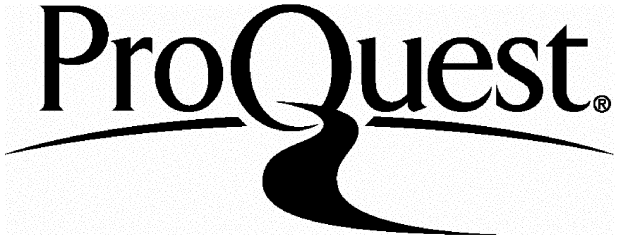
The ecology and genetics of speciation in *Heliconius* butterflies (Lepidoptera: Nymphalidae)

by

Christopher David Jiggins

A thesis submitted for the degree of
Doctor of Philosophy of the University of London

Department of Biology
University College London
The Galton Laboratory
4 Stephenson Way
London NW1 2HE



Promising indeed to the lovers of the marvellous is that land.....where the mightiest of rivers roll majestically through the primeval forests of boundless extent, concealing, yet bringing forth the most beautiful and varied forms of animal and vegetable existence; where Peruvian gold has tempted, Amazonian women have repulsed the unprincipled adventurer; and where Jesuit missionaries and luckless traders have fallen victim to cannibal Indians, and epicurean anacondas.

William H. Edwards (1847), *A Voyage Up the River Amazon*

ABSTRACT

Natural hybridisation between species offers an opportunity to study the processes of speciation. This thesis describes a study of hybridisation between *Heliconius himera*, which is endemic to dry forest in southern Ecuador and northern Peru, and *H. erato,* which is ubiquitous in wet forest throughout the neotropics. In a zone of contact in southern Ecuador hybrids are found at low frequency. Collections show that the contact zone is about 5 km wide, half the width of the narrowest clines between colour pattern races of *H. erato,* which implies that strong selection is maintaining the parapatric distributions of the species. Polymorphic protein and mitochondrial DNA markers were used to examine patterns of genetic differentiation and gene flow across the hybrid zone. Marked genetic differences between *himera* and *erato* are maintained in sympatry. Furthermore, analysis of linkage relationships between the allozyme markers showed that species differences are distributed widely across the genome. There was no evidence for any divergence in host plant ecology, but the hybrid zone between *himera* and *erato* was closely correlated with a habitat transition from wet to dry forest. Experiments showed that the barrier to gene flow was mainly due to strong assortative mating between *himera* and *erato.* Hybrid crosses showed no reduction in viability or fertility. Analysis of these broods showed that major gene control of pattern elements is similar to that found in previous studies of *H. erato* races, and the loci are homologous. Another species, *H. charitonia*, also has a genetically distinct sister species in the dry forests of Ecuador and Peru, *peruviana.* In both taxa, speciation is associated with divergence in habitat and warning colour, and in the case of *himera* and *erato* at least, there is a change in mating preferences but no evidence for genomic incompatibilities.

TABLE OF CONTENTS

Chapter 1

Chapter 2

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

Many thanks to Owen McMillan and Jim Mallet for invaluable supervision and collaboration. Roger and Sylvia Jiggins for butterfly cages, marmalade and everything. Instituto Nacional Ecuatoriano de Forestería y Areas Naturales (INEFAN) for granting permission to work in Ecuador. Fundación Arcoiris and in particular Pablo Lozano, Pablo Andrade and Fausto Lopez. Miguel Moreno and Germania Estévez at the Museo de Ciencias Naturales in Quito. Henri Descimon for finding Guayquichuma and Walter Neukirchen for photographs of hybrid butterflies. Peter Wilson for greasing the wheels of bureaucracy in Quito. For additional help in plant identification, Sandy Knapp; David Neill and Walter Palacios at the Herbario Nacional, Quito; Renato Valencia at the Universidad Católica and Bolívar Merino at the Herbario Reinaldo Espinosa in Loja. Daniel Falush, Gerardo Lamas, Keith Brown and Andrew Pomiankowski for useful comments. Angus Davison and Ashleigh Griffin, the only people in Ecuador to appreciate Mark E Smith. Yvonne Graneau and Peter King for assistance with protein electrophoresis. Roberto Carpio Ayala, José Carpio Ayala, María (de Vilcabamba) del Carmen Avila and María (de Loja) Arias Diaz for unstinting devotion to 'las orugas'. Andrew Brower for a favourable review and Neil Davies for unpublished sequence data. Keith Willmott and Durrell Kapan for collection data and company on a couple of field trips. Martin Brookes and Owen Rose for useful discussion of metapopulation structure. Jen Brady y el balcon. Francis Jiggins, Valerie and Cole McMillan, Ariane Crampton, Linda Dale, Grima & The Boyz, Elsa Avila, Ana, Kirsten Lauter, Rodrigo Tapia, Brian Bungle, Joy and Curtis Hofmann, CBs, La Siembra, The Green Triangle, Charlie Citrine and everyone else who has helped, with the possible exception of Fanny Bonilla and Transportes Loja. This project was funded by a BBSRC studentship and research grant (and latterly the Scotland Green Job Centre).

PREFACE

The tropical wet forests of south America are the most diverse in the world (Gentry, 1988). A single hectare of rainforest in upper amazonian Ecuador was found to contain 473 tree species (Vallencia et. al., 1994). In this thesis I describe a study which aims to understand the origins of this diversity. How does one species evolve into two or more daughter species? The study mainly concerns two butterfly species, *Heliconius himera* (Hew.) and *Heliconius erato* (Godt.), which hybridise in narrow zones of parapatric contact. By studying the ecological and genetic differences between taxa such as these, in the early stages of divergence, it should be possible to identify which factors are important in driving the formation of new species.

The first two chapters describe a hybrid zone between *himera* and *erato* in southern Ecuador, examining the distribution, ecology and genetics of overlapping populations. Here I show that the species boundary is extremely narrow, with no evidence for any mosaic distribution patterns. Furthermore colour pattern hybrids form just 5-10% of overlapping populations. This pattern is mirrored by genetic analysis in chapter 2, which shows that marked genetic differences between the species are maintained within the hybrid zone. Analysis of mitochondrial DNA, which might be expected to flow more freely across barriers to nuclear gene flow, also shows little evidence for introgression. Hence there is a virtually complete barrier to gene flow between the species, despite the occurrence of a fairly high frequency of hybrid individuals.

The following three chapters and the Appendix examine genetic, ecological and behavioural differences between *himera* and *erato.* The aim is to identify the factors which might be important in both driving the initial divergence of the species and in generating the barriers to gene flow which we observe today. Firstly I describe the genetics of wing colour pattern differences between *himera* and *erato*, by analysing the phenotypes of hybrid crosses. This analysis reveals a remarkably simple genetic system, whereby just two genetic loci are responsible for most of the variation seen in hybrid broods. There is good evidence that these loci are homologous with those responsible for much of the radiation of colour pattern races within *H. erato.*

There is a common assumption that the speciation of phytophagous insects is driven by divergence in larva host plant ecology. Contrary to this, in chapter 4, I show that there are no differences between *himera* and *erato* in their patterns of larval host plant use. However there are marked ecological differences between the species, and in chapter 5 I show that the hybrid zone is closely associated with a boundary between wet and dry forest. Thus ecological selection almost certainly determines the position of the cline, and may also contribute to the narrowness of the hybrid zone.

Of course, in order to make generalisations about how speciation occurs in *Heliconius*, we need to compare many species pairs. Chapter 6 describes a genetic and ecological study of another closely related species pair, *H. charitonia* (Linnaeus) and *H. peruviana* (Felder & Felder), which show many similarities with *himera* and *erato*. They are similarly differentiated genetically and also associated with a shift from wet to dry forest.

In the Appendix a series of experiments designed to investigate mating preferences and hybrid fitness in *himera* and *erato* are described. There was strong assortative mating between the species, with a 90% reduction in the probability of mating between *himera* and *erato*. A series of controlled crosses showed no evidence for any reduction in hybrid viability or fertility, compared to that of the parental species. This lack of any genomic incompatibilities between the species further highlights the importance of the ecological divergence described above. In conclusion, chapter 7 summarises the results and discusses their relevence to our understanding of speciation.

References

Gentry, A.H. 1988 Tree species richness of upper amazonian forests. *Proc. Natl. Acad. Sci., USA* **85**, 156-159.

Valencia, R., Baslev, H. & Mino, G.P. 1994 High tree alpha-diversity in Amazonian Ecuador. *Biodiv. Cons.* **3**, 21-28.

AUTHOR'S NOTE

Much of this thesis describes a collaborative project between myself and Owen McMillan. The chapters are all written by me, but will be published with, variously, Owen McMillan, Jim Mallet, Peter King, Pablo Lozano, Walter Neukirchen and Neil Davies as co-authors. Chapter 1 has already been published in the *Biological Journal of the Linnean Society* and provides a general introduction to the study system. In reading this chapter it should be noted that some of the aspects discussed are treated more thoroughly in subsequent chapters. Hence the colour pattern genetics of hybrids is analysed in Chapter 3. Host plant ecology is experimentally investigated in Chapter 4 and the habitat of the hybrid zone area, and a disussion of selection pressures implied by this analysis is described in further detail in Chapter 5. Chapters 2 and 4 have been submitted for publication to *Heredity* and *Ecological Entomology* respectively. The Appendix, senior authored by Owen McMillan, is nonetheless included here as I made a substantial contribution to the work and data analysis, and the results described have an important bearing on the rest of the thesis. It should be noted that appendices to individual chapters are referenced as (Appendix) whilst the main Appendix to the thesis is referenced as (McMillan et. al., 1997) throughout the text.

