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Hybrid incompatibility is consistent with a hybrid origin of *Heliconius heurippa* Hewitson from its close relatives, *Heliconius cydno* Doubleday and *Heliconius melpomene* Linnaeus

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Abstract

Shared ancestral variation and introgression complicates the reconstruction of phylogenetic relationships among closely related taxa. Here we use overall genomic compatibility as an alternative estimate of species relationships in a group where divergence is rapid and genetic exchange is common. *Heliconius heurippa*, a butterfly species endemic to Colombia, has a colour pattern genetically intermediate between *H. cydno* and *H. melpomene*: its hindwing is nearly indistinguishable from that of *H. melpomene* and its forewing band is an intermediate phenotype between both species. This observation has lead to the suggestion that the pattern of *H. heurippa* arose through hybridization. We present a genetic analysis of hybrid compatibility in crosses between the three taxa. *Heliconius heurippa* × *H. cydno* and female *H. melpomene* × male *H. heurippa* yield fertile and viable F_1 hybrids, but male *H. melpomene* × female *H. heurippa* crosses yield sterile F_1 females. In contrast, Haldane's rule has previously been detected between *H. melpomene* and *H cydno* in both directions. Therefore, *H. heurippa* is most closely related to *H. cydno*, with some evidence for introgression of genes from *H. melpomene*. The results are compatible with the hypothesis of a hybrid origin for *H. heurippa*. In addition, backcrosses using F_1 hybrid males provide evidence for a large Z(X)-chromosome effect on sterility and for recessive autosomal sterility factors as predicted by Dominance Theory.

Introduction

In groups of rapidly radiating species, resolution of phylogenetic relationships based on morphological or DNA sequence characters can be difficult. This can be due to low divergence between closely related species, such that there is not sufficient information in the data to reconstruct a reliable phylogenetic hypothesis. A more difficult problem to resolve is that genetic variation is often shared between species for long periods subsequent to speciation, leading to well-known problem of discordance between gene trees and species trees (Nichols, 2001; Hudson & Coyne, 2002). Even worse, hybridization likely occurs between lineages for considerable periods subsequent to divergence, such that different parts of the genome have distinct historical relationships (Machado *et al.*, 2002; Pääbo, 2003).

Hybridization can result in the flow of neutral loci between species (Takahata & Slatkin, 1984; Machado *et al.*, 2002). Furthermore, introgression might be favoured by natural selection if hybridization results in novel adaptive gene combinations. In plants, hybridization commonly leads to the establishment of novel species with a genome composed of large regions from two or more parental taxa (Rieseberg, 1997; Rieseberg *et al.*,

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2003). Although the production of such hybrid genomes seems to be rare in animals (Mayr, 1963; Bullini, 1994), it is likely that occasionally novel combinations of genes derived from hybridization play an important role in adaptive evolution, and perhaps even in speciation (Arnold, 1997; Dowling & Secor, 1997). Furthermore, it has recently been suggested that colonization of new habitats may increase rates of hybridization and introgression facilitating rapid adaptive radiation in many animal species (Seehausen, 2004). Thus, reconstructing relationships among populations and species is complicated in part by (i) shared ancestral variation, (ii) gene flow of neutral loci across species boundaries and (iii) the possible hybrid origin of novel adaptive gene combinations. Both (ii) and (iii) clearly violate the assumptions underlying the reconstruction of a bifurcating species tree.

It has been suggested that this problem can be resolved by studying the genealogical relationships of loci that cause sterility (Ting *et al.*, 2000). However there are two major problems with this approach. First, despite intensive study, only seven cases of loci causing species incompatibilities have been identified in animals (Wittbrodt *et al.*, 1989; Perez *et al.*, 1993; Hutter, 2002; Barbash *et al.*, 2003; Presgraves *et al.*, 2003; Tao *et al.*, 2003; Barbash *et al.*, 2004). Second, incompatibility is thought to result from the gradual accumulation of certain genes (Turelli & Orr, 1995, 2000; Turelli, 1998; Turelli & Orr, 2000; Orr & Turelli, 2001), and the genealogical history of any one such locus may not necessarily be representative of the genome as a whole. Instead, where species have recently diverged and hybridization is still common, an estimate of average divergence across the whole genome would be useful to determine species relationships.

We instead propose that an estimate of genomic divergence is possible using traditional crossing techniques. Comparisons of incompatibility with genetic distance show a relatively linear positive relationship in all taxa where sufficient data exists, implying that genomic incompatibility accumulates gradually and at an approximately constant rate between populations (Coyne & Orr, 1989, 1997; Sasa *et al.*, 1998; Orr & Turelli, 2001; Presgraves, 2002; Price & Bouvier, 2002). The accumulation of incompatibility is not expected to be truly linear, and theory predicts a 'snowball' effect whereby a linear accumulation of single gene differences would lead to an exponential increase in incompatibility (Orr, 1995). This is because the latter is caused by epistatic gene interactions rather than single gene effects. Nonetheless this accumulation of incompatibility has been termed a 'speciation clock' (Presgraves, 2002), and although it is clearly not truly clock-like, an estimate of the relative degree of incompatibility between taxa should give an estimate of their relative divergence times. We here aim to determine the relationships of three recently diverged taxa using such data.

