

Genetics of host plant use and life history in the comma butterfly across Europe: varying modes of inheritance as a potential reproductive barrier

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Abstract

Comma butterflies (Nymphalidae: *Polygonia c-album* L.) from one Belgian site and three Spanish sites were crossed with butterflies from a Swedish population in order to investigate inheritance of female host plant choice, egg mass and larval growth rate. We found three different modes of inheritance for the three investigated traits. In line with earlier results from crosses between Swedish and English populations, the results regarding female oviposition preference (choice between *Urtica dioica* and *Salix caprea*) showed X-linked inheritance to be of importance for the variation between Sweden and the other sites. Egg mass and growth rate did not show any sex-linked inheritance. Egg mass differences between populations seem to be controlled mainly by additive autosomal genes, as hybrids showed intermediate values. The growth rates of both hybrid types following reciprocal crossings were similar to each other but consistently higher than for the two source populations, suggesting a nonadditive mode of inheritance which is not sex-linked. The different modes of inheritance for host plant preference vs. important life history traits are likely to result in hybrids with unfit combinations of traits. This type of potential reproductive barrier based on multiple ecologically important traits deserves more attention, as it should be a common situation for instance in the early stages of population divergence in host plant usage, facilitating ecological speciation.

Introduction

In recent years there has been a strong increase in the interest in the possibility of ecological speciation, i.e. that differences in ecology between populations of the same species may be a driving force in incipient speciation (Schluter, 2001; Rundle & Nosil, 2005). The distinction between ecological and nonecological speciation (the latter due entirely to chance events such as mutation and drift, or to differences in nonecological traits) cuts across the classic geographical categories of speciation (Rundle & Nosil, 2005). Still, it has to a large degree received

attention because of its clear potential to initiate or at least complete speciation despite the presence of gene flow between the populations, i.e. in sympatry or parapatry (Via, 2001).

The study of evolutionary processes determining insect host plant preference is thus of great interest not only because of its many applications in fields such as pest management and conservation biology, but also because of its theoretical application in areas such as speciation theory and the origin of biodiversity. Already Darwin (1859) discussed intraspecific variation in food use as a prephase in speciation processes. Ever since, biologists have often used host-driven evolution as a foundation for discussions regarding speciation. Sympatric speciation is frequently modelled using the example of a stage involving a host plant shift and also discussions of parapatric and allopatric speciation processes often assume

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divergent selection pressures caused by different optimal (or available) food plants in different areas. For an overview of these discussions see e.g. Berlocher (1998).

For herbivorous insects, trade-offs between host plants are often assumed to be present; that is, different host plants require different specific adaptations, regarding for instance detoxification capacity (Agrawal, 2000). Specific adaptations to the host plant may also involve morphology, behaviour, and life history adaptations to host plant characteristics such as phenology (Bernays & Chapman, 1994). The selection regimes with respect to host plant adaptations will thus often differ between areas, for several reasons. An herbivorous species may have a total distribution which is larger than its major host plant, so that alternative hosts must be used in some areas. Use of alternative host plants can also be selected for if the host plant shows geographical variation between areas (Singer, 2002), or if a host plant is better relative to other host plants in one area but not in another, for instance because of climatic variation affecting the phenological timing of the host plant with the herbivore (Scriber, 2002a). Such geographical differences in selection regimes can be the necessary prerequisite for divergent selection, causing geographic variation and may also facilitate allopatric and parapatric speciation, as well as two-step speciation processes which are initiated in allopatry but completed in sympatry (Rundle & Nosil, 2005).

In particular, for a widely distributed species distributed over a great range of latitudes, the biotic and abiotic environment will change in many ways from high to low latitudes. These multiple differences in selective regimes may set the scene for genetic differentiation and, in some cases, for speciation. It is for this reason of great interest to extend the geographical range of genetic studies for insect-plant model systems which have already been intensively studied locally. One such model system is the polyphagous comma butterfly, *Polygonia c-album* L., and its relatives in the tribe Nymphalini of the family Nymphalidae. This system has been the focus of a series of investigations on the evolution of host plant range (e.g. Nylin, 1988; Janz *et al.*, 1994, 2001; Nylin *et al.*, 1996, 2005; Wedell *et al.*, 1997; Janz, 1998). Moreover, a role of host plants in the speciation of butterflies and other phytophagous insects has recently been implicated by evidence for higher net speciation rates in clades with wider host range, compared with sister clades, in the family Nymphalidae as a whole (Janz *et al.*, 2006) and in the genus *Polygonia* (Weingartner *et al.*, 2006).

In butterflies and moths, a disproportionately high number of traits that differ between sister species seem to be located on the X-chromosome, relative to the autosomes, than expected by chance alone (Sperling, 1994; Prowell, 1998). As in Lepidoptera females are the heterogametic sex (XY or ZW), the X-chromosome of females are inherited from the father. X-linkage can thus be demonstrated by female hybrids having trait values reflecting those of the paternal source population, in

both reciprocal crosses. The bias, approximately one-third instead of the expected 1 of 30 based on the number of autosomes, suggests that the X-chromosomes are somehow important in Lepidoptera speciation processes. Sperling (1994) further noted that in comparisons between more distantly related species this bias towards X-linkage disappears.

Female host plant preference is one of the traits that have been found to be strongly affected by the X-chromosomes when crossing closely related taxa of *Papilio* butterflies (Thompson, 1988; Scriber & Lederhouse, 1992). Jaenike (1989), commenting on Thompson's results, noted that studies on the genetics of preference differences *within* species would be of great interest in this context, and eventually an experiment crossing *P. c-album* populations from Sweden and England showed a within-species pattern where genetic control of differences in female host-plant preference between populations is also located on the X-chromosomes (Janz, 1998). In contrast, local genetic variation for host plant preference in *P. c-album* in the population around Stockholm (Sweden) was not found to be X-linked, but determined by one or a few major autosomal genes, possibly with some effect of the Y-chromosome (Nylin *et al.*, 2005, cf. Fig. 7).

It is now of interest to extend these investigations to a larger geographical area, taking into account more of the variation in host plant preference across Western Europe (Fig. 1). Is it a recurring pattern that large differences between populations of *P. c-album* are determined by sex-linked genes? The English population of *P. c-album* is known to have expanded from a small source population in recent years (Asher *et al.*, 2001) and may not be typical in its genetic composition. In addition, as mentioned above, it is of interest to determine the modes of inheritance for differences between populations of *P. c-album* regarding traits other than female host plant preference. Larval growth rate and egg mass are good candidates for being involved in co-adapted gene complexes with host plant preference, as host plants provide different larval environments which are likely to have different optimal growth rates (Wedell *et al.*, 1997) and a large egg mass, resulting in larger hatchlings, may be more important for hatchling success on some hosts than on others (Braby, 1994).