CHAPTER 1

Biological Journal of the Linnean Society (1996), *59*: 221–242. With 4 figures

What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae)

CHRIS D. JIGGINS[1], W, OWEN McMILLAN[1], WALTER NEUKIRCHEN[2] AND JAMES MALLET[1]

[1] *The Galton Laboratory, Department of Biology, University College London, 4 Stephenson Way, London NW1 2HE*

[2] *Fuchsiensweg 23, 12357 Berlin, Germany*

Received 25 October 1995, accepted for publication 19 January 1996

To understand speciation we need to study the genetics and ecology of intermediate cases where interspecific hybridization still occurs. Two closely related species of *Heliconius* butterflies meet this criterion: *Heliconius himera* is endemic to dry forest and thorn scrub in southern Ecuador and northern Peru, while its sister species, *H. erato*, is ubiquitous in wet forest throughout south and central America. In three known zones of contact, the two species remain distinct, while hybrids are found at low frequency. Collections in southern Ecuador show that the contact zone is about 5 km wide, half the width of the narrowest clines between colour pattern races of *H. erato*. The narrowness of this cline argues that very strong selection ($s \approx 1$) is maintaining the parapatric distributions of these two species. The zone is closely related with a habitat transition from wet to dry forest, which suggests that the narrow zone of parapatry is maintained primarily by ecological adaptation. Selection on colour pattern loci, assortative mating and hybrid inviability may also be important. The genetics of hybrids between the two species shows that the major gene control of pattern elements is similar to that found in previous studies of *H. erato* races, and some of the loci are homologous. This suggests that similar genetic processes are involved in the morphological divergence of species and races. Evidence from related *Heliconius* supports a hypothesis that ecological adaptation is the driving force for speciation in the group.

ADDITIONAL KEY WORDS: — parapatry – *Passiflora* – refugium biogeography – hybridization.

CONTENTS

Correspondence to Chris Jiggins. email: c.jiggins@ucl.ac.uk

0024–4066/96/011221 + 22 $25.00/0

INTRODUCTION

Speciation links evolution within populations to the generation of biological diversity on a wider scale, but the genetic processes involved are still poorly understood. Perhaps the best way to understand the genetics and ecology of speciation is to study intermediate cases. The majority of such studies have been carried out in hybrid zones where hybrids are abundant. Many of these cases have been stable over huge periods of evolutionary time, although abundant hybrids are still produced (Endler, 1977; Barton & Hewitt, 1989; Hewitt, 1989; Harrison, 1993). These studies have been extremely useful for understanding the interactions of gene flow and selection. However, inferences about speciation from these studies have been limited, because, under most definitions of species, speciation has not happened. For example hybrid zones in *Bombina* separate forms differing in mating call, warning coloration, life history, preferred habitat, enzymes and mtDNA, and are maintained by hybrid inviability at many loci (Szymura & Barton, 1986; 1991). However hybrids are in Hardy-Weinberg equilibrium and the zone itself has been stable, probably since secondary contact about ten thousand years ago. Moreover, genetic differences between forms suggests that divergence has been continuing for 3–4 million years without leading to speciation.

To understand the genetics and ecology of speciation it will be valuable to study examples where hybrids are produced, but are rarer than expected under random mating. In these situations, the two parental forms have reached that stage in speciation where they can maintain their genetic integrity in the face of gene flow, but have diverged sufficiently recently that genetic and ecological differences between them are likely to be involved both in current species maintenance, and in the initial divergence which led to speciation. Whether such forms are considered to be 'good' species depends on the species definition which one chooses to invoke. Under a genotypic cluster definition (Mallet, 1995), maintenance of the genetic integrity of taxa in sympatry is used as the criterion for species. Under the biological species concept the occurrence of any hybrids may be taken to imply that speciation is incomplete (Dobzhansky, 1937; Mayr, 1940). Whatever definition one uses, taxa which hybridize occasionally in sympatry are clearly important for the study of speciation as they have begun to acquire some, if not all, of the characteristics of species. Furthermore the existence of hybrids allows genetic analysis of species differences through the study of naturally occurring and laboratory produced crosses.

Although taxa meeting this criteria have been studied in the laboratory (Coyne & Orr, 1989), there has been little work done in the wild. This is unfortunate for two reasons. First, interspecific hybridization in animals is much commoner than has been thought: approximately 9% of bird species are known to hybridize (Grant & Grant, 1992) and the proportion is similar or even higher in other groups, for example European butterflies and coral reef fishes (Guillaumin & Descimon, 1976;

Pyle & Randel, 1994). Second, it is important to study interspecific hybridization in the wild, in order to identify factors maintaining the genetic integrity of hybridizing forms in a natural context. Interspecific hybrid zones are particularly useful as many of the factors which are important in maintaining such zones are also important in speciation; for example assortative mating, ecological adaptation, and hybrid sterility or inviability.

Since Dobzhansky (1937) most genetic work on reproductive isolation between species has concentrated on sibling species of *Drosophila.* Speciation in butterflies of the genus *Heliconius* provides an interesting contrast to *Drosophila.* In many continental *Drosophila* speciation has occurred with little ecological radiation, producing groups of reproductively isolated sibling species which are almost identical morphologically (Dobzhansky, 1937). In contrast *Heliconius* species show a remarkable diversity of colour pattern, which is evident both within and between species. Together with its sister genera, *Eueides* (12 spp.) and *Neruda* (3 spp.), *Heliconius* (40 species) appears to have undergone a recent radiation, compared to more basal genera of heliconiines with far fewer species, for example *Dryas* (1 sp.), *Agraulis* (1 sp.), *Dione* (3 spp.) and *Podotricha* (2 spp.) (Brown, 1981). In the *Heliconius* radiation this may be associated with the unusual ecological adaptations of the genus, such as pollen feeding, traplining of food and oviposition resources and longevity of adults (Gilbert, 1975; Brown, 1981).

Moreover, within *Heliconius* there is a range of intermediate steps on the road to speciation. Closely related species pairs such as *Heliconius clysonymus* and *H. erato* overlap in parapatry without hybridizing (Benson, 1978). At the other end of the spectrum, *Heliconius* species often consist of a large number of colour pattern races, which are stable over wide geographic areas, but hybridize freely where they abut. Some are separated by broad clines at a few colour pattern loci, others by narrow hybrid zones where more loci and greater pattern differences are involved (Turner, 1971; Benson, 1982; Mallet, 1986; 1993; Mallet et al., 1990). These hybrid zones are probably maintained by frequency-dependent mimetic selection against rare colour patterns (Brown, Sheppard & Turner, 1974; Mallet & Barton, 1989b). The extraordinary diversity of mimetic geographic races, and the fact that closely related species pairs nearly always show divergent wing colour patterns suggests that adaptive radiation of colour patterns preceded, or at least accompanied speciation in this group (Turner, 1976).

Between these extremes of geographic races and good, non-hybridizing species, are forms which produce hybrids, but which are stable to introgression when in contact. Hybridization in sympatry is regularly found, especially among the species closely related to *H. melpomene* (e.g. *H. melpomene, H. cydno* and the 'silvaniforms' *H. numata, H. ethilla, H. hecale*; Ackery & Smiles, 1976; Brown, 1976; Holzinger & Holzinger, 1994). Parapatric hybridization between *Heliconius* species is known only between *H. erato* and *H. himera* (Descimon & Mast de Maeght, 1984; Konig, 1986; Mallet, 1993). Descimon and Mast de Maeght suggested a more complete study of hybridization between these forms would be of great interest, although "one may cast doubts about the observational and experimental facilities offered by populations flying in remote parts of southern Ecuador". We attempt such a study here.

In this paper the biogeography, ecology and genetics of hybridization are investigated in the wild. Distributions and contact zones of *H. erato* and *H. himera* are mapped in detail. The study concentrates on one of these regions of contact in southern Ecuador where the habitat and food plant preferences of the two species are

investigated for evidence of ecological divergence. The width and structure of the hybrid zone are determined, yielding clues to the levels and types of selection. All wild-caught hybrids known from the zone are recorded and the commonest phenotypes figured in colour. The genetic basis of colour pattern differences deduced from the phenotypes of these hybrids is used to infer relationships between *H. himera* and other races of *H. erato*. Descimon & Mast de Maeght (1984) proposed that there was a deficit of hybrids, especially females (Haldane's [1922] Rule), compared with that expected under Hardy-Weinberg; this is tested more fully with the larger collections now available. Levels of hybridization seen in nature are used to assess the taxonomic status of the forms and the extent of gene flow between them. This paper concerns only the field data; further papers in preparation deal with mate choice, hybrid inviability and sterility, and introgression of molecular markers across the contact zone between the two species.

METHODS AND MATERIAL

Southwest Ecuador and northern Peru were visited at various times between 1984 and 1995. The distribution of *Heliconius* species was investigated over a wide area and especially in the provinces of Loja, El Oro, Zamora-Chinchipe (Ecuador), and the Departments of Amazonas, Cajamarca, Piura and Lambayeque (Peru). The study concentrated on the zone of contact found between *H. himera* and *H. erato* in southern Ecuador described by Descimon & Mast de Maeght (1984). A number of sites were visited on the Guayquichuma to Zambi road, which runs along the Río Yaguachi valley, and in the vicinity of Buenavista and Chaguarpamba in the adjacent valley. Collections were made during both dry and wet seasons.

A 'species index' was calculated for each site as an indirect indicator of gene frequencies across the zone, using colour pattern markers to identify parental and hybrid genotypes. Each individual collected was scored as follows *H. himera* = 0, *H. erato* = 1, putative F1 hybrid = 0.5 and putative backcrosses 0.25 and 0.75 respectively. The hybrid genetics were inferred from field collections, but have since been confirmed in laboratory hybridizations and allozyme studies of field-caught hybrids. The mean species index value for each site was calculated. A line was drawn along the Río Yaguachi valley to form the Guayquichuma transect line. This line was chosen as the forest remnants in this valley form a naturally linear habitat. Perpendiculars were dropped from each site along the valley onto the line, and a graph of species index against transect distance was then plotted. The width (w) of the cline was determined by drawing a tangent to the steepest section of the cline and measuring the distance which this line projects onto the x-axis.