In *Heliconius* butterflies, inter-specific hybridization is known in many taxa (Mallet *et al.*, 1998; Gilbert, 2003). Wild-caught hybrid specimens are known between several species in the *Heliconius melpomene* Linnaeus and *Heliconius cydno* Doubleday (Lepidoptera: Nymphalidae) complex, and there is also shared variation among these taxa at both mtDNA (C. A. Salazar, C. Jiggins, E. Bermingham & M. Linares, unpubl. data) and nuclear genes (Beltrán *et al.*, 2002). One member of this group, *Heliconius heurippa* Hewitson, is unusual in that its colour pattern appears to share distinct genetic elements derived from two putative parental species. Crossing experiments have shown that elements of the *H. heurippa* pattern breed true when hybridized with either *H. melpomene* or *H. cydno*. Such crosses provide some evidence for genetic homology of pattern elements (Fig. 1; M. Linares, unpubl. data), as if similar phenotypes were independently derived in each species then one might expect some disruption of their development in hybrid individuals. Such breakdown does occur in certain crosses in *Heliconius*, providing evidence for similar yet independently derived phenotypes in other species (Sheppard *et al.*, 1985). In the case of *H. heurippa* (Fig. 1b), the hindwing is nearly indistinguishable from that of the subspecies *Heliconius melpomene melpomene* Linneaus (Fig. 1c) and breeds true when they are crossed (Fig. 1e; M. Linares, unpubl. data), although the yellow forewing band is closer in appearance to the subspecies *Heliconius cydno cordula* Neustetter (Fig. 1a) and similarly breeds true (Fig. 1f; M. Linares, unpubl. data). Furthermore, in the forewing the pattern of a distal red band and a proximal yellow band is extremely similar to that seen in *H. melpomene* × *H. cydno* hybrids, suggesting that the red forewing band could be generated by genes homologous to the red forewing in *H. m. melpomene* (Fig. 1e; Gilbert, 2003; Naisbit *et al.*, 2003; M. Linares, unpubl. data). Thus, based strictly on wing colour pattern genetics *H. heurippa* appears to be intermediate between *H. c. cordula* and *H. m. melpomene* and perhaps somewhat closer to the latter (see dotted diagram in Fig. 1).

In addition, a colour pattern virtually identical to that of *H. heurippa* can be created in the laboratory after just a few generations of hybridization between *H. c. cordula* and *H. m. melpomene* (Fig. 1d), subspecies that occur in sympatry near to the current range of *H. heurippa*, on the eastern slope of the Colombian Andes near Villavicencio (Fig. 2). This has lead to the hypothesis that *H. heurippa* has arisen as a result of hybridization between *H. melpomene* and *H. cydno* (K. S. Brown & L. Gilbert, pers. com.; M. Linares, unpubl. data). Alternatively, the *H. heurippa* pattern elements may be ancestral to the group, or they may have arisen multiple times using similar developmental pathways. These alternatives are difficult to distinguish, but the hybrid origin hypothesis is clearly supported by the fact that among all the diversity of colour pattern races in both *H. melpomene* and *H. cydno*, the two phenotypes that give rise to the *H. heurippa* pattern are those that occur closest to its current geographic range. Unsurprisingly, given the intermediate

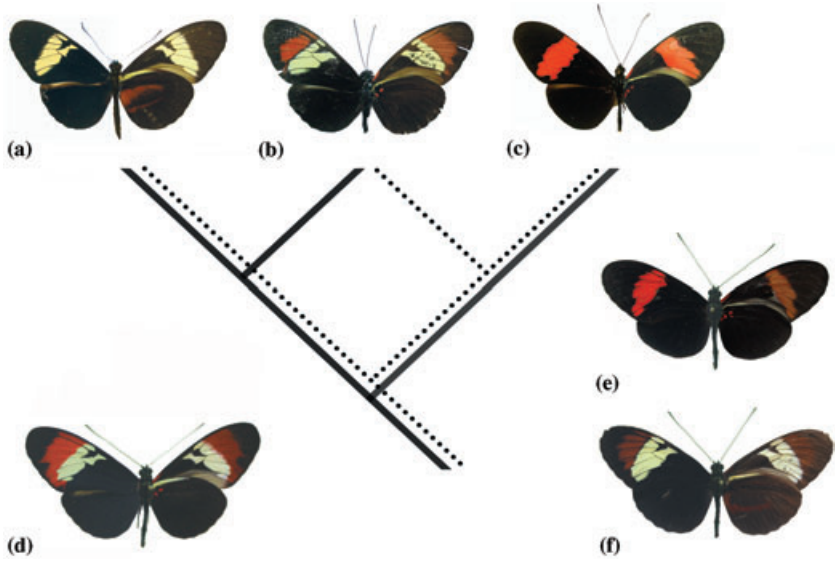

Fig. 1 Species used in this study. (a) *Heliconius cydno cordula*, (b) *H. heurippa*, (c) *H. melpomene melpomene*, (d) A hybrid derived from a cross between offspring of two C × (C × H) backcross families, (e) F_1 (M × H) hybrid and (f) F_1 (C × H) hybrid, showing a close resemblance to the species *H. heurippa*. All pictures show dorsal (left) and ventral (right) view. The cladogram shows proposed relationships based on the present study, whereby *H. heurippa* is most closely related to *H. cydno*, but may have arisen through introgression of colour pattern gene(s) from *H. melpomene* (dotted line). However, note that four colour pattern traits, the red forewing band, short forewing red line, red hindwing spots and absence of hindwing brown mark all suggesting *H. heurippa* is more closely related to *H. melpomene*.