In herbivores with a parasitic larval lifestyle, such as butterflies, the growing larva often completes its whole development on a single host plant species; hence, a set of genes functioning well together on one host could be an expected evolutionary outcome under some circumstances (Janz, 2003). Such co-adapted gene assemblages could, in turn, facilitate speciation as hybrids between the genetic forms would often have genetic combinations which are less fit on any host and females may even show a preference for a host to which other parts of the genome are not well adapted (Forister, 2005). To determine the genetic structure behind geographical variation in host

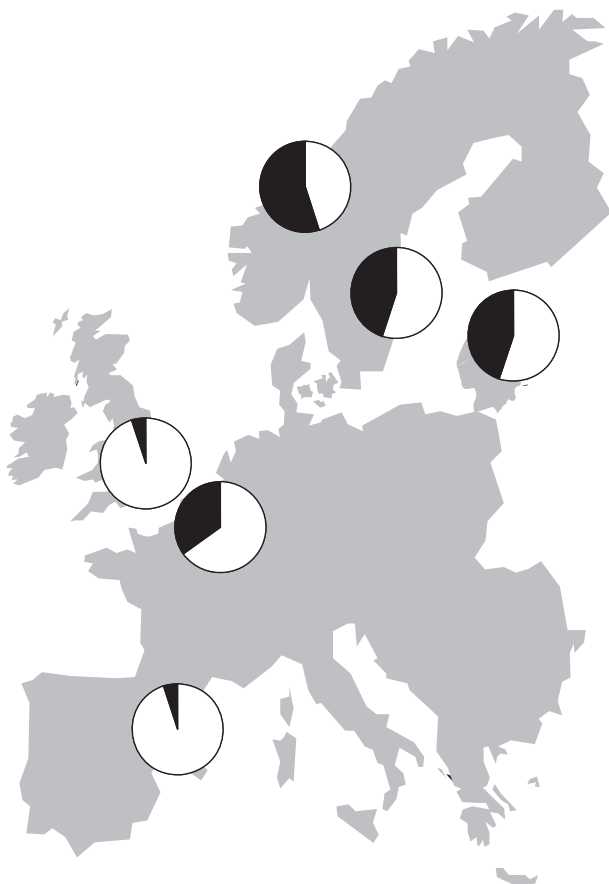


Fig. 1 A schematic illustration of female host plant preferences in the spring generation of *Polygonia c-album*, across Europe. Black portion of pie charts correspond approximately to the percentage of eggs that a female would lay on *Salix caprea* when given a simultaneous choice of *Urtica dioica* or another urticalean rosid plant (white portion). Females in Sweden, Estonia, Norway and Belgium are less specialized on urticalean rosids than females from England and Spain. Based on Nylin, 1988; Janz *et al.*, 1994, Janz 1998; R. Krogen, N. Janz, G. H. Nygren, S. Nylin & C. Stefanescu, unpublished.

plant preference, growth rate and egg mass, we crossed populations of *P. c-album* from Sweden with populations from Belgium and Spain (several populations from different altitudes in Catalonia) and investigated hybrids together with pure stock. We show that the modes of inheritance vary among all three investigated traits and discuss the probable implications for hybrid fitness, reproductive barriers and models of ecological speciation.

Methods

Study organism and populations studied

Polygonia c-album is a widespread butterfly species of the family Nymphalidae. The butterfly is found all over

Eurasia, from England to Japan and from northern Sweden to North Africa. Hibernation diapause is in the adult stage and mating and oviposition takes place in the spring, and in southern populations again in two or three subsequent generations. The larvae of the most polyphagous populations, such as the Swedish one, routinely utilize host plants from several taxa: the 'urticalean rosids' *Urtica*, *Humulus* and *Ulmus* and the distantly related *Salix* (Salicaceae), *Ribes* (Grossulariaceae), *Betula* and *Corylus* (Betulaceae) (Nylin, 1988); hence the species has a wide host range for a butterfly, although it is by no means an indiscriminate generalist.

We sampled *P. c-album* populations from three distinct latitudes (countries): Sweden, Belgium and Spain (cf. Fig. 1). The Spanish stock came from four different altitudes in Catalonia. The first laboratory-reared generation of adults from Belgium and Spain were crossed with Swedish stock to investigate the inheritance of oviposition preference (choice between *Urtica dioica* and *Salix caprea*), egg mass and larval growth rate. The latitudinal samples were from widely separated areas and direct gene-flow between them can thus be assumed to be very low:

Sweden (vicinity of Stockholm) 59°N. This is a univoltine population (one generation per year) known to have a very wide host plant range, with spring generation females having only a weak average preference for *U. dioica* over *S. caprea* in choice tests (Fig. 1; Nylin, 1988; Janz *et al.*, 1994). Second-generation females can be produced by manipulating the photoperiod (Nylin, 1992 and see below) and such females have a stronger preference for *U. dioica* (e.g. Nylin *et al.*, 2005). Eight females were collected in the wild in 2002 and their offspring used for crossings with Spanish populations. Five females were collected in 2003 and their offspring used for crossings with the Belgian stock.

Belgium (vicinity of Antwerp) 51°N. This is a partially bivoltine population with intermediate host plant range, with females showing only slightly higher preference for *U. dioica* than Swedish females (G. H. Nygren, S. Nylin, C. Stefanescu & R. Krogen, unpublished). Three females of this population were collected in the wild in 2003.