Ecological effects on the contact zone were assessed by records of larval food plants and a general plant survey. Passifloraceae are virtually the sole host plants for *Heliconius* (Gilbert, 1975; Brown, 1981). To determine which hosts are utilized, a search was made at all sites visited for *Passiflora* and associated larvae or eggs, which were reared to determine species and sex. The distributions of all *Passiflora* and *Heliconius* species were also recorded. Five common tree species were recorded across the area to give general information on vegetation. Plant specimens were collected and deposited in the Herbario Nacional in Quito and in the herbarium of the Universidad Nacional, Loja. Vouchers of the butterflies were deposited in the Museo de Ciencias Naturales in Quito.

RESULTS

Geography of the hybrid zones

H. erato is a widespread species occurring from south Texas to southeastern Brazil and Argentina where it is generally found in gaps and disturbed areas of wet or gallery forest. *H. himera* replaces *H. erato* in a restricted area centred on the western slopes of the Andes in southern Ecuador and northern Peru and the Marañon valley in north eastern Peru (Fig. 1). There are three known contact zones between *H. himera* and different races of *H. erato* and hybrids have been found, albeit rarely, in all of these (Fig. 1; Descimon & Mast de Maeght, 1984; Konig, 1986; Mallet, 1993). The contact zone studied here is in the Río Puyango drainage, Loja and El Oro provinces, Ecuador (Fig. 2). The east-west extent of the contact zone is restricted by the Andes to the east, and mountains and coastal desert to the west. Across this region there is an abrupt transition from *H. erato cyrbia* to *H. himera*. The majority of collections are from the Guayquichuma transect, which follows a linear forest habitat running along the Río Yaguachi valley floor and in tributary valleys (Table 1A). Across the transition there is a smooth, monotonic cline with no evidence of a mosaic pattern. The width of this cline is approximately 5 km (Fig. 3). Collections in the Chaguarpamba region are less complete but show a similar pattern (Table 1B). The only inconsistency is at site 17 which is a pure *himera* site that lies between two mixed sites (Fig. 2). However, this is probably a sampling problem, as only 14 individuals were collected here; otherwise the transition occurs over approximately the same distance as in the Guayquichuma transect, and a comprehensive sampling of the whole region would almost certainly show a simple cline across the whole zone of contact.

Ecology of the hybrid zones

Collections of *Heliconius* larvae and eggs in the hybrid zone area show that the primary host plants for *H. himera* and *H. e. cyrbia* are *Passiflora rubra* and *P. punctata* (Table 2). These plants are both relatively common throughout the hybrid zone (Table 3). There is no evidence that either of the two primary host plant species is preferred by either butterfly species. In cage experiments *H. himera* and *H. erato* females lay eggs freely on *P. rubra, P. punctata* and a third species, *P. auriculata*, and the larvae of both species survive well on all three (Jiggins, McMillan & Mallet, 1996). *Passiflora auriculata* is known to be an important host of *H. erato* in other areas (Brown, 1981) and although there are currently no records of larvae or eggs on this species from this area, it is likely that *H. erato* does utilize it here as well. *Passiflora auriculata* only occurs in the wetter areas alongside *H. erato* and so is largely unavailable to *H. himera.*

H. himera and *H. erato* have divergent altitudinal ranges. Museum specimens and field experience indicate that *H. himera* is abundant up to 2000 m, with a lower limit of around 400 m where contact with *H. erato lativitta* occurs in the Marañon valley (Mallet, pers. obs.), although larval host plants occur both higher and lower. On the pacific slopes the lower limit of *H. himera* is restricted by contact with *H. erato* in Ecuador, and by desert in Peru. In contrast, *H. erato* rarely occurs above 1500 m but descends to sea level where suitable habitat occurs (Brown, 1979; pers. obs.).

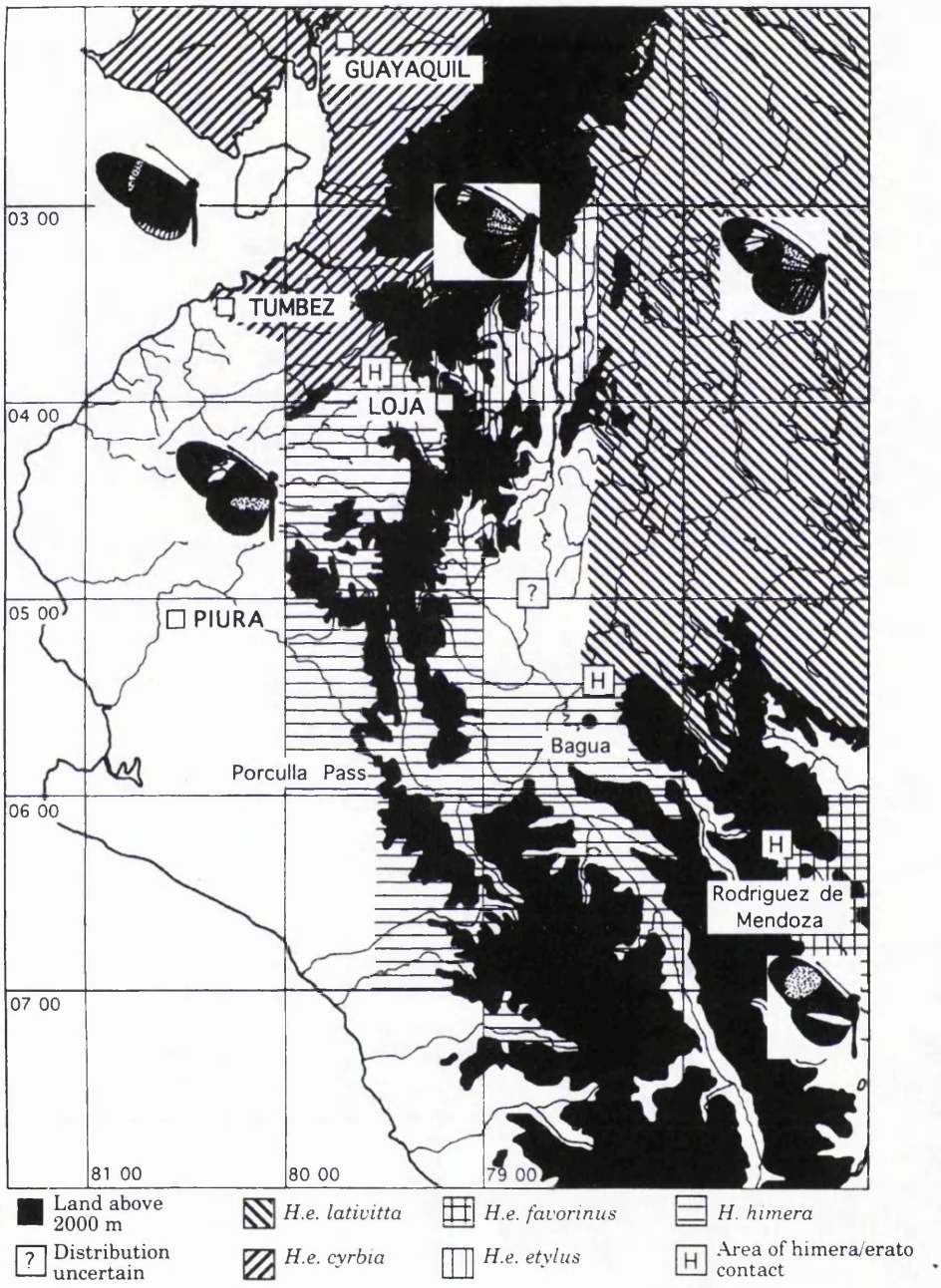


Figure 1. Distribution of *Heliconius himera* and surrounding races of *Heliconius erato*. Data from collections by the authors and quarter degree grid square data from Brown (1979). On the butterflies, stippled shading represents red/orange and white is yellow/white.

The two species are associated with very different habitats (Table 3). North of the contact zone, where *H. erato* is common, the forest vegetation is dominated by species such as *Cecropia sp.* and *Ochroma pyramidale.* These are large leaved, 'light demanding' species typical of secondary wet forest. At the other extreme, in areas where *H. himera* is common, the vegetation is thorn scrub dominated by *Acacia macracantha*, a small-leaved xerophytic species. In the area of contact there is a patchwork of secondary and disturbed primary forest remnants surrounded by open pasture grazed by cattle, which is devoid of *Heliconius*. Associated with this transition are changes in other *Heliconius* and their host plants. *Heliconius melpomene cythera*, which mimics *H. e. cyrbia*, and *H. sara* drop out as the habitat becomes dryer, the latter presumably because its primary host-plant *P. auriculata* is not found in dry forest (see above). We have no information on the host plants of *H. melpomene* as it is extremely rare in this region compared to *H. erato*. The *himera/erato* hybrid zone correlates well with the centre of this transition, but the habitat change appears to be considerably broader than the cline between the butterflies.