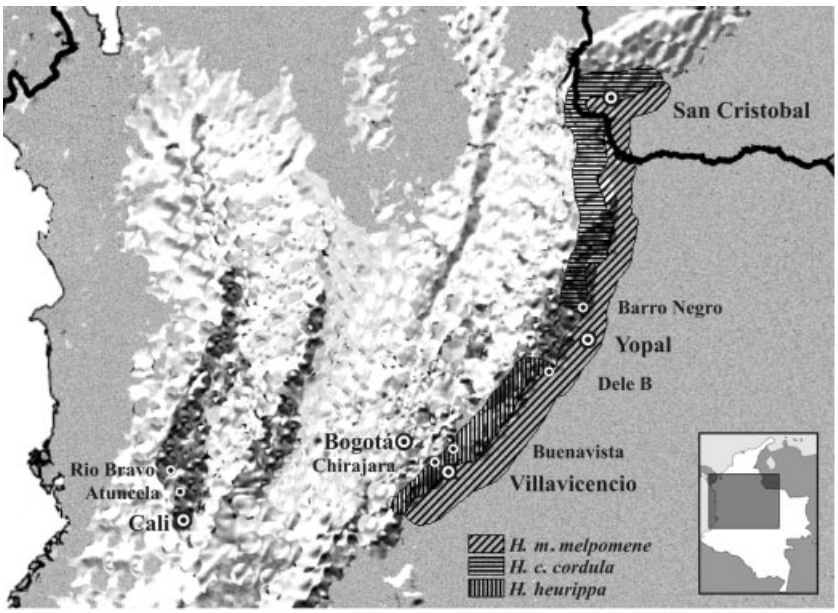

Fig. 2 Geographic distribution of the study species in the eastern Andes. The eastern slopes of the Colombian Andes are shown, with partial distributions of *H. melpomene* and *H. cydno*, complete known distribution of *H. heurippa* and sample localities (black dots). *Heliconius melpomene* is found throughout the lowlands from Central America to Brazil, whereas *H. cydno* is found throughout the Andes. *Heliconius melpomene* is broadly sympatric with both *H. heurippa* and *H. cydno* in the Andean foothills. *Heliconius cydno* and *H. heurippa* are probably parapatric around Yopal, but this region remains poorly explored.

nature of *H. heurippa*, its phylogenetic position is unclear. It has been considered a close relative of *H. melpomene* based on colour pattern and genitalia (Emsley, 1965) and to *H. cydno* according to mitochondrial DNA (Brower, 1994; Beltrán *et al.*, 2002).

In order to further unravel the evolutionary origin of *H. heurippa*, we here describe crosses designed to investigate the hybrid incompatibility of *H. heurippa* with respect to both *H. cydno* and *H. melpomene*. The aim of this study is (i) to examine the degree of hybrid compatibility between these three taxa in order to determine more precisely the relationship of *H. heurippa* with respect to its putative parental species, and (ii) conduct backcross experiments to investigate whether the genetic incompatibilities among these taxa show any evidence for recessive factors and a large Z(X) effect.

Materials and methods

Collection localities are given in Table 1 and Fig. 2. Crosses involving the three species were performed in La Vega, 50 km northwest of Bogotá, Colombia. *Heliconius cydno* were collected to the west of the Cauca Valley, in the Dagua region and in the foothills of the eastern slope of the Andes. In crosses between these two *H. cydno* populations no detectable reduction in hybrid fertility or viability was

Table 1 Collection localities for the *Heliconius* races used in these crosses.

Species	Locality	Latitude, longitude and altitude
H. melpomene melpomene (M)	Chirajara, Cundinamarca	4°12′48″N, 73°47′70″W, 1150–1450 m
H. melpomene melpomene (M)	Dele B (RíoCharte), Casanare	5°25′5″N, 72°31′20″W, 1150 m
H. heurippa (H)	Chirajara, Cundinamarca	4°12′48″N, 73°47′70″W, 1150–1450 m
H. heurippa (H)	Buenavista, Cundinamarca	04°10′30″N, 73°40′41″W, 1270 m
H. cydno (C)	Barro Negro, Casanare	6°01′6″N, 72°05′47″W, 1050 m
H. cydno (C)	Atuncela, Valle del Cauca	3°44′3″N, 76°41′53″W, 1400 m
H. cydno (C)	Río Bravo, Valle del Cauca	3°54′13″N, 76°38′18″W, 1000 m

Each species has been given a one-letter code used in Tables 2–3. All localities are in Colombia.

observed (Linares, 1997; C. A. Salazar, unpubl. data). Stocks of *H. m. melpomene* and *H. heurippa* were established from individuals collected near Villavicencio. In all cases, recently emerged virgin females were presented to sexually mature males in order to maximize the probability of obtaining a successful mating. Sexually mature (2 or 3 days old) females show a low mating probability with males that are not from their own species (C. A. Salazar & M. Linares, unpubl. data).