Spain (Catalonia) 42°N, sampled in 2002. All the sampled Spanish populations have partial second or third generations (C. Stefanescu, personal observation). The populations have been found to be highly specialized on *U. dioica* in choice tests with *S. caprea* (G. H. Nygren, S. Nylin, C. Stefanescu & R. Krogen, unpublished). In the field they have also been observed to frequently utilize other urticalean rosid plants such as *Humulus lupulus* and *Ulmus* spp. (C. Stefanescu, personal observation). The samples came from the altitudes of:

- 1 1000 m a.s.l. (El Puig; five females collected)
- 2 200–320 m a.s.l. (Can Liro; five females)
- 3 350 m a.s.l. (Sallent; one female)

4 Sea level (El Cortalet; three females). Only the pure-stock first laboratory generation was investigated for this population, but some results are included here for completeness of preference and life history data from Catalonia

Overview of procedures

Adult butterflies, reared from eggs of the wild-caught females collected in the laboratory, were placed in flight cages of approximately 1 m³ in size. Provisions were made, when allocating adults to cages, to attempt to obtain mated females from each of four categories: the two pure stocks and two categories of hybrids representing the reciprocal crosses with the male parent (and thus the X-chromosome of offspring females) originating either from Sweden or from the other studied population. Individuals were marked so that the category of mating could be assessed and each individual was mated only once, producing the families of offspring that were used as data points for growth rate data to investigate differences between the categories. Pairs remain *in copula* for several hours, so mating pairs can easily be isolated and the individuals and stock involved in the mating noted. Variation among categories in host plant preference and egg mass was also studied in this F1 generation (first generation of offspring where hybrids were present), using females as data points.

Butterflies from El Cortalet arrived late in the season to the laboratory and no crossing experiment could be made with this population, but we include some results from a female preference study and egg masses taken from the first adult generation – which was raised in a common garden experiment together with the F1 generation of the remaining Spain–Sweden samples in 2002.

Rearings and measurements

The crossings and rearings were performed with Swedish stock originating from females collected in the same years, as described above (Spain–Sweden 2002, Belgium–Sweden 2003). A common-garden design was used where all categories of larvae of the F1 generation were reared side-by-side. Larvae were individually reared in plastic jars with fresh supplies of *U. dioica*; pupal mass and development time were noted for each individual to provide a measurement of larval growth rates. As life history traits are known to differ strongly between the two developmental pathways (direct development or development to hibernation diapause; Nylin, 1992) two treatments were used to measure larval growth rates. All larvae were initially (first 2 weeks) reared at 17 °C at 12 h light/12 h dark. After this period half of the larvae were moved to another room with 22 °C, 22 h light/2 h dark, a treatment which has previously successfully been used to produce the directly developing morph (dd treatment in the following) in the Swedish population

(Nylin, 1992). This generation has light-coloured wing undersides. Butterflies remaining in short-day conditions until eclosion developed into the easily distinguished much more dark-coloured overwintering morph (Dark treatment in the following). All food plants were checked daily and replaced if wilted or consumed.

Two days after pupation the pupal mass was measured, using a kern 410 electro balance. After eclosion, males and females were individually marked and held together for mating as described above, to produce mated F1 females for preference tests and egg mass determination.

Mated females were placed in cages (0.5 m³) with a sugar-solution soaked sponge for feeding in the middle and two equally sized and randomly chosen host plants (*U. dioica* and *S. caprea*) presented at a random position in the cage. Allocation of plant pairs to cages were decided by rolling of a dice and flipping of a coin thereafter decided the orientation (left or right) for the plant pair inside the cages. Flight cages were illuminated by a 75 W light bulb, hanging at a distance of approximately 3 dm above the transparent upper part of the cage. Mated female butterflies were arbitrarily placed in the cages; hence a mixture of categories was present in the lab simultaneously. After 8 h of light the two host plants were removed and eggs counted. If ten or more egg had been laid, the preferences shown on this day was included in the calculation of average preference assessments for the individual female.

The eggs from one female at a time were then poured out on a white paper and ten eggs from the centre of the paper were arbitrarily chosen and weighed together (on a Cahn C-30 Microbalance; Cahn Instruments, Cerritos, CA, USA) to obtain a good estimate of average egg mass on this day of egg-laying. Several such estimates were obtained per female and the egg masses reported are averages of such averages.

The Belgian larval life history study was performed in July and the preference study in August 2003. The Spanish experiment was performed later in the season, the larval life history study was initiated in the middle of August and ended in the middle of September; the preference study started in the middle of September and ended in the middle of October 2002.

The high levels of preference for *U. dioica* throughout this study (even in the case of Swedish females, cf. Fig. 1) are a result of the fact that the F1 generation, where hybrid females were present, could be tested only late in the season. As mentioned above, preferences of *P. c-album* butterflies changes dramatically through the year, with increasing specialization on *U. dioica* (Nylin *et al.*, 2005). To compensate for this trend in the even later Spanish–Swedish experiment, we positioned the *U. dioica* approximately 1 dm lower and further from the light source than the *S. caprea* in the cages, to achieve a higher encounter rate with *S. caprea*. This procedure was necessary to avoid a situation where all eggs were laid on *U. dioica* (so that no measurable preference variation would have been present). As all females in this experi-

ment was treated the same, results from comparisons between populations are still clear, but absolute preferences should not be directly compared among different experiments.

Results

Female egg-laying preference

The variance of the female preferences (averages over the oviposition period for each female) showed highly different values between the individuals (Levene's test for homogeneity of variances: Belgium–Sweden $n = 46$, $P < 0.01$; Spain–Sweden $n = 58$, $P \ll 0.001$); hence statistical analyses were made with nonparametric tests (STATISTICA '99 edition). We used Mann–Whitney U -tests for two-way comparisons and Kruskal–Wallis ANOVA for one test with more than two categories.

The host plant preferences of summer-generation Belgian females were nearly the same as for the Swedish population (Fig. 2a; average of averages for pure-stock Swedish females 94.4% eggs on *U. dioica* and for Belgian 97.3%). Despite these similarities, crossing the populations revealed some significant genetic patterns. The pure-stock females from Belgium and Sweden did not differ significantly in host plant preference (Mann–Whitney U -test, $n = 19$, $U = 40.0$, $P = 0.68$). The biggest difference was instead found between the two categories of hybrids, which is of interest because they differ from each other both in the X- and the Y-chromosomes (Mann–Whitney U -test, $n = 32$, $U = 55.5$, $P < 0.01$). Females of the hybrid category with a Swedish female parent and a Belgian male parent (Sw + Be in Fig. 2a) very strongly preferred *U. dioica* and rejected *S. caprea*. In this high degree of specialization they showed a significant difference from the pure Belgian stock (with which it shares X-chromosome origin but not Y-chromosome; Mann–Whitney U -test, $n = 24$, $U = 34.5$, $P < 0.05$) and a tendency to differ also from the pure Swedish stock (from which it differs in X-chromosome but not in Y-chromosome origin; Mann–Whitney U -test, $n = 25$, $U = 43.0$, $P = 0.076$). The other hybrid category was less specialized and showed no significant differences to the pure populations (Mann–Whitney U -test, Sw + Sw vs. Be + Sw, $n = 27$, $U = 79.0$, $P = 0.76$ and Be + Sw vs. Be + Be, $n = 26$, $U = 64.5$, $P = 0.52$).