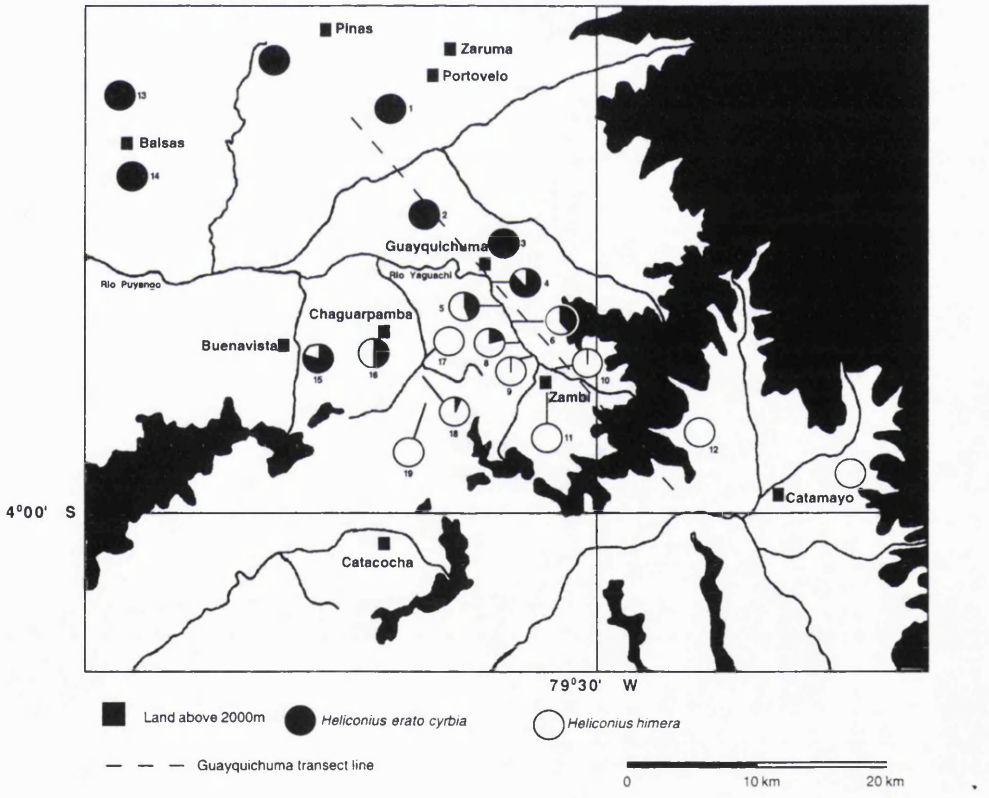


Figure 2. Distribution of phenotypes in the contact zone between *Heliconius himera* and *H. erato cyrbia* in southern Ecuador. Pie diagrams represent 'species index' values for each site (Tables 1A, B).

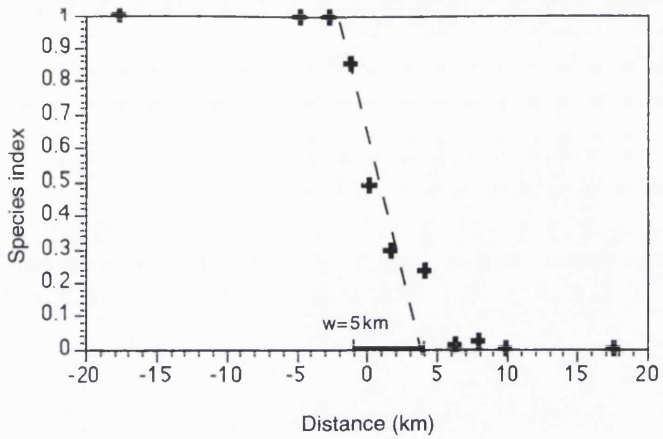


Figure 3. The cline between *H. himera* and *H. erato* along the Guayquichuma transect in southern Ecuador (Table 1A; Fig 2). The width (w) of the cline is determined by drawing a tangent to the steepest section of the cline and measuring the distance which this line projects onto the x-axis.

TABLE 1(A). Guayquichuma transect. Data collected along the road between Portovelo (site 1) and Zambi (site 11). Transect distance refers to the distance along a straight line drawn through the zone when a perpendicular is dropped from the site onto this line. Distances are measured towards Zambi (positive) and towards Portovelo (negtative) with zero at the site closest to a 0.5 species index value. This line and all sites are marked on Fig. 2. Descimon & Mast de Maeght (1984) collected at site 4, which is 8–10 km south of Guayquichuma. Numbers indicate individuals collected or marked. Hybrid classifications are according to colour pattern phenotype; BC = backcross to *H. e. cyrbia* and BH = backcross to *H. himera*. See text for details of 'species index' calculations. The individuals of unknown sex are those collected by Descimon & Mast de Maeght (1984)

Transect distance (km)		-17.7	-4.9	-2.9	-1.3	0	1.5	2.5	3.95	6.2	7.8	9.8	17.5
Site number		1	2	3	4	5	6	7	8	9	10	11	12
Altitude (m)		1150	800	1000	950	1000	1050	1100	1100	1150	1250	1250	1800
Latitude	South 3°	42.7	48.0	48.8	50.0	50.7	51.5	52.0	52.6	53.6	54.2	55.7	56.5
Longitude	West 79°	39.6	34.5	33.8	34.2	34.3	33.9	33.7	33.2	32.5	31.7	31.6	26.8
H. himera	males				15	13	13	1	6	25	48	14	7
	females					1	2		2	8	3	12	15
	sex unknown				11								
Hybrids													
F1	males				7	4	2		1	1			
	females												
BC	males		1		4	1	1	1					
	females			1	1								
BH	males				2	1	1	1					
	feales				1	1							
H.e.cyrbia	males	16	16	15	104	11	2		1		1		
	females	7	14	1	38	3	3		1				
	sex unknown				31								
Species index		1	0.99	0.99	0.85	0.49	0.29	0.33	0.23	0.01	0.02	0	0

TABLE 1(B). Chaguarpamba transect. Data collected in the vicinity of Chaguarpamba (site 16), Buenavista (near sites 15) and Balsas (sites 13 & 14). Details as in (A)

Site number		13	14	15	16	17	18	19
Altitude (m)		1000	900	1100	1100	1350	1275	1500
Latitude	South 3°	43.6	46.5	53.5	52.0	53.6	54.4	56.1
Longitude	West 79°	50.4	48.0	41.8	39.8	37.7	38.5	38.2
H. himera	males			10	8	8	16	16
	females			1		6	6	8
Hybrids								
F1	males							
	females			1				
BC	males			1				
	females							
BH	males							
	females							
H.e.cyrbia	males	13	10	37	5		1	
	females	3	2	7	3		1	
Species index		1	1	0.79	0.50	0	0.08	0

TABLE 2. Host plant records from the hybrid zone and surrounding areas. This tables shows the number of wild collected larvae and eggs reared to emergece from each host plant species (letters represent the phenotype of the adult raised from the collected egg or larva; h = *Heliconius himera*, c = *H. erato cyrbia*, F1 = F1 hybrid, BH = backcross to *H. himera* and BC = backcross to *H. e. cyrbia*). Site numbers correspond to those in Fig. 2. The 'X' marks the fact that *P. punctata* was not found in Vilcabamba, although cultivated plants of both species were readily used by wild *H. himera* there. *P. sanguinolenta* is a closely related form to *P. rubra* and the two are difficult to distinguish when sterile, so records from these species have been combined. Vilcabamba lies 25 km to the south of Loja and Alluriquín is on the road from Quito to Santo Domingo. All of the *Passiflora* species shown are in the subgenus Plectostemma

Site	*P. rubra*	*P. punctata*
Celica		1 h
Vilcabamba	9 h	X
19	1 h	
11	1 h	1 h
10	2 h	
9		1 h
8		1 h
7		1F1, 1BC
6	2 h	
5	1BH	
4		4c
2	1c	
Balsas	1c	4c
Alluriquin	3c	2c

Genetics of hybrids

A total of 43 field-caught hybrids are now known from the S. Ecuador contact zone (Appendix), the most known from any of the three *himera/erato* contact zones. The commonest hybrid phenotype is intermediate to the two parental species (Fig. 4A); it has the *himera* red hindwing bar and *cyrbia* red forewing band on a black background with a faint bluish iridescence (*cyrbia* has strong blue iridescence, *himera* has none). The shape of the forewing band is intermediate to the parental types and there is sometimes a trace of the *himera* yellow band in the forewing. These are interpreted as F1 types; this implies that the red *cyrbia* forewing band and red *himera* hindwing bar are both dominant, and the yellow *himera* forewing band is recessive. All other cyrbia traits (blue iridescence, white hindwing fringe, yellow underside hindwing bar) are largely recessive. The remaining hybrid phenotypes are interpreted as backcrosses (Fig. 4 B–H), and can be used to test deductions made from the F1s, as well as demonstrating whether loci act independently or show linkage. The interpretations of hybrid phenotypes made here are confirmed by allozyme studies of hybrids and the results of laboratory crosses (in prep.).

The inheritance of the colour pattern appears surprisingly simple, consisting of six major loci, each of which controls a simple pattern element with nearly complete dominance. Using the nomenclature of Sheppard *et al.* (1985), two putative loci are: *R* controlling the *cyrbia* red forewing band (present-dominant); and *Cr* controlling the presence of *cyrbia* yellow underside hindwing bar (absent, or shadow-dominant). At least four further loci can be assigned to explain the other pattern elements: these are

TABLE 3. Habitat data from the Guayquichuma hybrid zone. Data is shown from six hybrid zone sites across the transition. For comparison there are a further two sites where each of the two parental species is common (*H. himera*: Catamayo and Vilcamba, *H. erato cyrbia*: Balsas and Piñas). Vilcabamba lies 25 km to the south of Loja. The presence/absence of five common tree species shown. Also shown are collections of other *Heliconius* species and host plant (*Passiflora*) species. Only one of three *Heliconius* species, two of four *Passiflora* species and none of the tree species, are shared by the sites at either end of the transition

	Balsas	Piñas							Catamayo	Vilcabamba
Site number			2	4	5	7/8	9	10	12	
Altitude (m)	950	1000	800	950	1000	1100	1150	1250	1800	1600
species index	1	1	0.99	0.85	0.49	0.24	0.02	0	0	0
Tree species										
Ochroma sp.	+	+		+						
Inga sp.	+	+	+	+	+	+				
Cecropia sp.	+	+	+	+	+	+				
Ficus cf. maxima				+	+	+	+	+	+	+
Acacia macracantha								+	+	+
Other *Heliconius* species										
H. charithonia peruviana	+	+	+	+	+	+	+	+	+	+
H. sara	+	+	+	+	+					
H. melpomene cythera	+			+						
Passiflora species										
P. rubra	+	+	+	+	+	+	+	+	+	+
P. punctata	+	+	+	+	+	+	+	+	+	
P. adenopoda		+	+							
P. auriculata	+	+	+	+	+					