Mated females were kept individually in $1 \times 1 \times 2$ m outdoor insectaries with access to sufficient pollen sources (*Lantana* and *Psiguria*) and artificial nectar (10% sugar solution). Plants were provided for oviposition, mainly *Passiflora edulis* and *P. oerstedii*. *Heliconius heurippa*, *H. m. melpomene* and *H. c. cordula* from slopes of the Colombian Andes seem to be oligophagous. The three species lay eggs with a similar frequency on all plant species presented to them (including, *P. edulis*, *P. oerstedii*, *P. maliformis*, *P. ligularis* and *P. arborea*) in the insectaries. Also the different larval instars grow equally well on all the *Passiflora* species that were provided to them as food, mainly *P. edulis* and *P. oerstedii* (C. A. Salazar & M. Linares, unpubl. data). Eggs were collected every 8 days and kept individually in plastic pots with food. Larvae were reared on *Passiflora* plants grown directly in the insectaries. After pupation they were transferred to baskets for eclosion. The number of eggs laid, hatch rates, and number of enclosing butterflies were recorded. F_1 males, F_1 females and female offspring of backcrosses were tested for fertility with respect to control broods reared under the same conditions.

Statistical analysis

Hatch rate data were compared using likelihood-ratio tests implemented in the BETABINO program (Freeware development by Ziheng Yang, UCL-UK; see Jiggins *et al.*, 2001a; Naisbit *et al.*, 2002a). Differences between classes could be established with analysis of variance. However, this method can cause heteroscedasticity when the data show nonnormal distributions and the sample size is different between classes. This is the case in the hatch rate data collected here. We wish to compare these rates between broods of different types, taking into account real differences in hatch rates between replicates because of genetic or environmental variation. A better way to analyse our data, is to use a binomial parameter that changes across replicate broods within each cross type according to a β distribution. The β-binomial distribution can fit the skewed (e.g. L-shaped, reverse L-shaped) or bimodal distributions expected in extreme cases of variation within brood classes (e.g. crosses with sterility segregation). Then comparisons can be carried out between crosses using the likelihood parameters of mean, variance, and their standard errors. Five alternative models can be fitted with the program when a data set contains replicate broods of several classes: (i) a classical binomial parameter for each class, which assumes a zero brood to brood variance; (ii) a single β mean and variance for the entire data set; (iii) different means for each class but a single variance; (iv) a single mean but different variances; and (v) a different mean and variance for each class. Fitting the models on only part of the data set allows for specific hypotheses to be tested using likelihood ratio tests. For more details about use and implementation see ftp://abacus.gene.ucl.ac.uk/pub/ and the Appendix in Jiggins *et al.*, 2001a). Contingency tables for the segregation of sterility phenotypes and genetic marker loci were tested for heterogeneity using the program 'Monte Carlo R × C v2.0' designed by W. Engels, University of Wisconsin. When the expected data in any cell is <5, the program uses the Lewontin & Felsenstein (1965) algorithm, that takes a large number of random tables with the same marginal sums as the observed data, and simultaneously asks if each table deviates from the expected as much or more than the observed data. The ratio of the number of trials with log likelihood ratio test (LLR) less than or equal to the observed data to the total number of trials can be used as a true probability. All analyses were carried out with 20,000 trials.

Polymerase chain reaction methods for *Tpi* and *Apterous*

Total genomic DNA was extracted from tissue preserved in alcohol with a QIAGEN DNeasy tissue kit (QIAGEN,

Hilden, Germany) and the offspring of broods genotyped for two Z(X)-linked marker loci. Intron 3 of the sex (Z)-linked *Triose phosphate isomerase* (*Tpi*) gene was amplified using primers situated in the surrounding exons. Primer sequences, Polymerase chain reaction (PCR) conditions and evidence for sex linkage were described previously (Jiggins *et al.*, 2001a; Beltrán *et al.*, 2002). This intron contains a 33 bp insertion in populations of *H. heurippa* that is absent in *H. melpomene melpomene* from the Colombia Andes. This length variation was used to follow segregation of *Tpi* in backcross broods. Alleles were separated on 2% Metaphor (QIAGEN) agarose gels run for 4 h at 125 V and stained with ethidium bromide. All broods proved informative with regard to the segregation of alleles in female offspring, having F_1 fathers heterozygous for the *Tpi* insertion. Primers for the *Apterous* (*ap*) gene were developed for *Heliconius* and shown to be Z(X)-linked in *H. melpomene* broods (C. D. Jiggins, unpubl. data). Sequences for these primers are 5′-TGAATCCTGAATACCTGGAGA-3′ (forward) and 5′GGAACCATACCTGTAAACCC-3′ (reverse), which amplify a 228 bp region of coding sequence in the *ap* gene, corresponding to position 184–412 in a reference sequence of *Precis coenia* (GENBANK Accession number L42140). Female individuals from Brood 959 were sequenced to search for diagnostic restriction enzyme sites. One site, 31 bp from the end of the amplification fragment, was found to be different between alleles derived from *H. heurippa* and *H. melpomene*. The difference is because of a single base-pair substitution corresponding to a restriction site recognized by the enzyme Dde1 (5′ to 3′ CTNAG). The *ap* fragment was amplified using a PCR profile of 94 °C for 3 min, followed by 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min for 35 cycles. Amplified DNA was digested with Dde1 (0.025 units/μl) for 3 h at 37 °C. Digestion products were separated by electrophoresis on 2% Metaphor agarose gel and stained with ethidium bromide. As the grandparents of brood 959 were not available for genotyping, two wild-caught individuals each of *H. m. melpomene* and *H. heurippa* were genotyped to confirm that the cut site allele segregating in our cross was derived from the *H. melpomene* population. This marker was informative in the broods backcrossed to *H. melpomene*, but not those to *H. heurippa*, presumably because the restriction site was polymorphic in the *H. melpomene* population from which parental stocks were derived.