Females from the four Spanish areas were highly specialized and showed no significant differences in preference among each other (Fig. 2b; Kruskal–Wallis ANOVA, $n = 14$ females, $H_3 = 3.67$, $P = 0.30$: El Puig 100%, Sallent 100%, Can Liro 99.5% and El Cortalet 100% eggs on *U. dioica*), hence, they were pooled in the preference study (Fig. 2c). Despite the higher position of the *S. caprea* in the cages employed in this experiment (see Methods) females of the Spanish populations laid almost all their eggs on *U. dioica*.

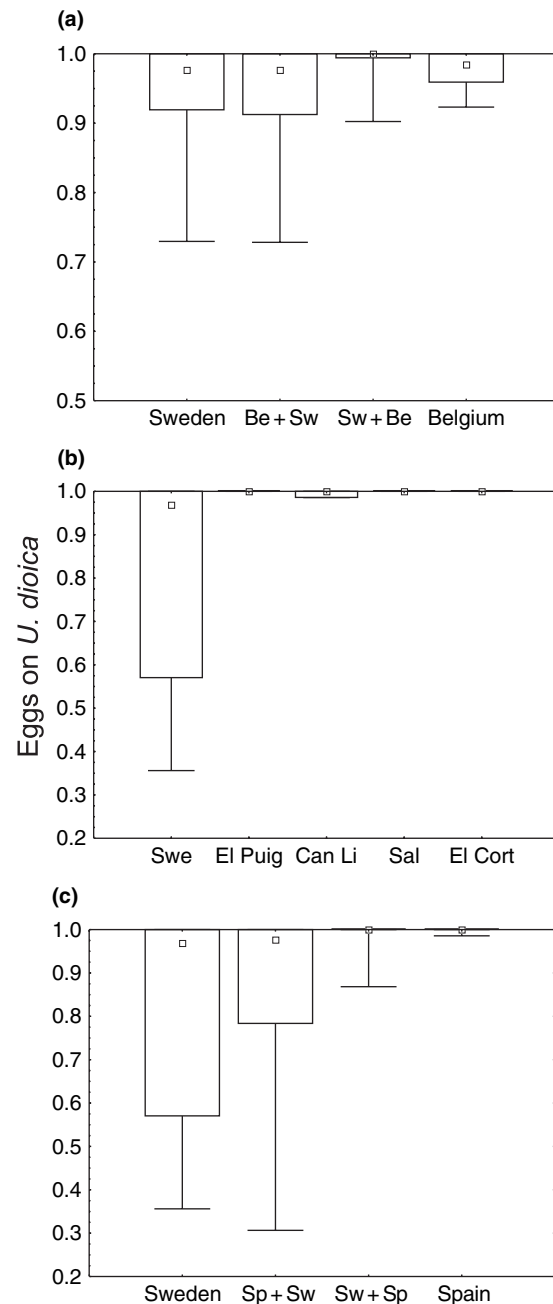


Fig. 2 Host plant preferences (proportion of eggs laid on *Urtica dioica*) for summer-generation females of *Polygonia c-album* in a choice test between *U. dioica* and *Salix caprea*. (a) Belgium–Sweden crossing experiment, (b) the Swedish population and stock from four different areas in Spain and (c) Spain–Sweden crossing experiment (with the Spanish areas pooled). Hybrid groups in (a) and (c) are named after origin of female parent followed by origin of male parent (mother + father), thus hybrid groups are presented side by side to the source population according to origin of the X-chromosomes. Figures show medians, 25–75% percentiles (boxes) and total range (whiskers).

The Spanish pure stock differed significantly from the Swedish, although the experiment was performed with the more *Urtica*-specialized summer-generation females (Mann–Whitney *U*-test, $n = 21$, $U = 16.0$, $P \leq 0.05$). The Swedish butterflies were clearly affected by the higher position of the *U. dioica* in the cages and laid on average 16.9% of the eggs on *S. caprea*, an unusually high proportion at this late time of the year. Unlike the Belgium–Sweden crossing, the two Spanish–Swedish hybrid categories did not show larger differences to each other than found between the pure stock and showed only a tendency to differ (Mann–Whitney *U*-test, $n = 37$, $U = 109.5$, $P = 0.062$). Comparisons showing the effect of the X-chromosome on preference (i.e. with male parent from different populations, female parent from the same population) were significant in both cases (Mann–Whitney *U*-test, Sw + Sw vs. Sw + Sp, $n = 26$, $U = 32.0$, $P < 0.05$ and Sp + Sw vs. Sp + Sp, $n = 32$, $U = 67.5$, $P < 0.05$) but no effect of the Y-chromosomes could be found in any of the comparisons where the origin of female parent instead differed (Mann–Whitney *U*-test, Sw + Sw vs. Sp + Sw, $n = 25$, $U = 56.0$, $P = 0.67$ and Sw + Sp vs. Sp + Sp, $n = 33$, $U = 112.5$, $P = 0.46$).

To further evaluate these patterns, we also performed a nonorthodox statistical analysis where we pooled the females according to origin of the sex-chromosomes. Thus the effect of male parent (and hence origin of the X-chromosome in F1 females) was studied by grouping all F1 females with Swedish male parents (pure or hybrid stock) and testing them against the group with non-Swedish male parents and vice versa for female parents (and origin of Y-chromosomes in F1 females). The statistics should be taken with some caution, but the results may serve to illustrate the differences between the two crossing experiments. By testing for sex-chromosomal effects in this manner both of the sex-chromosomes had significant effects in the Belgian experiment (Mann–Whitney *U*-test, male parent Sw + Sw and Be + Sw against Sw + Be and Be + Be, $n = 51$, $U = 203.0$, $P < 0.05$; female parent Sw + Sw and Sw + Be against Be + Sw and Be + Be, $n = 51$, $U = 219.0$, $P < 0.05$) but only the effect of male parent (X-chromosome) was significant in the Spanish experiment (Mann–Whitney *U*-test, male parent Sw + Sw and Sp + Sw against Sw + Sp and Sp + Sp, $n = 58$, $U = 225.0$, $P < 0.01$; female parent Sw + Sw and Sw + Sp against Sp + Sw and Sp + Sp, $n = 58$, $U = 415.0$, $P = 0.99$).