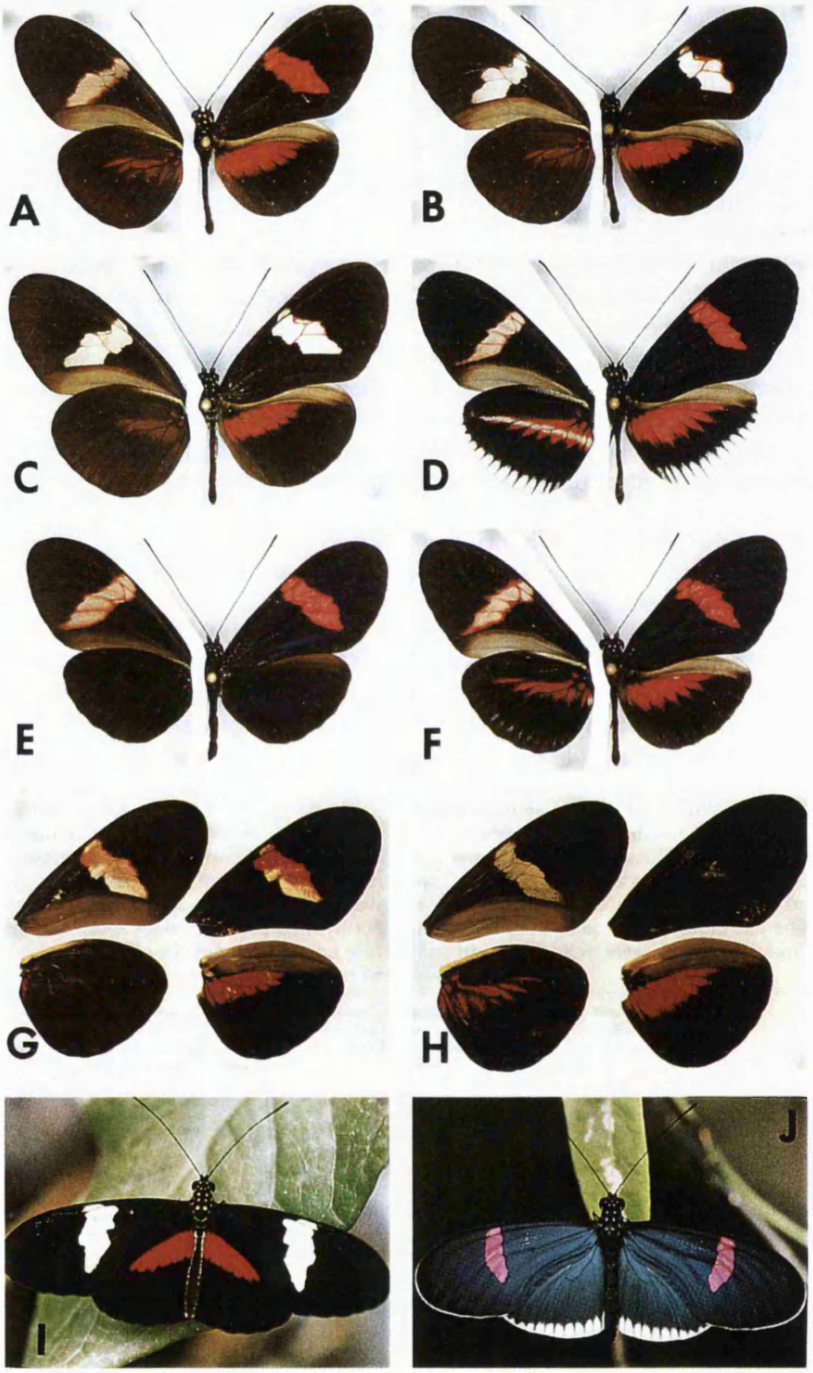


Figure 4. Hybrid and parental phenotypes. All hybrids shown were collected in the wild and full details are listed in the appendix (see ref. nos.). Proposed hybrid categories: F1, BH = backcross to *himera* and BC = backcross to *cyrbia*. Proposed colour pattern loci (see results) *cyrbia* red forewing band, *R*, (present-dominant); *cyrbia* yellow underside hindwing bar, *Cr*, (absent-dominant); blue *cyrbia* iridescence, *Ir*, (absent-dominant); *cyrbia* white hindwing margin, *We*, (absent, or trace-dominant); *himera* red hindwing bar, *Rb* (present-dominant); and *himera* yellow forewing band, *Y* (absent-dominant). A, F1, ref. no. 11; Rr, Crcr, Irir, Wewe, Rbrb, Yy; B, BH, ref. no. 10; R-, Cr-, Ir-, We-, Rb-, yy; C, BH, ref. no. 15, R-, Cr-, Ir-, We-, Rb-, yy; D, BC, ref. no. 17, R-, crcr, irir, wewe, Rb-, Y-; E, BC, ref. no. 14, R-, Cr-, irir, We-, rbrb, Y-; F, BC, ref. no. 12, R-, Cr-, irir, We-, Rb-, Y-; G, BH, ref. no. 41, R-, Cr-, Ir-, We-, Rb-, yy; H, BH, ref. no. 25, rr, Cr-, Ir-, We-, Rb-, Y-; I, *Heliconius himera*, rr, CrCr, IrIr, WeWe, RbRb, yy. J, *Heliconius erato cyrbia*, Rr, crcr, irir, wewe, rbrb, YY.

blue *cyrbia* iridescence controlled by *Ir* (absent, or trace-dominant); *cyrbia* white hindwing margin, controlled by *We* (absent, or trace-dominant); *himera* red hindwing bar, *Rb* (present-dominant); and *himera* yellow forewing band, *Y* (absent, or trace-dominant). The necessity for two loci controlling the forewing band is shown by two hybrids with almost completely black forewings (Appendix Nos. 25 & 31; Fig. 4h). This is impossible under a simple single locus or tightly linked supergene system, such as that seen in other races of *H. erato* (Sheppard et al., 1985; Mallet, 1989), where no recombinants are known.

Although the genetics is simple, hybrids show some disruption of pattern elements suggesting additional polygenic effects. When the forewing band is yellow in hybrids, some red scales are often present, especially around the edge of the band (Fig. 4B,C), presumably indicating the expression of homozygous *yy* on an *R-* background. Both the hindwing margin (Fig. 4E,F) and the yellow underside bar can be present as a faint shadow, although for the purposes of this analysis they are considered absent. Hybrids also show a slight 'raying' of the *himera* red hindwing bar (Fig. 4D,F), which could represent the effect of the genetic background of *erato*, in which there are some races with red-rayed hindwings. The red hindwing bar also shows through on the underside of the hindwing in hybrids to a far greater extent than in *H. himera* (Fig. 4D,F.H). Iridescence also seems more continuously variable than expected under a single locus hypothesis.

Almost every possible F1 and backcross phenotypic combination is seen in the wild (Table 4). This suggests independence of action, as well as a lack of strong linkage. The combinations we have not seen in the field are mostly those combining recessive phenotypes from both species, which can only be produced by F2 or other hybrid × hybrid crosses. These are very unlikely to be seen given that even F1

TABLE 4. Combinations of colour pattern traits seen in wild caught hybrids. There are more data points than hybrids as any one individual can feature a number of times. Dominant/dominant combinations are produced in F1, backcross and F2 broods; dominant/recessive combinations can be produced in backcross and F2 type broods whilst recessive/recessive combinations could only be produced by F2 or other interhybrid crosses. Recessive-recessive combinations are not seen and are likely to be extremely rare due to the low frequency of hybrids in the contact zone. Also absent are recombinants between *cyrbia* yellow bar and *cyrbia* white edge which may imply linkage between loci affecting these traits

himera traits	*dominant* red fw; present **R-**	*dominant* yellow fw; absent **Y-**	*recessive* red hw bar; absent **rbrb**	*recessive* yellow hw bar; present **crcr**	*recessive* blue iridescence; present **irir**	*recessive* white hw edge; present **wewe**	
recessive **rr** red fw band; absent	XX	2	0	0	0	0	Recessive /recessive combinations
recessive **yy** yellow fw band; present	7	XX	0	0	0	0	
dominant **Rb-** red hw bar; present	15	10	XX	6	6	6	
dominant **Cr-** yellow hw bar; absent	16	11	7	XX	9	0	
dominant **Ir-** iridescence; absent	10	5	1	3	XX	3	
dominant **We-** white margin; absent	16	11	7	0	9	XX	
	Dominant/dominant combinations						

hybrids are rare. Other combinations not found are recombinants between *cyrbia* yellow hindwing bar and *cyrbia* white hindwing margin, which suggests these two loci are tightly linked. Rare recombinants are known from our laboratory crosses (in prep.), so two loci have been assigned rather than one. In *H. erato* the allele that determines hindwing marginal 'cream rectangles' in S.E. Brazil also determines yellow bar, hence the name *Cr* for the locus we have assigned for the yellow hindwing bar (Sheppard *et al.*, 1985; Mallet, 1989).

Descimon & Mast de Maeght (1984) have suggested that there are fewer hybrids in this *himera* × *erato* zone than expected given random mating and full viability of hybrids. This holds for the more extensive data now available. Random collections from the km −1.3 site total 26 *H. himera*, 173 *H. e. cyrbia* and 15 hybrids of which 7 can be interpreted as F1, 3 as backcrosses to *himera* and 5 as backcrosses to *cybria* (Table 3). Frequencies of *cyrbia* alleles estimated assuming Hardy-Weinberg from this site give $q_R = 0.6383$, $q_{cr} = 0.9069$, $q_{ir} = 0.9069$, $q_{we} = 0.9069$, $q_{rb} = 0.9069$,$q_Y = 0.9056$; so the average *cyrbia* allele frequency can be estimated as $q = 0.8619$. Hybrids are deficit, so Hardy-Weinberg estimates of dominant allele frequencies will be incorrect — this is why q_R is estimated to be so much lower than the other loci. However, the estimate obtained is very similar to the average frequency based on the proportions of pure types, which gives $q = 173/(173 + 26) = 0.8693$. If all five loci are in linkage equilibrium, we expect 0.004 pure *himera* phenotypes, 69.8 pure *cyrbia* phenotypes, and 134.6 intermediates. We actually find 26:173:14, giving $G_1 = 709.1$, $P < 0.001$. Of course, this result is in part due to gametic correlations (linkage disequilibrium), rather than merely an absence of heterozygotes. Linkage disequilibrium is expected in hybrid zones as a consequence of gene flow (Barton & Gale, 1993), even between unlinked loci. However, this cannot be the whole story in this case: even if the whole pattern were inherited at a single gene, we still expect many more intermediates (50.9, all of which are expected to be F_1 in phenotype), together with fewer pure phenotypes, 4.1 *himera* and 159.0 *cyrbia*. This has $G_1 = 88.59$, $P < 0.001$, showing that there is a shortage of hybrid phenotypes even if there is strong linkage disequilibrium.