Results

Crosses between *H. heurippa* and *H. melpomene*

Female hybrids between *H. heurippa* (H) and *H. melpomene* (M) from the Eastern Andean foothills show asymmetrical sterility. The female offspring of a cross between female *H. heurippa* and male *H. melpomene* (H × M) were completely sterile, either failing to lay eggs or laying eggs that never hatched (Table 2). Sterile eggs in the F_1 generation were consistently smaller in size compared with eggs laid by controls (C.A. Salazar, pers. obs.), similar to phenotypes observed in previous crosses involving *H. melpomene* from French Guiana (Jiggins *et al.*, 2001a). In contrast, the reciprocal cross (M × H) produced female offspring that laid fertile eggs with a hatch rate of 0.56 ± 0.076, not significantly different to the controls, 0.53 ± 0.059 for M and 0.66 ± 0.06 for H ($G_2 = 2.38$, n.s.; Table 2). Male hybrids were fully fertile in both crosses, with hatch rates of 0.69 ± 0.115 and 0.52 ± 0.09 respectively, following Haldane's rule (Table 2). The average proportion of females was 0.49 ± 0.05 and did not differ significantly in seven cross classes ($G_7 = 8.75$, n.s.), four F_1 and three backcross broods (a total of 76 broods, 795 adults): (i) M × M, (ii) H × H, (iii) H × M, (iv) M × H, (v) (M × H) × (H/F_1/M), (vi) (H/M) × (M × H) and (vii) (H/M) × (H × M). This observation suggests an absence of Haldane's rule for viability.

Crosses were carried out to distinguish between sterility caused by genomic incompatibility and cytoplasmic factors. Here, the sterile F_1 females have cytoplasm and W chromosome from H, but a Z(X) chromosome from M. The female offspring of a fertile F_1 female (M × H) backcrossed to an M male, possesses the cytoplasm, Z(X)-chromosome and on average 75% autosomes from M. These females should be fully fertile if F_1 sterility results from cytoplasmic effects, but in fact this cross shows segregation of sterility phenotypes, ranging from fully fertile to partially sterile, although never with complete sterility (Fig. 3a). This suggests that interactions between the M Z(X)-chromosome and H autosomal genes are the cause of F_1 sterility. The same type of M Z(X)-chromosome-H autosomal interaction could be responsible for the segregation of sterility observed in F_2 (M × H) × (M × H) broods: of nine females tested from two broods, five were fertile (0.658 ± 0.183; $G_2 = 2.36$, n.s.) and four were sterile (Table 2). In addition, seven males tested were fully fertile. However, autosomal homozygous interactions (H_2 sensu; Turelli & Orr, 2000) through homozygous H and/or M autosomes cannot be ruled out as the main cause of sterility in these F_2 broods.

To verify the importance of the Z(X)-chromosome in causing sterility and to test for autosomal recessive or dominant sterility factors, fertile F_1 males were backcrossed to females of the two parental species. In crosses in both directions, female offspring were recovered showing a complete range of fertility, but with pronounced bimodality [Appendix A1(a,b); Fig. 3b,c]. In the backcross to *H. heurippa* 26 females were tested from seven broods, with a ratio of eight sterile to 18 fertile females, when all classes showing some degree of fertility were combined. Some of these sterile females laid eggs whereas others did not. There was a highly significant association between sterility and Z(X)-linked *Tpi* in female offspring from the backcross to *H. heurippa* [Appendix A1(a); Fig. 3b]. All seven females that were

Table 2 Hatch rate of control, F_1 and backcross broods.