Egg mass

The variances in egg mass showed no significant differences, in all the experiments together or in analyses of each of the crossings separately (Levene's test for homogeneity of variance: all classes $P = 0.61$, the crossings separately $P = 0.79$ – 0.12). Hence, parametric tests were

used (GLM ANOVAS and for *post hoc* tests of specific differences between two categories we used 'Unequal N HSD', a modification of Tukey's HSD as implemented in STATISTICA).

Egg mass of the Swedish population differed between the 2 years (compare Fig. 3a with Fig. 3b–d; Sweden-02 on average 320 μg , Sweden-03 348 μg ; GLM ANOVA on averages of egg mass for females, $n = 16$ females, $F = 7.74$, $P < 0.05$). As noted in Methods the two experiments were not performed at the same time in the summer and the difference was thus probably caused by a degeneration of host plant quality throughout the season, affecting female adult mass and eventually egg mass. Thus, egg mass should only be compared within the same common-garden experiment, where differences seen between categories are more likely to have a genetic basis.

Egg mass differed between the categories of F1 females (GLM ANOVA, $n = 43$, $F_{3,39} = 16.67$, $P < 0.001$). Belgian females laid on average lighter eggs than the Swedish females (Fig. 3a; 291 vs. 346 μg ; Unequal N HSD, $n = 18$ females, $P < 0.001$), whereas hybrids were intermediate. The pure Swedish stock also laid significantly heavier eggs than any of the hybrid categories (Fig. 3a; comparison with Be + Sw $n = 23$ females, $P < 0.01$; with Sw + Be $n = 20$ females, $P < 0.001$). The only indication of sex-linkage was that the Belgian pure stock differed from the hybrid category with a Swedish male parent (Be + Sw, $n = 23$ females, $P < 0.05$) but not from the other hybrid category (Sw + Be, $n = 20$ females, $P = 0.54$). However, the hybrid categories did not differ significantly from each other ($n = 25$ females, $P = 0.24$). Similarly, in a model analysing the effect of parental origin for each female, the effects of female parent and male parent were both significant, but the female \times male parent interaction was not (GLM ANOVA on average of female egg mass; $n = 43$, $F = 13.77$, $P_{(\text{female parent})} < 0.001$; $F = 40.12$, $P_{(\text{male parent})} < 0.001$; $F = 2.78$, $P_{(\text{female} \times \text{male parent})} = 0.10$). Hence, egg mass differences between Sweden and Belgium are likely to be inherited in a mostly additive fashion.

Egg masses of the butterflies from the four Spanish areas were overall lower than for Swedish eggs (Fig. 3b–d; GLM ANOVA with Spanish areas pooled, $n = 45$, $F = 8.10$, $P < 0.01$): El Puig on average 302 μg , Can Liro 312 μg , Sallent 261 μg and El Cortalet 289 μg . The significant difference was mostly caused by low egg mass for the Spanish area Sallent (cf. Fig. 3b–d).

The crossings of the El Puig and Can Liro populations with the Swedish population (Fig. 3b,c) showed no significant differences in egg mass among categories (GLM ANOVA; Sweden-El Puig: $n = 22$, $F_{3,18} = 0.77$, $P = 0.52$; Sweden-Can Liro: $n = 28$, $F_{3,24} = 0.18$, $P = 0.91$). This is perhaps not surprising as the differences in egg mass between Sweden and these two areas were small and egg masses similar to the source populations are consistent with additive inheritance. However, the

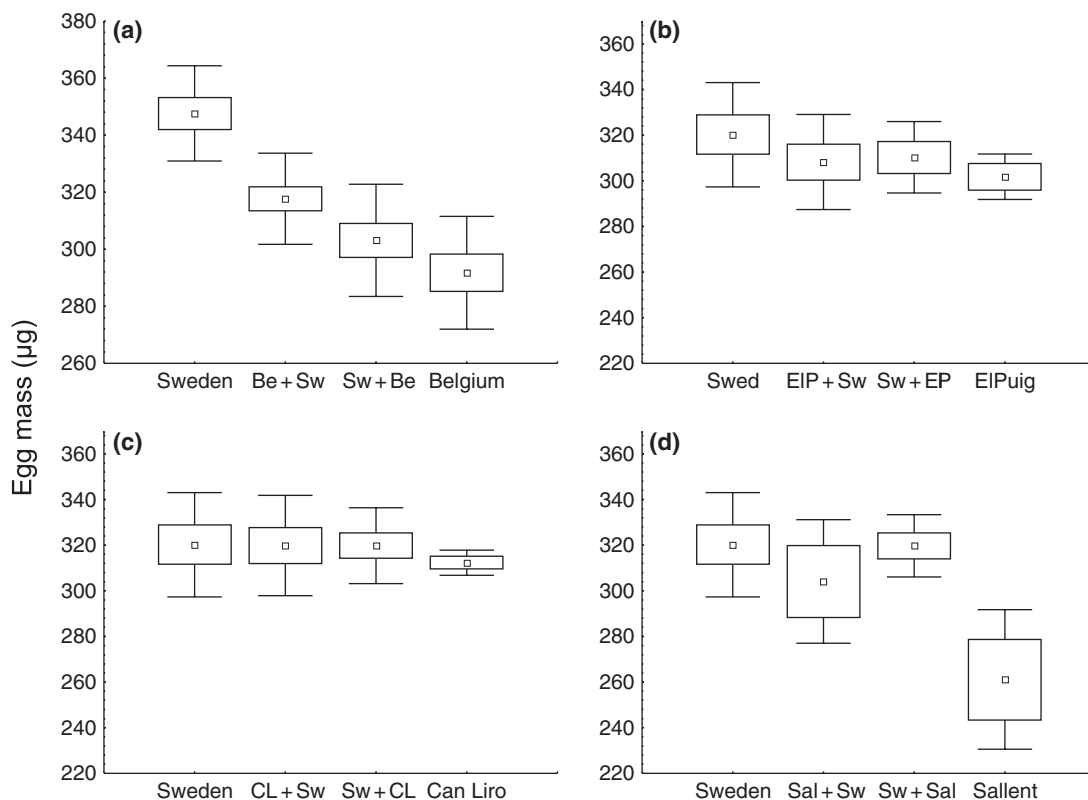


Fig. 3 Egg mass in experiments with crossings between different populations of *Polygona c-album*. (a) Belgium–Sweden crossing, (b) El Puig–Sweden crossing, (c) Can Liro–Sweden crossing and (d) Sallent–Sweden crossing. Hybrid groups are named after origin of female parent followed by origin of male parent (mother + father). Figures show means, standard errors and standard deviations.

crossing Sweden–Sallent differed (Fig. 3d) and showed significant variation among categories (GLM ANOVA, $n = 19$, $F_{3,15} = 5.73$, $P < 0.01$). In a model analysing the effect of parental origin for each female, female parent showed a significant effect, and both male parent and the female \times male parent interaction showed a tendency to affect egg masses (GLM ANOVA, $n = 19$, $F = 11.56$, $P_{(\text{female parent})} < 0.01$; $F = 3.91$, $P_{(\text{male parent})} = 0.067$; $F = 3.75$, $P_{(\text{female} \times \text{male parent})} = 0.072$). Hence the Sweden–Sallent crossing may have revealed some nonadditive genetic differences located on the sex-chromosomes (note that this stock originated from a single wild-caught female, however, so it may not be representative of the Sallent population *per se*). As noted in Methods, no crossing experiment could be made with the El Cortalet stock.