It is common in cases of hybrid incompatibility to observe sex ratio biases. This is often greater inviability or sterility in the heterogametic sex, an effect known as Haldane's rule (Haldane, 1922; Coyne & Corr, 1989). Descimon & Mast de Maeght (1984) caught only male hybrids and suggested that Haldane's rule may apply in this case. A total of 7 female and 36 male hybrids are now known in all hybrid classes. This is not significantly different from the ratio of 81 females to 269 males among parental types at the hybrid sites ($G_1 = 1.109$, NS). Similarly there is no significant excess of males when only F1s are considered (1 female: 18 males; $G_1 = 2.193$, NS).

DISCUSSION

Taxonomic status of H. himera

For many years *Heliconius himera* has been considered a race of *H. erato* (Eltringham, 1916; Lamas, 1976; Brown, 1979; but see Kaye, 1916 and Emsley, 1965). However, our exhaustive collections in southern Ecuador make it clear that despite some hybridization, *H. himera* and *H. erato* maintain their genetic integrity in contact zone

populations. In the centre of the hybrid zone parental phenotypes are common and hybrids rare. Moreover studies of mtDNA and allozyme loci show a similar genetic break in the hybrid zone, with little evidence for introgression of genetic markers between *H. erato* and *H. himera* (in prep.). This pattern contrasts with collections from the centres of interracial hybrid zones which are composed mainly of hybrids, with colour pattern loci approximately in Hardy-Weinberg equilibrium (Turner, 1971; Benson, 1982; Mallet, 1986; Mallet *et al.*, 1990). This pattern of random mating is reflected in the lack of genetic differentiation across interracial hybrid zones at mtDNA (Brower, 1994) and allozyme loci (Turner, Johnson & Eanes, 1979).

We consider *himera* and *erato* to be separate species because of the strong hybrid deficit (see also Descimon & Mast de Maeght, 1984). Nonetheless, the two forms are geographic replacements and, with the exception of wing color pattern, are virtually identical morphologically, with only minor differences in genitalic structure (Emsley, 1965) and wing shape (Fig. 4). This species pair represents an important intermediate stage in the evolution of parapatric geographic races into a pair of species that overlap in broad sympatry. The remainder of this discussion focuses on the conclusions that can be drawn from our data on the maintenance of the extremely narrow cline between these two forms and, more generally, about speciation in *Heliconius*.

Structure and maintenance of the hybrid zone

Perhaps the most striking feature of the *erato/himera* hybrid zone is its narrowness (Fig. 3). At only 5 km, it is considerably narrower than interracial zones in *Heliconius erato*, which vary between 10 km (Mallet *et al.*, 1990), where colour pattern differences are greatest, to several hundred kilometres where pattern differences are small (Brown & Mielke, 1972; Mallet, 1986). Most clines and hybrid zones can be explained as a dynamic balance between selection and migration. In such cases the width of the hybrid zone should be approximately proportional to $\sigma/\sqrt{s}$ (σ is dispersal distance, s is selection pressure). Although we have no direct estimate of dispersal distance across our study area, it seems reasonable to assume that it is similar to that observed in other *H. erato* hybrid zones. Thus, a reduction in zone width from 9.6 km, the mean width over three loci in Mallet *et al.* (1990), to 5 km represents a four-fold increase in selection. As $s \approx 0.5$ in the interracial hybrid zone this means that s must be close to 1, the maximum possible, in this case. This calculation assumes that the *himera/erato* zone is maintained via warning colour selection, as in the case of *H. e. lativitta* and *H. e. favorinus* (Mallet & Barton, 1989b). This may not be the case, but other selection models yield similar results (Mallet & Barton, 1989a; Barton & Gale, 1993); selection must be intense, however caused.

Selection on colour pattern

The narrow hybrid zones between colour pattern races of *Heliconius* can be explained by purifying frequency dependent selection on mimetic colour patterns. This type of selection in hybrid zones can be very strong ($s \approx 0.5$ as shown in a release-recapture experiment [Mallet & Barton, 1989b]; $s > 0.01$ per locus from linkage disequilibria [Mallet *et al.*, 1990]). The strength of frequency dependent selection depends critically on colour pattern differences between forms (as perceived by predators) and levels of predation. The pattern differences between *himera* and

erato cyrbia on the one hand, and *erato favorinus* and *erato lativitta* on the other both appear similarly great to our eyes, although *H. erato cyrbia* is somewhat unique in its iridescent blue background colour, very distinct from the black, yellow and red colours of *himera*. This could contribute to the narrower width of this zone if predators could more easily distinguish *himera* from *erato cyrbia* than *favorinus* from *lativitta*. However, there is reason to suppose that levels of predation may be lower in S. Ecuador, as jacamars (Galbulidae) have not been sighted in the contact zones. Jacamars are probably a major selective agent acting on *Heliconius* wing patterns, although other potential predators such as motmots (Motmotidae) and flycatchers (Tyrranidae) could also be important, and are present near Guayquichuma. Selection on colour patterns could be important in this hybrid zone, but it is hard to believe that it explains a four-fold increase in selection over that found in interracial hybrid zones.

Frequency dependent selection will also be critically dependent on the local abundance of other species in the same mimicry ring. However the only known mimic of either *H. erato cyrbia* or *H. himera* in the contact zone area is the *cyrbia* mimic, *H. melpomene cythera*. This species is extremely rare in the area. During the entire study only four individuals of *H. melpomene* were collected, two at site 4 and two in Balsas. This suggests that mimicry does not play a major role in this hybrid zone.

Assortative mating and hybrid inviability

Other selection pressures, not found in interracial zones, are almost certainly important in this hybrid zone. Neither the deficit of hybrids nor the correlation of the zone with ecological variables can be explained by predator selection on colour patterns alone. The hybrid deficit must be caused either by assortative mating (premating isolation) or selection against hybrids (postmating isolation). Behavioural and breeding experiments suggest that this is mainly a result of mating preferences rather than hybrid inviability (in prep.). The relative abundance of backcross hybrids (Table 1A, B) also suggests a lack of hybrid inviability or sterility, at least in the F1 generation. Assortative mating can explain the rarity of hybrids, but on its own cannot maintain a stable hybrid zone unless there is a "rare mate disadvantage" (Sanderson, 1989). Assortative mating could explain the hybrid deficit when coupled with some other form of selection that maintains the position and narrowness of the hybrid zone.

Habitat-dependent selection

The *himera/erato* hybrid zone studied here shows a clear association with the ecotone between wet and dry forest. Similar associations are found in the other *himera/erato* contact zones known. To the east of Bagua in the Río Marañon valley, *H. e. lativitta* replaces *H. himera* at a similar transition from dry thorn forest to wet forest (Konig, unpubl.; Mallet, 1993; Fig. 1). Also in northeastern Peru, *himera/erato* hybrids are known from the wet forest around Rodriguez de Mendoza where *H. e. favorinus* is common (Konig, 1986; Fig. 1); here, hybrids presumably result when *H. himera* flies over a mountain pass from Río Marañon valley dry forest to the wet forest habitat of *H.e. favorinus*.

The evidence for ecological association of the hybrid zones is supported by the wider scale biogeography of the two species. The range of *H. himera* (Fig. 1) has a dry climate and distinctive cactus/thorn scrub or gallery forest vegetation, in marked contrast to the tropical wet forest found further to the north and on the eastern slopes

of the Andes, where *H. erato* occurs. This dry forest region is a centre of endemism for both flora and fauna and has been described as a Pleistocene refugium where speciation occurred (Brown, 1979, 1981, 1982, Lamas, 1982). It is actually unlikely that this was a rainforest refuge as is claimed for other centres of endemism in the neotropics: it is more likely that the endemism is driven by adaptation to the distinctive habitat. Other butterfly species/races characteristic of the area include *Heliconius charithonia peruviana, H. clysonymus tabaconas*, the ithomiines *Hyalyris coeno latilimbata, Mechanitis mazaeus* ssp. nov., *Elzunia pavonii* and *Scada kusa* and the papilionid *Parides erlaces chinchipensis* (Brown, 1979; Lamas, 1982).

Distribution patterns across a hybrid zone can give clues as to the importance of habitat in structuring the cline. In some cases the association is so strong that a mosaic distribution pattern is found. A good example of this is between the crickets *Gryllus pennsylvanicus* and *G. firmus* (Rand & Harrison, 1989) in which an interlocking patchwork distribution of the two forms is strongly related to soil type. Such a pattern might be expected where forms with different ecological adaptations colonize habitat types distributed in a mosaic, and where each patch is sufficiently large relative to the dispersal of the organism (Harrison, 1990). In S. Ecuador, there is little evidence that such a pattern exists for *Heliconius*, and the clines appear to form simple monotonic transitions. This is probably due to the relative high per-generation dispersal rate in *Heliconius* which is sufficient to overcome the effect of habitat patchiness. Habitat patches would presumably have to be on the order of two hybrid zone widths ($\geq$ 10 km wide) to allow significant local adaptation. The *Heliconius* pattern is more like that in high-dispersal animals such as birds, where mosaic patterns are not found and hybrid zones are monotonic; these also commonly show correlations with habitat, but on a broader scale. Moore & Price (1993) found several bird hybrid zones in the Great Plains of N. America that correlated well with the forest/savannah boundary, including that between the northern flicker races (*Colaptes*).