Cross type	Maternal genotype	Paternal genotype	No. of broods	No. of eggs	Mean hatch rate	SE	Variance	SE
H. melpomene × *H. heurippa* crosses								
Pure	M	M	17	701	0.533	0.059	0.051	0.016
	H	H	22	710	0.664	0.060	0.077	0.019
F_1 interspecific	H	M	4	128	0.695	0.144	0.085	0.05
	M	H	2	182	0.765	0.22	0.11	0.096
Sterile backcrosses	H × M	H/F_1/M	12	127	0	–	–	–
	H × M	H/F_1/M	6	0	0	–	–	–
Fertile backcrosses	M × H	H/F_1/M	17	522	0.564	0.076	0.089	0.021
	H/M	M × H	7	308	0.522	0.09	0.044	0.03
	H/M	H × M	7	302	0.695	0.115	0.101	0.04
	(M × H) × M	M	25	722	0.504	0.055	0.068	0.015
F_2 interspecific	(M × H) × (M × H)	M/H	4	85	0.658	0.183	0.114	0.054
	(M × H) × (M × H)	M	4	51	0	–	–	–
	(M × H) × (M × H)	H	1	0	0	–	–	–
	H/M	(M × H) × (M × H)	7	220	0.602	0.109	0.082	0.031
H. cydno × *H. heurippa* crosses								
Pure	C	C	27	1206	0.665	0.053	0.071	0.016
	H	H	22	710	0.664	0.060	0.077	0.019
F_1 interspecific	C	H	6	616	0.461	0.086	0.037	0.020
	H	C	5	258	0.59	0.135	0.091	0.04
Backcrosses	C × H	F_1/C/H/F_2	12	185	0.69	0.053	0.014	0.011
	H × C	H/C/F_1	10	228	0.66	0.067	0.025	0.015
	H/C	H × C	3	351	0.50	0.214	0.098	0.066
	(C × H) × (C × H)	F_2/C	4	99	0.70	0.143	0.082	0.048
	(C × H) × C	C/F_2/H	4	119	0.78	0.144	0.090	0.065
	(C × H) × H	F_2	2	24	0.876	0.090	0.0003	0.027

Individuals of hybrid genotype are coded as maternal × paternal genotype where M is *H. melpomene melpomene*, H is *H. heurippa* and C is *H. cydno*. Mean hatch rate, the variance in hatch rate and their standard errors were calculated using the program BetaBino (see Methods). Note that in some brood classes hatch rate may have a bimodal, L or reverse-L shaped distribution.

completely sterile lacked the H *Tpi* insertion and therefore had the M Z(X) chromosome whereas most females that showed some fertility had the H Z(X) chromosome ($G_1 = 8.43$, $P < 0.05$).

In the backcross to *H. melpomene* the H Z(X)-chromosome is being introgressed into a largely *H. melpomene* autosomal background. The H Z(X)-chromosome is associated with complete fertility in the F_1 generation, so we did not expect significant sterility in this cross. Nonetheless, of 34 females tested from three broods, 12 were sterile and 22 were fertile. In all cases sterile females laid eggs that failed to hatch. In this cross we scored two Z(X)-linked markers, *Tpi* and *ap* that showed six recombinant genotypes in 30 individuals scored, a recombination rate of 0.2. There was no significant association of Z(X)-chromosome genotype with sterility either for each marker individually [*Tpi*, $G_1 = 0.67$, n.s. and *ap*, $G_1 = 1.64$, n.s.; Fig. 2c; Appendix A1(b)] or both markers together ($G_3 = 2.30$, n.s.; Fig. 2c; Appendix A2). Nonetheless the fertility of females was more common when both *Tpi* and *ap* alleles belong to *H. melpomene*: of 21 fertile females, 13 had both *ap* and *Tpi* alleles from *H. melpomene*. This result suggests that there may be a weak effect of the H Z(X)-chromosome on sterility too. We also demonstrated segregation of sterility phenotypes among various F_2 individuals, although the small sample size means that these crosses are not very informative with respect to the genetics of sterility (Table 2).

Crosses between *H. heurippa* and *H. cydno*

Hybrids between *H. heurippa* (H) and *H. cydno* (C) were completely fertile and viable. The female offspring of a cross female *H. heurippa* × male *H. cydno* (H × C) laid eggs with a hatch rate of 0.66 ± 0.067; the reciprocal cross (C × H) had a hatch rate of 0.69 ± 0.053. In all F_1 and backcross broods tested between *H. heurippa* and *H. cydno*, fertility was not significantly different to controls (Table 2; $G_9 = 7.90$, n.s.).

In general, F_1 and backcross broods involving *H. heurippa* × *H. cydno* and *H. heurippa* × *H melpomene* crosses showed similar sex ratios. The average proportion of females was 0.52 ± 0.05 and did not differ significantly in seven cross classes ($G_7 = 11.15$, n.s.), four F_1 and three backcross broods (a total of 85 broods, 1258 adults): (i) C × C, (ii) H × H, (iii) C × H, (iv) H × C, (v) (C × H) × (F_1/C/H/F_2), (vi) (H × C) × (H/C/F_1) and (vii) (H/C) × (H × C). This observation suggests an absence of Haldane's rule for viability in these crosses.