Growth rate

No differences in larval growth rate between the sexes could be found in any of the crossing experiments in any of the two treatments (GLM ANOVAS, $P = 1.00$ – 0.34). Hence, the sexes were pooled together. Levene's test for homogeneity of variance showed no significant differ-

ences in variance of growth rates between the categories in the direct development (dd) treatment. In contrast, all but one (Belgium) of the overwintering (Dark) treatments showed significant differences in variance between the categories, which remained after transformation (LN, LOG10, Square root, 1/X or EXP). For this reason we chose to perform statistical analyses of the crossing results only on growth rate results from the dd groups, except for the Belgium–Sweden Dark treatment experiment. However, the relative order of growth rates between the categories was on the whole the same in both the dd and the Dark treatments for all the crossings (Fig. 4).

The total sample size for growth rate measurements was 946 individually reared larvae. However, statistical analyses were performed at the level of average growth rate for families (offspring of a female).

Growth rates were much higher for the dd treatment (Fig. 4); this is mainly a result of the higher temperatures during late larval development in this treatment, but larvae destined for direct development also are more time-stressed and grow faster even in mixed broods at the same temperature (Nylin, 1992). Note from Fig. 4 the consistent pattern of similar growth rates when compar-

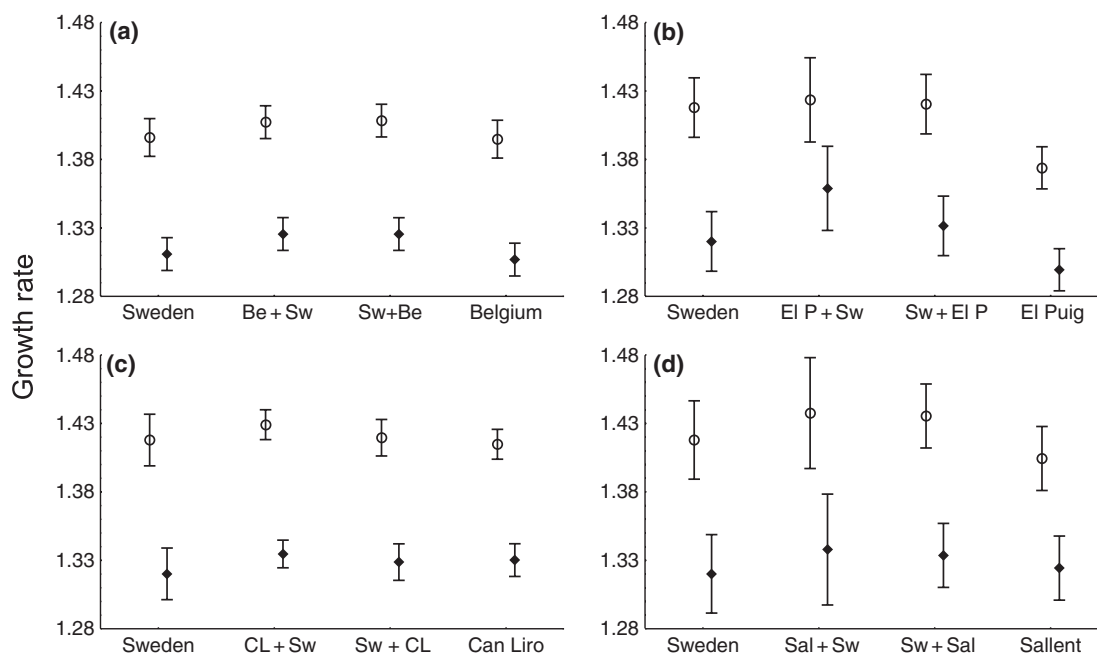


Fig. 4 Growth rate (LN mg day⁻¹) in experiments with crossings between different populations of *Polygonia c-album*. (a) Belgium–Sweden crossing, (b) El Puig–Sweden crossing, (c) Can Liro–Sweden crossing and (d) Sallent–Sweden crossing. Both the light morph treatments (open circles) and the dark morph treatments (filled squares) are presented in the graphs, with means and standard errors. Hybrid groups are named after origin of female parent followed by origin of male parent (mother + father).

ing pure stock in each crossing, but higher growth rates for both types of hybrids. In the statistical tests this pattern was clearly reflected by weak and nonsignificant effects of source populations for parents (as main factors) coupled with strong and often significant interactions between origin of female and male parents. In the Belgium vs. Sweden dd treatment (Fig. 4a), the female \times male parent interaction was borderline significant (GLM ANOVA, $n = 14$; $F = 0.0$, $P_{(\text{female parent})} = 0.84$; $F = 0.0$, $P_{(\text{male parent})} = 0.99$; $F = 5.0$, $P_{(\text{female} \times \text{male parent})} = 0.050$). A similar pattern was found in the Dark treatment (GLM ANOVA, $n = 16$; $F = 0.1$, $P_{(\text{female parent})} = 0.75$; $F = 0.1$, $P_{(\text{male parent})} = 0.75$; $F = 7.1$, $P_{(\text{female} \times \text{male parent})} < 0.05$). In a complete model of the Belgium–Sweden growth rate data, analysing both treatments simultaneously, the effect of treatment was significant and so was the interaction female \times male parent, but not the parental factors alone (GLM ANOVA, $n = 30$; $F = 444.1$, $P_{(\text{treatment})} < 0.001$; $F = 0.2$, $P_{(\text{female parent})} = 0.67$; $F = 0.1$, $P_{(\text{male parent})} = 0.78$; $F = 13.5$, $P_{(\text{female} \times \text{male parent})} < 0.01$).

