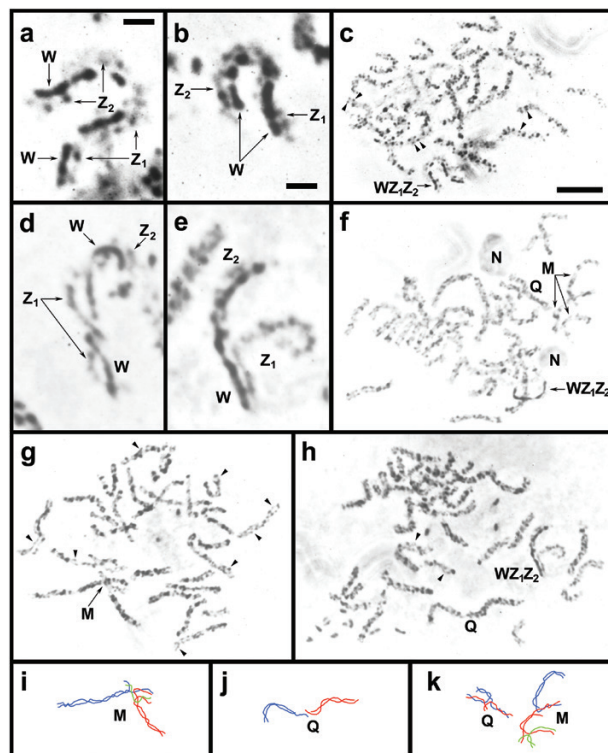

Figure 1. Phase-contrast micrographs of light microscopic preparations of pachytene oocytes in *Yponomeuta cagnagella*, *Yponomeuta padella* and their F_1 and backcrosses stained with lactic acetic Orcein. Abbreviations: W, female-specific sex chromosome (heterochromatic); Z_1 (long) and Z_2 (short) sex chromosomes showing chromomere pattern. Scale bars: 2 μm (A, B, D, E) or 10 μm (C, F, G, H). A, detail of the WZ_1Z_2 trivalent from a *Y. cagnagella* female. B, detail of the WZ_1Z_2 trivalent from a *Y. padella* female. C, pachytene nucleus from a *Y. cagnagella* female; arrowheads indicate examples of pairs of homologous chromomeres in autosome bivalents. D, detail of the WZ_1Z_2 trivalent from an F_1 hybrid (*Y. padella* × *Y. cagnagella*) female; the trivalent is not synapsed and shows only partial pairing of the W chromosome from the *Y. padella* mother with the two paternal *Y. cagnagella* Z chromosomes. E, detail of the WZ_1Z_2 trivalent in a backcross (BC) hybrid *Y. cagnagella* × F_1(*Y. padella* × *Y. cagnagella*) female; the Z_2 chromosome is well paired with the W, and the Z_1 shows only partial pairing at one distal segment. F, pachytene complement in a BC hybrid *Y. cagnagella* × F_1(*Y. padella* × *Y. cagnagella*) female showing a quadrivalent (Q) and a multivalent (M) composed of six chromosomes; see panel K for schematic interpretation of Q and M. Abbreviation: N, nucleous. G, pachytene complement in a BC hybrid *Y. cagnagella* × F_1(*Y. padella* × *Y. cagnagella*) female showing one multivalent (M) composed of five chromosomes as illustrated in panel I and a number of pairing irregularities (arrowheads), such as loops and weakly paired segments. H, pachytene complement in a BC hybrid *Y. cagnagella* × F_1(*Y. padella* × *Y. cagnagella*) female showing the unusual
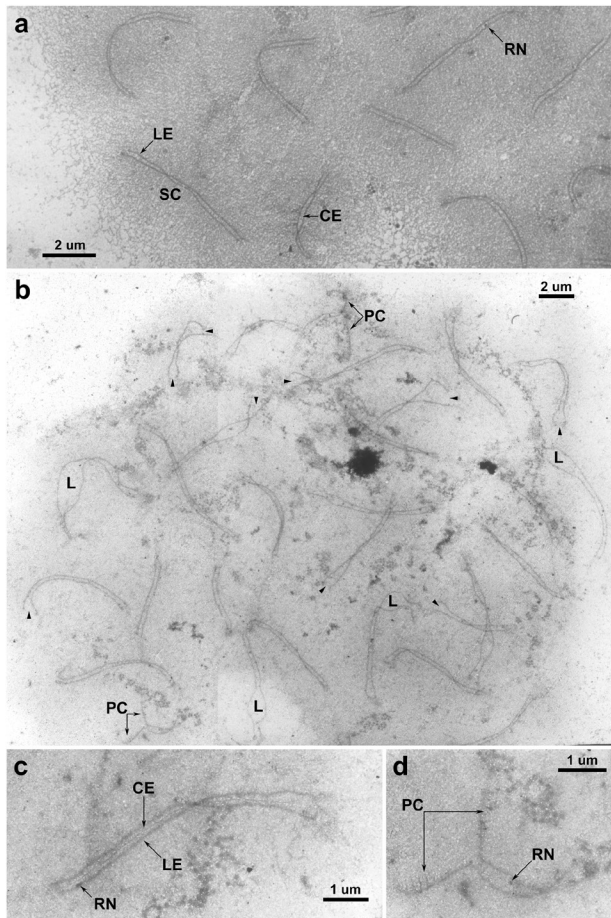
Figure 2. Electron microscopic microspread preparations of pachytene spermatocytes of *Yponomeuta cagnagella*, *Yponomeuta padella* and F$_1$ hybrid males. Abbreviations: CE, central element of the SC; LE, lateral element of the SC; PC, polycomplex; RN, recombination nodule; SC, synaptonemal complex. A, part of pachytene nucleus from *Y. padella* male showing well-formed SCs (even CEs are seen); note a bivalent with a readily visible RN located between two LEs. B, late zygotene nucleus from F$_1$ hybrid (*Y. padella* × *Y. cagnagella*) male showing, besides regular SCs, many pairing abnormalities, such as loops (L), indicating inversions, lack of sypapsis at distal ends of the SC (arrows), and PCs associated with LEs. C, detail of one well-formed SC from the above nucleus showing a clear CE (one distal end is not fully synapsed, probably owing to a nucleolar organizer region 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.