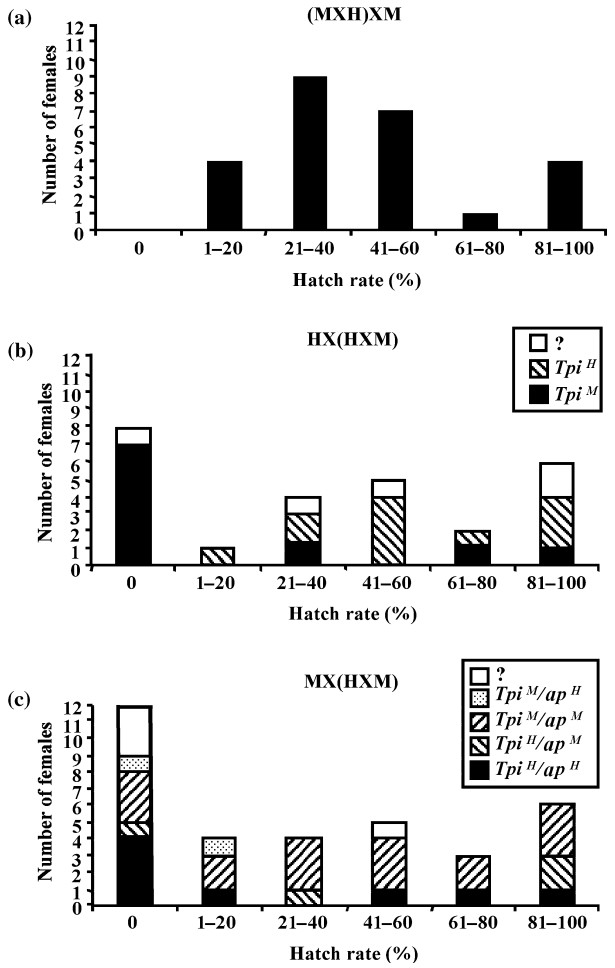

Fig. 3 Segregation of sterility phenotypes in backcross broods. The distribution of hatch rates for the female offspring of each cross type is shown. Shading indicates genotypes at *Tpi* and *ap* marker loci with superscripts showing the alleles; M: *H. melpomene*, H: *H. heurippa* and ?: for individuals that were not analysed. Female genotypes are shown first.

Discussion

Sterility and the status of *H. heurippa*

Heliconius heurippa is entirely compatible with *H. cydno*, but shows sex-specific hybrid sterility following Haldane's rule, when crossed with *H. melpomene*. Clearly, *H. heurippa* is much more closely related to *H. cydno* than to *H. melpomene*. This does not rule out a role for hybridization in the origin of *H. heurippa*, and implies that, if hybridization did occur it must have involved introgression of *H. melpomene* colour pattern allele(s) into a largely *H. cydno* genetic background, rather than an even hybrid mixing of the two species. In agreement with this hypothesis, COI-COII and *Tpi* gene sequences group *H. heurippa* with *H. cydno* (Brower, 1994; Beltrán *et al.*, 2002). However, given the hybridization and gene flow known to occur between these taxa, estimates of relationships based on single gene loci are likely to be subject to error. Indeed, a third nuclear gene, *Mpi*, shows almost identical alleles shared between individuals of *H. melpomene* and *H. cydno* (V. Bull, M. Beltran, E. Bermingham, C. Jiggins, O. McMillan & J. Mallet, unpubl. data). The estimate of relationships based on genomic compatibility presented here is a useful complement to previously published gene genealogies and morphological studies, and is perhaps more likely to reflect similarity across the whole genome of the three species.

Recessivity and the genetics of hybrid sterility

Given the relatively few phylogenetically independent comparisons supporting Haldane's rule (Read & Nee, 1991), and the great preponderance of studies in male heterogametic taxa such as *Drosophila*, there is a clearly a need to study hybrid breakdown in Lepidoptera and birds in order to determine whether there is a common genetic basis to the phenomenon (Laurie, 1997; Orr, 1997; Jiggins *et al.*, 2001a; Naisbit *et al.*, 2002a). With the asymmetry for sterility between *H. melpomene* and *H. heurippa* found in this study, there are now 30 cases in Lepidoptera of female sterility and 56 of female inviability, following Haldane's rule (Presgraves, 2002). In contrast, in *Drosophila* male sterility is far more frequent than male inviability (Laurie, 1997; Orr, 1997) and this pattern has been explained by dominance theory combined with faster male evolution (Orr, 1997; Johnson, 2000). The contrast between *Drosophila* and Lepidoptera could be caused by the effect of faster-male evolution, but this remains to be demonstrated. Faster-male evolution would act against the faster evolution of sterility in the female heterogametic sex in Lepidoptera (Tao & Hartl, 2003). In our crosses, there is evidence for a large Z(X) effect, but as has been discussed, such an effect is largely a result of the backcross design and does not necessarily indicate a greater accumulation of sex-linked vs. autosomal sterility factors.

The large X effect is consistent with Dominance Theory, but more convincing support would come from the demonstration of autosomal recessive sterility factors as predicted by the theory (Turelli & Orr, 1995, 2000; Turelli, 1998). There is some evidence for such factors in our backcross to *H. melpomene*. This cross involves introgression of the H Z-chromosome into an *H. melpomene* genetic background, a combination that is associated with complete fertility in the (M × H) F_1. The H autosome by M Z-chromosome interaction that causes sterility in the F_1 is also expressed in this brood, but in at most 25% of the offspring. Hence, the observed segregation of sterile and partially sterile phenotypes (<60% hatch rate) in 25 of the 34 offspring (Fig. 3c) would seem to suggest additional sterility factors. This must result from H_2 interactions (sensu Turelli & Orr, 2000) between

homozygous *H. melpomene* autosomal alleles and the H Z-chromosome, or heterozygous H_1 interactions between autosomal *H. heurippa* and homozygous *H. melpomene* alleles. At present, evidence for a role of the Z(X)-chromosome is inconclusive. Of the 16 individuals from this backcross with both *ap* and *Tpi* M alleles, 13 were fertile but only 3 were sterile, clearly in the direction expected if the Z chromosome had an effect on sterility. However, a test for heterogeneity on the data is not significant (Appendix A2; $G_3 = 2.30$, n.s.). Therefore, it seems likely that some combination of H_1 and H_2 interactions are causing sterility in this cross. Although our conclusions are clearly limited by the small sample sizes obtainable in *Heliconius* crossing experiments, the results nevertheless give some indication of the presence of recessive sterility interactions in agreement with Dominance Theory, and suggest means by which this hypothesis could be further tested.