Interracial *Heliconius* hybrid zones are also monotonic and in general show little association with habitat. Benson (1982) presents some evidence for a relationship between colour pattern races and aridity in *H. erato*. Rayed races tend to be found in the dense amazonian forests, while forms with red forewing and yellow hindwing bands (known as 'postman races') are often found in more open habitats of S. Brazil and the llanos of Colombia and Venezuela. Despite these broad associations there are plenty of exceptions to the pattern such as *H. e. favorinus*, a postman race which occurs in dense wet forest in eastern Peru (Mallet, 1993). There is not much evidence that hybrid zones so far studied between *H. erato* races show much correlation with habitat variables (Mallet, 1986, 1993; but see Benson, 1982). The ecological correlates of the *himera/erato* hybrid zones contrast strongly, and highlight the ecological differences between *H. himera* and the other races of *H. erato*.

Given the strong and consistent association of *H. himera* with dry regions, it seems likely that both the position and width of the transition zones between the two species are largely determined by ecological selection. What features of the habitat might cause this selection? There is no host plant specialization between the species and the primary host plants occur right across the hybrid zone (Table 2; Jiggins, McMillan & Mallet, 1996). This is good evidence that host plant adaptations have not diverged. More likely are adaptations to altitude or water stress. *H. himera* reaches some 400–500 m higher than *H. erato*, and this may be associated with physiological adaptations to altitude and in particular to lower temperatures. *H. himera* may be able to reach higher altitudes than *H. erato* simply because of the greater daytime

temperatures in the arid habitat. While altitude may be important, there is relatively little altitudinal variation across the hybrid zone (Table 1A, B), which is much more clearly associated with aridity and vegetation changes (Table 3). This suggests that selection associated with the wet/dry transition is more important. A possible candidate might be desiccation tolerance.

Competitive interactions

Interspecific competition is known to be important in determining species boundaries (Bull, 1991; Hoffmann & Blows, 1994). The classic studies where this has been demonstrated experimentally are in sessile organisms such as plants (Santelmann, 1991) and intertidal organisms (Connell, 1961); however there are also examples in more dispersive organisms such as between red and arctic foxes, *Vulpes vulpes* and *Alopex lagopus*. The fox species boundary is believed to be an interaction between body size and interspecific competition for food (Hersteinsson & Macdonald, 1992). If, as argued above, the *Heliconius* contact zone is determined by ecological adaptations to different habitats, competitive exclusion of the two ecologically similar species should take place where their respective habitats meet along an ecotone. This is perhaps a result of competition for the primary host plant species, *P. rubra* and *P. punctata* (Table 2). A switch of competitive advantage, from one species to the other, should occur at some point across the ecotone. This might give rise to the observed pattern even if the ecotone itself were considerably wider than the contact zone.

Multiple selection pressures

It seems probable that all five proposed selective forces, i.e. habitat, competitive exclusion, frequency-dependent selection on colour patterns, assortative mating and hybrid inviability (in approximately that order), may act together to maintain the narrowness and position of this contact zone. Although it will be hard to separate all of these effects, the geographic correlations discussed above give clear evidence that ecology plays a major role. Whatever the exact nature of selection, it must be very strong to explain the abrupt transition from *H. erato* to *H. himera*.

Genetics of species differences

To understand the evolutionary origins of the two species we need to compare the genetic basis of differences between them with those between races of *H. erato*. This study shows that there are at least six major loci, each of which codes for a different colour pattern element. Genetic studies of a number of races of both *H. erato* and *H. melpomene* have also shown major locus inheritance of colour patterns (Sheppard *et al.*, 1985). For example hybrid zones near Tarapoto in Peru involve three colour pattern loci in *H. erato* and four in *H. melpomene* (Mallet, 1989). Although the loci found here are similar to those known in *H. erato*, not all are homologous. In particular the loci controlling the yellow *himera* forewing band and red *erato* forewing band are independent; in *H. erato* races, no recombinants have been produced (Sheppard *et al.*, 1985; Mallet, 1989). The divergence in colour pattern between *H. himera* and *H. erato* is similar to, but more extensive than that between geographic races of *H. erato*. This suggests that the divergence of colour pattern between species and races involves the same genetic processes.

Mode of speciation

While the colour pattern differences between *H. himera* and *H. erato* are like those between geographic races, the strong ecological differences between the two species are not. The importance of ecological divergence in speciation is supported by evidence from other species closely allied to *H. erato*, which almost invariably show strong habitat differences. *Heliconius clysonymus* and *H. telesiphe* both occasionally overlap with *H. erato*, but are found at higher altitudes (800–2000 m as opposed to 0–1500 m). There is evidence for competitive exclusion between *H. clysonymus* and *H. erato* (Benson, 1978). Another close relative, *H. charithonia*, is found in similar forest edge and secondary growth habitats to those of *H. erato* (Table 3). Although *H. charithonia* feeds on most host plants used by *H. erato*, it also commonly feeds on *Passiflora adenopoda* whose hooked trichomes kill *H. erato* larvae placed on the plant (Gilbert, 1971). *Heliconius charithonia* lays larger clutches of smaller eggs, and is also more mobile, being the only member of the genus that regularly colonizes Caribbean Islands, and as far north as central Texas and Florida. In Panama and Costa Rica it is commonest in seasonally suitable cloud forest and savannah habitats; but is rare in habitats that are permanently suitable such as the Osa Peninsula, Costa Rica (Gilbert, 1991). This suggests that *H. charithonia* relies more on colonization of seasonally available habitats than the other species, in addition to its host plant divergence. Similarly, *H. hermathena*, a close relative of *H. charithonia*, is found in patches of savannah in the central Amazon, very different habitats to the surrounding rainforest where *H. erato* is found. According to Brown & Benson (1977), *H. hermathena* may have diverged in dry habitats surrounding Pleistocene forest refugia, so that its range has contracted into a few small pockets now that the rainforest has expanded. There is therefore substantial evidence among the species most closely related to *H. erato* that habitat-dependence is important in speciation, but is involved much less, if at all, in the divergence of races (see above under *Habitat-dependent selection*).

Within other clades in the genus, different ecological factors may be important. For example within the *H. melpomene* group, related species are often sympatric and apparently avoid competition by means of strong differences in host plant specificity and microhabitat (Smiley, 1978; Mallet & Gilbert, 1995). In conclusion, whereas geographic race formation does not involve much adaptation to habitat or host plants, speciation in *Heliconius* is strongly associated with such ecological changes.

Geographic context of speciation

The limited geographic extent of contact between *himera* and *erato* might at first sight suggest that divergence and speciation occurred in allopatry. However this seems somewhat unlikely as the wet and dry forest habitats of the two species would always have been in contact. Ecological traits can easily diverge in parapatry, providing habitat patches are sufficiently large relative to dispersal (Haldane, 1948).

Clines for a wide variety of different characters often occur together; for example the northern flicker hybrid zone is correlated with a habitat boundary, but was initially identified in a plumage trait, which is presumably sexually selected and not itself directly related to ecology (Moore, 1987; Moore & Price, 1993). In the *himera/*

erato hybrid zone, clines of colour pattern, ecological adaptation, mtDNA, allozymes, and mate choice all occur together. This has been used as *prima facie* evidence for secondary contact (Barton & Hewitt, 1985). However, a genetic barrier to gene flow is likely to accumulate further differences on each side, just as with a geographic barrier (Hewitt, 1989; Mallet, 1993). In *Heliconius*, strong adaptation to an ecological gradient could have prevented other traits, such as colour patterns, mate choice, and genetic markers spreading across the zone. As each selected trait became fixed, the genetic barrier would become more intense, and further accumulation of differences would become possible. Parapatric differentiation is at least as likely as allopatric on the available evidence.

CONCLUSION

The *himera/erato* hybrid zone is a good example of an intermediate stage in speciation. We propose that these two forms have already speciated because, although they hybridize, hybrids are rare in the contact zone, and gene flow does not result in homogenization. The contact zone is extremely narrow compared with interracial hybrid zones of *H. erato*, suggesting that selection is intense, $s \approx 1$. Although the hybrid zone between *himera* and *erato* conforms to a classical idea of a zone of secondary contact, being limited in extent and consistent at many genetic clines, it is clear that ecological adaptation in parapatry could have produced similar results. The strong habitat differences between *H. erato, H. himera* and other species in the clade, coupled with little ecological differentiation between geographic races, implicates ecological adaptation as a prime mover of speciation in *erato* group *Heliconius*. This study shows that considerable genetic, ecological, and behavioural information relevant to understanding speciation can be deduced directly from field data collected in an interspecific hybrid zone. Examples such as this are clearly more useful than allopatric species, where it is unclear whether, given sympatry, genetic integrity would be maintained or dissolve.

ACKNOWLEDGEMENTS

We would like to thank all those in Ecuador who have helped the project, in particular INEFAN for granting permission to carrying out research in the country; the Museo de Ciencias Naturales and Peter Wilson in Quito and Fundación Arcoiris in Loja for their support. Angus Davison, Ashleigh Griffin, Pablo Losano, Bolivar Medino and Pablo Andrade helped with fieldwork and plant identification. We are also grateful to Henrí Descimon, Andrew Brower, Keith Brown and Gerardo Lamas for advice and comments. This project is funded by a BBSRC research grant and studentship.

REFERENCES

Ackery PR, Smiles RL. 1976. An illustrated list of the type-specimens of the Heliconiinae (Lepidoptera: Nymphalidae) in the British Museum (Natural History). *Bulletin of the British Museum (Nat. Hist.), Entomology* **32:** 171–214.

Barton NH, Gale KS. 1993. Genetic analysis of hybrid zones. In: Harrison RG, ed. *Hybrid Zones and the Evolutionary Process*. New York: Oxford University Press, 13–45.

Barton NH, Hewitt GM. 1985. Analysis of hybrid zones. *Annual Review of Ecology and Systematics* **16:** 113–148.