The results were similar for the El Puig vs. Sweden crossing (Fig. 4b), where only the dd treatment was analysed (as explained above). This was the only growth rate data set with a tendency for significance for origin of parents as main factors, but the interaction was even stronger (GLM ANOVA, $n = 9$; $F = 4.4$, $P_{(\text{female parent})} = 0.091$; $F = 5.8$, $P_{(\text{male parent})} = 0.060$; $F = 7.1$, $P_{(\text{female} \times \text{male parent})} < 0.05$). The other Spain vs.

Sweden crossings showed similar but weaker and nonsignificant patterns (Fig. 4c,d). This was true for Can Liro vs. Sweden (GLM ANOVA, $n = 18$; $F = 0.2$, $P_{(\text{female parent})} = 0.66$; $F = 0.8$, $P_{(\text{male parent})} = 0.39$; $F = 1.2$, $P_{(\text{female} \times \text{male parent})} = 0.28$) and for Sallent vs. Sweden (GLM ANOVA, $n = 9$; $F = 0.1$, $P_{(\text{female parent})} = 0.74$; $F = 0.2$, $P_{(\text{male parent})} = 0.65$; $F = 2.5$, $P_{(\text{female} \times \text{male parent})} = 0.18$).

Discussion

The results of the crossings performed in this study suggest three different modes of inheritance for variation in the three investigated traits at the large geographical scale investigated.

Regarding female host plant preference, the present investigation between populations of *P. c-album* complements the results from the crossing experiment between the Swedish and English population performed by Janz (1998); see also Janz, 2003) and from a local genetic study of the Swedish population (Nylin *et al.*, 2005). The Sweden–England study demonstrated that the difference in female host plant preference between these populations is almost totally determined by genes located on the X-chromosome, whereas the variation within one Swedish population did not seem to be X-linked, but be caused by a few other major genes (of which one may be Y-linked; cf. Fig. 7 in Nylin *et al.*, 2005) and by plasticity.

In the present study crosses between the Swedish population, which is strongly polyphagous in the spring generation and the Spanish stock, which (like the English) is much more specialized on urticalean rosid plants (*Urtica*, *Humulus* and *Ulmus*) likewise show a strong X-chromosomal effect. Females of the Belgian populations are apparently only slightly more specialized than Swedish females, which constrains the possibilities of detecting the genetic background of the differences. This is particularly true in the summer generations studied here, where even Swedish females are relatively specialized. Importantly, however, the two hybrid categories from reciprocal crosses between the Belgian and Swedish populations differed significantly in preference, although the source populations did not and they did so in a manner consistent with an X-chromosomal effect. It seems, therefore, that this is the geographical scale at which the X-linked differences between Sweden and the other areas become detectable (cf. Fig. 1).

The total evidence would thus seem to suggest that one or more genes on the X-chromosome cause the larger differences in preference between the studied populations, whereas heritable preference variation at the more local scale is because of other genes. We believe that the larger geographical variation is connected to patterns of voltinism, with specialization on the urticalean rosids being selected for more strongly in the southern bi- or trivoltine populations. These preferred hosts support faster growth and at least in the case of the herbaceous *Urtica* and *Humulus* also deteriorate less in quality over the season than alternative hosts (Nylin, 1988; Janz *et al.*, 1994; G. H. Nygren, S. Nylin, C. Stefanescu & R. Krogen, unpublished). It now seems possible that the northern univoltine area (here represented by Stockholm, Sweden) is of one major X-chromosomal 'type' (i.e. individuals carry a characteristic fixed allele or alleles on the X-chromosome with major influence on preference), and Spain and England of another, with Belgium as an intermediate and genetically mixed population of great interest for future studies.

Our results from the Belgium vs. Sweden experiment suggest an additional influence on host plant preference from the Y-chromosome, which is consistent with the earlier results from the Stockholm population noted above (Nylin *et al.*, 2005). The Y-chromosome may thus carry a gene with weaker effects on female host plant preference than the X-linked gene, one that has its strongest influence within populations – against a background of X-linked genes that have been driven to fixation.

Butterfly species can show autosomal genetic migration between species but low levels of sex-chromosomal interchange (Scriber, 1994, 2002b). Similarly, an investigation of two closely related bird species showed fairly high migration of autosomal genes between the two species but no migration of sex-linked genes (Saetre *et al.*, 2003). If this is a common phenomenon, the

specific importance of genes carried on sex chromosomes would be implicated in the context of speciation processes (Bachtrog *et al.*, 2006). As noted in the Introduction, the bias towards genetic differences between sister species of butterflies and moths being carried on the X-chromosome (Sperling, 1994; Prowell, 1998) does suggest that (at least in the Lepidoptera) traits coded for by genes on sex chromosomes are more likely to be important for speciation than other traits. The reasons are unclear and several possibilities have been discussed (Janz, 2003; Nylin *et al.*, 2005). Here, we focus more on multi-trait combinations between such sex-linked traits and traits with other modes of inheritance.

Egg mass differences between the studied populations seem to be controlled mainly by additive autosomal genes. Interestingly, all continental multivoltine populations had a lower average egg mass than the Swedish univoltine population. It could be speculated that the higher egg mass of Swedish stock is related to the importance of tough-leaved bushes and trees as host plants in this population and the corresponding need for large hatchlings that can survive the establishment phase (cf. Braby, 1994). This is however contradicted by the difference in egg mass between Sweden and Belgium despite small differences in preference and by the importance of trees in *Ulmus* as hosts for the Spanish populations (C. Stefanescu, personal observation). The primary reason for this pattern should thus perhaps rather be sought in life history adaptations (Wiklund *et al.*, 1987; Garcia-Barros, 1994), although it remains likely that there are secondary effects of egg mass on early larval survival on tough-leaved hosts.

The elevated larval growth rates in both categories of hybrids in the crossing experiments suggest a nonadditive and autosomal genetic component affecting the growth rates. Interestingly, higher growth rate of both hybrid categories was found also in the Sweden–England *P. c-album* crossing experiment performed by Janz (2003). Such patterns do not suggest sex-linkage and the lack of difference between the sexes regarding growth rates of hybrid larvae support this impression.

Higher growth rate for F1 hybrids can be seen as an example of hybrid vigour (heterosis). This is a common phenomenon, but the underlying mechanisms are elusive (Syed & Chen, 2005). One classic explanation involves a reversal of inbreeding depression, through a masking of deleterious recessive alleles (that have accumulated in an isolated stock) by dominant alleles. *Polygonia c-album* is however a highly mobile species with an open population structure (Nylin *et al.*, 2005), which makes inbreeding depression an unlikely explanation.