Using the 'Speciation Clock' to study relationships

Our results are perhaps of greater interest in terms of the light that they shed on diversification in *Heliconius*. In particular we are interested in *H. heurippa* because of its possible hybrid origin. The crosses show that *H. heurippa* is completely compatible with *H. cydno*, but shows asymmetrical hybrid female sterility with *H. melpomene*. However if *H. heurippa* resulted from introgression of genes from *H. melpomene*, we might also expect somewhat less incompatibility in *H. heurippa* × *H. melpomene* crosses as compared with *H. cydno* × *H. melpomene*. There is evidence that this is indeed the case. In one direction of cross *H. melpomene* and *H. heurippa* produce fertile F_1 females, but the corresponding *H. melpomene* × *H. cydno* cross is known to produce sterile females. The only published analysis of F_1 females in an M × C cross was a brood that produced three completely sterile females and four females with a 20% hatch rate (Naisbit *et al.*, 2002a). In the same analysis, evidence from backcrosses to *H. melpomene* provided further indirect evidence that M × C crosses would produce sterile F_1 females, as the Z-chromosome was strongly associated with sterility, in contrast to the lack of association found in our broods (Fig. 3c). Hence, the available evidence is consistent with the prediction that *H. heurippa* is genetically intermediate between *H. cydno* and *H. melpomene*.

The accumulation of genes that cause genomic incompatibility is presumably a stochastic process (Orr & Turelli, 2001). Hence, when the number of genes that differ between taxa is very low, an estimate of divergence based on compatibility is likely to be subject to a large stochastic error. It is therefore important to determine whether the incompatibility observed in our crosses could be explained by just one or very few loci, as this might cast doubt on our conclusions regarding the relationship of these three taxa. First, if a single sex-linked locus were causing sterility we would probably expect a bimodal 1 : 1 ratio of completely sterile to fertile phenotypes in the backcross to *H. heurippa*. Hence, the segregation of many distinct sterility phenotypes in this backcross implies that many loci are involved. Second, the presence of sterility in the backcross to *H. melpomene* shows that there are divergent autosomal loci between *H. melpomene* and *H. heurippa*, in addition to the Z and autosomal loci that interact to cause sterility in the F_1 generation. Although the asymmetry of sterility in the F_1 generation implies that divergence is recent, there is nonetheless evidence that a significant number of sterility factors have accumulated between *H. heurippa* and *H. melpomene*, in contrast to the complete fertility observed between *H. heurippa* and *H. cydno*.

Given the complete compatibility between *H. heurippa* and *H. cydno*, it could be argued that *H. heurippa* is best considered a sub-species of *H. cydno*. Nonetheless, it is known that speciation in *Heliconius* does not always involve hybrid incompatibility. *Heliconius erato* and *H. himera* remain distinct in hybrid zones maintained by strong ecological selection against hybrids and assortative mating, with no reduction in viability or fertility of interspecific hybrids (Jiggins *et al.*, 1996; McMillan *et al.*, 1997). In *H. cydno* and *H. melpomene* disruptive sexual selection accompanied by mimicry forms a barrier of >99.9%, whereas genomic incompatibilities lead to isolation of just 70% (Naisbit *et al.*, 2002a,b). Unfortunately, good collections are not available from zones of sympatry between *H. heurippa* and *H. cydno* in order to test whether the two species do indeed maintain their distinctness in sympatry. However, *H. heurippa* females show strong assortative mating when tested against both *H. melpomene* and *H. cydno*, which likely results in a strong barrier to gene flow in the wild (C. A. Salazar & M. Linares, unpubl. data), suggesting that *H. heurippa* is correctly considered a good species.

In the case of *H. melpomene* and *H. cydno*, it is known that premating isolation results from mate preferences based on colour pattern (Jiggins *et al.*, 2001b). In that case, a switch in colour pattern driven by selection for mimicry was the most likely first step in speciation. In contrast, *H. heurippa* is not mimetic, and instead we have suggested that its colour pattern has arisen through the establishment of a hybrid pattern resulting from introgression of genes from *H. melpomene* into *H. cydno* (Fig. 1; M. Linares, unpubl. data). If this novel colour pattern also led to strong premating isolation with *H. cydno*, then *H. heurippa* may represent a case of speciation initiated by hybridization, a rather unusual phenomena in animals.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/jeb/jeb839/jeb839sm.htm

Appendix A1. Hatch rate of eggs laid by the female offspring of backcross broods.
Appendix A2. Segregation of sterility phenotypes and marker genotypes in backcross to *H. melpomene* broods.

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