sex chromosome trivalent (see detail in panel E), possibly a quadrivalent (Q), indicating a translocation between two autosomes, as illustrated in panel J, and at least two small loops (arrows), indicating inversions and/or deletions.
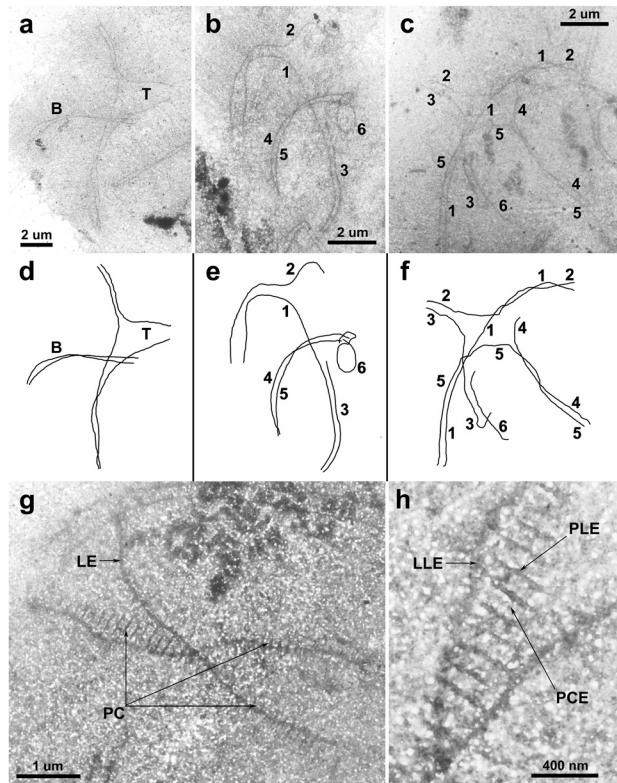

Figure 3. Electron microscopic microspread preparations of pachytene spermatocytes from backcross (BC) hybrid males *Y. cagnagella* × F$_1$(*Y. padella* × *Y. cagnagella*). A, trivalent (T) supposedly associated with a bivalent (B); see panel D for schematic interpretation. B, multivalent configuration 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.

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 of the same individual (Fig. 3A–C). In some nuclei, one or two bivalents were not synapsed, and their lateral elements were decorated with PCs (Fig. 3D, E).

STERILITY OF F$_1$ HYBRIDS (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 F_1 hybrids ($G_3 = 1.40$, $P = 0.70$ for crosses with *Y. cagnagella*, and $G_4 = 6.29$, $P = 0.178$ for crosses with *Y. padella*; Table 1A, B). There was also no difference between the reciprocal F_1 hybrid crosses ($G_1 = 0.60$, 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% ($G_4 = 1.61$, n.s.). For F_1 hybrids crossed with *Y. padella*, only one of the four hybrid crosses, *Y. padella* × F_1(pc), differed from the intraspecific cross (57.7 vs. 91.7%, $G_4 = 9.738$, $P = 0.045$; Table 1B); crosses with F_1(pc) as a parent consistently showed a lower percentage of pairs that produced offspring than those involving the reciprocal F_1(cp) hybrid (Table 1A–C).

Lifetime fecundity

With *Y. padella* as the backcross species, significant differences in lifetime fecundity were found ($F_4 = 4.45$, $P = 0.0026$). However, three out of four possible crosses with F_1 hybrids did not differ significantly from the intraspecific cross. The difference was caused by F_1(pc) × *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 ($F_3 = 3.26$, $P = 0.027$), and again only F_1(pc) × *Y. cagnagella* differed from the intraspecific cross ($F_4 = 3.257$, $P = 0.027$), 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 F_1 hybrids × *Y. cagnagella* crosses.

When comparing all crosses in which F_1 hybrids were involved, the F_1(pc) females in backcrosses with *Y. cagnagella*, *Y. padella* and F_1(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 F_1(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 F_1 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* × 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 × BC(pc×c), 88%; BC(c×pc) × c, 75.6%; and BC(pc×c) × 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 × (p×pc), p × (pc×p) and (p×pc) × 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 involving BC(p×pc) females and BC(pc×p) 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(pc×p) × *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) × *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(pc×p) × *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(pc×p) females were low, reducing the statistical power of the tests.

DISCUSSION

Chromosomal rearrangements and gene incompatibilities are the two evolutionary mechanisms

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 synapsis 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 *Y. cagnagella*. A very limited number of crosses [*Y. padella* × F_1(pc), BC(c×pc) × *Y. cagnagella* and BC(pc×p) × *Y. padella*] showed negative effects.

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; Raijmann *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_1. 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_1 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_2 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 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, 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 MIGITATE 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 suggests 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 regions 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; Bendall *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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