Two other theoretical explanations for heterosis involve overdominance or epistasis. The first suggests that heterozygosity *per se* at a given locus promotes fitness, the second that heterosis results from epistatic interactions among alleles at different loci. Evidence for

overdominance has always been scant (Futuyma, 1998), whereas the epistasis theory is gaining momentum (Routman & Cheverud, 1997; Syed & Chen, 2005). Epistasis is also now seen as the major explanation for the reverse of heterosis, hybrid inferiority (Burke & Arnold, 2001). In this context it is worth pointing out that higher growth rates should not necessarily be seen as increasing fitness, as insect growth rates are generally not maximized but optimized to local conditions (Abrams *et al.*, 1996; Nylin & Gotthard, 1998). Whatever its causes, the diverging growth rates in hybrids of *P. c-album* demonstrate that although growth rates in the present study often are similar in the source populations, they actually have different genetic backgrounds (see also Carroll *et al.*, 2001) and this suggests that they, like host preference and egg mass, are the product of local adaptation.

Empirical studies and theory of ecological speciation in phytophagous insects have focused mainly on sympatric speciation via host races (Via, 2001; Berlocher & Feder, 2002; Coyne & Orr, 2004). Such modes of speciation are facilitated if there is a close linkage between genes for preference for and performance on, a given host plant so that host races can form more easily (Ortíz-Barrientos *et al.*, 2002; Coyne & Orr, 2004). However, another type of ecological speciation process, one which has received much less attention, may also be acting in for instance phytophagous insects (Forister, 2005). This process does not involve linked traits but rather the opposite: a break-up of co-adapted gene complexes by recombination and/or by differences in the mode of inheritance. This has historically most often been treated as an obstacle to ecological speciation, but if such adaptive trait combinations have already evolved in allopatry the very fact that they will be broken in hybrids – making them unfit – could instead aid speciation by creating a reproductive barrier that prevents further recombination. This would be an example of the two-stage process of ecological speciation envisaged by e.g. Rundle & Nosil (2005), who suggest that speciation in nature may usually involve both an allopatric and a sympatric or parapatric phase. Recent evidence suggests that this may have been the case even in classic cases of supposedly sympatric speciation in phytophagous insects, such as *Rhagoletis* flies (Feder *et al.*, 2005).

A graphic example of the potential for such a reproductive barrier was recently provided by Forister (2005), who studied host races/incipient species of *Mitoura* butterflies (*M. nelsoni* and *M. muiri*). These butterflies show dominant inheritance of both female host plant preference and larval performance (including survival), but with dominance in opposite directions. The traits are unlinked and crossings of the host races resulted in hybrid females that preferred to oviposit on the plant where its hybrid offspring had lowest survival. This should contribute to reproductive isolation between the host races in much the same way as hybrid inviability because of developmental defects (Porter & Johnson,

2002) but as an explicit effect of ecology – in the sense that it is the environment chosen by the female for her offspring which is incompatible with offspring traits.

We suggest that it is very likely that preference and performance genes will often be unlinked and that their mode of inheritance will often differ (Thompson *et al.*, 1990; Bossart, 2003; Forister, 2005 and further references in Ortíz-Barrientos *et al.*, 2002; Janz, 2003). If so, the new combinations have a high likelihood of being less fit than the original co-adapted ones. This reasoning can and should be extended also to traits that affect performance in less obvious ways. Successful utilization of a particular plant as host entails not only larval survival but also that the insect's entire life history and seasonal adaptations fit the characteristics of the plant (Bernays & Chapman, 1994; Wedell *et al.*, 1997; Carroll *et al.*, 2001). In the case of *P. c-album*, for instance, crossing a Swedish-type male with a Spanish-type female would produce hybrids with a relatively high preference for *Salix* and probably for other bushes and trees, as in the Swedish population, but coupled with a lower egg mass (smaller hatchlings) and an attempt at a higher larval growth rate. Although this remains to be explicitly tested, we believe that this would be a poor life history strategy on bushes and trees (Wedell *et al.*, 1997; G. H. Nygren, S. Nylin, C. Stefanescu & R. Krogen, unpublished).

The results of the present study and of Forister (2005) thus point to the importance of an ecological speciation process based on a multi-trait model of a reproductive barrier between incipient species. This is not a new idea; discontinuity along clines, caused by breakdown of co-adapted gene complexes, has probably always been at least implicit in ecological models of parapatric or alloparapatric speciation (Bush, 1975). Nevertheless, in their recent book on speciation, Coyne & Orr (2004) list a large number of types of reproductive barriers, probably the most exhaustive such list attempted to date, but the ecological multi-trait type is not mentioned. We suggest that it is best included in the type of barrier that Coyne & Orr (2004) term 'ecological inviability', covering low fitness in hybrids for reasons having to do with ecological fit. However, Coyne & Orr (2004) list only single-trait cases where hybrids are intermediate and thus 'fall between parental niches'. The same type of reasoning lead Rundle & Nosil (2005) to state that 'ecologically dependent reductions in hybrid fitness require phenotypes that are intermediate between parental forms', and to imply that dominance and epistasis is therefore a problem for such models. We suggest that finding these modes of inheritance for differences between incipient species may well be a problem for applying single-trait models, but would instead make the multi-trait ecological model even more likely to apply.

In conclusion, a role for host plants has recently been implicated in the speciation of *Polygonia* butterflies (Weingartner *et al.*, 2006) and other nymphalid butterflies (Janz *et al.*, 2006) and sex-linked genes seem to

play some general role in Lepidoptera speciation (Sperling, 1994; Prowell, 1998). In butterflies, effects of sex-chromosomes seem to be important for host preference differences between sister species (Thompson, 1988; Scriber, 1994). It is thus interesting to note that the earlier found pattern of genes for host preference differences within *P. c-album* being located on the sex-chromosomes (Janz, 1998), holds true also over a larger geographical scale. We do not find it likely that such genetic differentiation would proceed to speciation without geographical isolation in a highly mobile species such as *P. c-album*. Nevertheless, the situation in this species may provide a model of the earliest stages of divergence because of local adaptation of host preference and life history; a type of divergence which particularly after a longer period in allopatry might well contribute to ecological speciation. We suggest that an important component of speciation processes may be the formation of unfit hybrids at secondary contact between incipient species, because of differing modes of inheritance for different ecologically important traits which need to be co-adapted, such as preference and performance.

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