Barton NH, Hewitt GM. 1989. Adaptation, speciation and hybrid zones. *Nature* **341:** 497–503.

Benson WW. 1978. Resource partitioning in passion vine butterflies. *Evolution* **32:** 493–518.

Benson, WW. 1982. Alternative models of infrageneric diversification in the humid tropics: tests with passion vine bufferflies. In: Prance GT ed. *Biological Diversification in the Tropics*. New York: Columbia University Press, 608–640.

Brower AVZ. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proceedings of the National Academy of Science*, USA **91:** 6491–6495.

Brown KS, Mielke OHH. 1972. The heliconians of Brazil (Lepidoptera: Nymphalidae). Part II. Introduction and general comments, with a supplementary revision of the tribe. *Zoologica, New York.* **57:** 1–40.

Brown KS. 1976. An illustrated key to the silvaniform *Heliconius* (Lepidoptera: Nymphalidae) with descriptions of new subspecies. Transactions of the American Entomological Society **102:** 373–484.

Brown KS. 1979. *Ecologia Geográfica e Evolução nas Florestas Neotropicais.* 2 vols. (Livre de Docencia) Universidade Estadual de Campinas, Campinas, Brazil.

Brown KS. 1981. The biology of *Heliconius* and related genera. *Annual Review of Entomology* **26:** 427–456.

Brown KS. 1982. Paleoecology and regional patterns of evolution in neotropical forest butterflies. In: Prance GT, ed. *Biological Diversification in the Tropics*. New York: Columbia University Press, 255–308.

Brown KS, Benson WW. 1977. Evolution in modern Amazonian non-forest islands: *Heliconius hermathena. Biotropica* **9:** 95–117.

Brown KS, Sheppard PM, Turner JRG. 1974. Quaternary refugia in tropical America: evidence from race formation in *Heliconius* butterflies. *Proceedings of the Royal Society, London (B)* **187:** 369–378.

Bull CM. 1991. Ecology of parapatric distributions. *Annual Review of Ecology and Systematics* **22:** 19–36.

Connell JH. 1961. The influence of interspecific competition and other factors on the distribution of the barnacle *Chthamalus stellatus. Ecology* **42:** 710–723.

Coyne JA, Orr HA. 1989. Two rules of speciation. In: Otte D, Endler JA, eds. *Speciation and its Consequences.* Sunderland, MA: Sinauer Associates. 180–207.

Descimon H, Mast De Maeght J. 1984. Semispecies relationships between *Heliconius erato cyrbia* Godt. and *H. himera* Hew. in southwestern Ecuador. *The Journal of Research on the Lepidoptera* **22:** 229–239.

Dobzhansky T (ed.). 1937. *Genetics and the origin of Species.* Columbia University Press.

Eltringham H. 1916. On specific and mimetic relationships in the genus *Heliconius. Transactions of the Entomological Society, London,* **1916:** 101–148.

Emsley MG. 1965. Speciation in *Heliconius* (Lep., Nymphalidae): morphology and geographic distribution. *Zoologica, N.Y.* **50:** 191–254.

Endler JA. 1977. *Geographic Variation, Speciation and Clines.* Princeton, N.J: Princeton University Press.

Gilbert LE. 1971. Butterfly-plant coevolution: has *Passiflora adenopoda* won the selectional race with heliconiine butterflies? *Science* **172:** 585–586.

Gilbert LE. 1975. Ecological consequences of a coevolved mutualism between butterflies and plants. In: Gilbert LE, Raven PR. eds. *Coevolution of Animals and Plants.* Austin, TX: University of Texas Press, 210–240.

Gilbert LE. 1991. Biodiversity of a Central American *Heliconius* community: pattern, process, and problems. In: Price PW, Lewinsohn TM, Fernandes TW, Benson WW eds. *Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions.* New York: John Wiley & Sons. 403–427.

Grant PR, Grant BR. 1992. Hybridization of bird species. *Science* **256:** 193–197.

Guillaumin M, Descimon H. 1976. La notion d'espèce chez les Lépidoptères. In: Bocquet C, Génermont J, Lamotte M, eds. *Les Problèmes de l'Espèce dans le Règne Animal,* Vol. 1. Paris: Société zoologique de France, 129–201.

Haldane JBS. 1922. Sex ratio and unisexual sterility in hybrid animals. *Journal of Genetics* **12:** 101–109.

Haldane JBS. 1948. The theory of a cline. *Journal of Genetics* **48:** 277–284.

Harrison RG. 1990. Hybrid zones: windows on evolutionary processes. In: Futuyma D, Antonovics J, eds. *Oxford surveys in Evolutionary Biology. Vol. 7.* Oxford: Oxford University Press, **7:** 69–128.

Harrison RG. 1993. Hybrids and hybrid zones: historical perspective. In: Harrison RG, ed. *Hybrid Zones and the Evolutionary Process.* New York: Oxford University Press, 3–12.

Hersteinsson P, Macdonald DW. 1992. Interspecific competition and the geographical distribution of red and arctic foxes *Vulpes vulpes* and *Alopex lagopus. Oikos* **64:** 505–515.

Hewitt GM. 1989. Divergence and speciation as viewed from an insect hybrid zone. *Canadian Journal of Zoology* **68:** 1701–1715.

Hewitt GM. 1989. The subdivision of species by hybrid zones. In: Otte D, Endler JA, eds. *Speciation and its Consequences.* Sunderland, MA: Sinauer Associates, 85–110.

Hoffmann AA, Blows MW. 1994. Species borders: ecological and evolutionary perspectives. *Trends in Ecology and Evolution* **9:** 223–227.

Holzinger H, Holzinger R. 1994. *Heliconius* and related Genera. Lepidoptera: Nymphalidae. The Genera *Eueides, Neruda* and *Heliconius.* Sciences Nat., Vennette, France, 328 pages.

Jiggins CD, McMillan WO, Mallet J. 1996. Host plant adaptation has not played a role in the recent speciation of *Heliconius himera* and *Heliconius erato* (Lepidoptera: Nymphalidae). *Evolution.* submitted.

Kaye WJ. 1916. A reply to Dr. Eltringham's paper on the genus *Heliconius*. *Transactions of the Entomological Society, London* **1916:** 149–155.

König F. 1986. Ein *Heliconius erato himera* — Hybrid aus Nord-Peru (Lepidoptera, Heliconiiae). *Zeitschrift der Arbeitsgemeinschaft Österreichischen Entomologen* **38:** 49–50.

Lamas G. 1976. Notes on Peruvian butterflies (Lepidoptera). II. New *Heliconius* from Cusco and Madre de Dios. *Revista Peruana Entomologia* **19:** 1–7.

Lamas G. 1982. A preliminary zoogeographical division of Peru based on butterfly distributions (Lepidoptera, Papilionoidea). In: Prance GT ed. *Biological Diversification in the Tropics*. New York: Columbia University Press. 336–357.

Mallet J. 1986. Hybrid zones in *Heliconius* butterflies in Panama, and the stability and movement of warning colour clines. *Heredity* **56:** 191–202.

Mallet J. 1989. The genetics of warning colour in Peruvian hybrid zones of *Heliconius erato* and *H. melpomene*. *Proceedings of the Royal Society, London (B)* **236:** 163–185.

Mallet J. 1993. Speciation, raciation, and color pattern evolution in *Heliconius* butterflies: evidence from hybrid zones. In: Harrison, RG ed. *Hybrid Zones and the Evolutionary Process*. New York: Oxford University Press, 226–260.

Mallet J. 1995. A species definition for the Modern Synthesis. *Trends in Ecology and Evolution* **10:** 294–299.

Mallet J, Barton NH. 1989a. Inference from clines stabilized by frequency-dependent selection. *Genetics* **122:** 967–976.

Mallet J, Barton NH. 1989b. Strong natural selection in a warning colour hybrid zone. *Evolution* **43:** 421–431.

Mallet J, Barton NH, Lamas G, Santisteband J, Muedas M, Eeley H. 1990. Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *Heliconius* hybrid zones. *Genetics* **124:** 921–936.

Mallet J, Gilbert LE. 1995. Why are there so many mimicry rings? Correlations between habitat, behaviour and mimicry in *Heliconius* butterflies. *Biological Journal of the Linnean Society* **55:** 159–180.

Mayr E. 1940. Speciation phenomena in birds. *American Naturalist* **74:** 249–278.

Moore WS. 1987. Random mating in the Northern flicker hybrid zone: implications for the evolution of bright and contrasting plumage patterns in birds. *Evolution* **41:** 539–546.

Moore WS, Price JT. 1993. Nature of selection in the northern flicker hybrid zone and its implications for speciation theory. In: Harrison, RG ed. *Hybrid Zones and the Evolutionary Process*. New York: Oxford University Press, 196–225.

Pyle RL, Randel JE. 1994. A review of hybridization in marine angelfishes (Perciformes, Pomacanthidae). *Environmental Biology of Fishes* **41:** 127–145.

Rand DM, Harrison RG. 1989. Ecological genetics of a mosaic hybrid zone: mitochondrial, nuclear, and reproductive differentiation of crickets by soil type. *Evolution* **43:** 432–449.

Sanderson N. 1989. Can gene flow prevent reinforcement? *Evolution* **43:** 1223–1235.

Santelmann MV. 1991. Influences on the distribution of *Carex exilis*: an experimental approach. *Ecology* **72:** 2025–2037.

Sheppard PM, Turner JRG, Brown KS, Benson WW, Singer MC. 1985. Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. *Philosophical Transactions of the Royal Society, London (B)* **308:** 433–613.

Smiley JT. 1978. Plant chemistry and the evolution of host specificity: new evidence from *Heliconius* and *Passiflora*. *Science* **201:** 745–747.

Szymura JM, Barton NH. 1986. Genetic analysis of hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata* near Cracow in Southern Poland. *Evolution* **40:** 1141–1159.

Szymura JM, Barton NH. 1991. The genetic structure of the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*: comparisons between transects and between loci. *Evolution* **45:** 237–261.

Turner JRG. 1971. Two thousand generations of hybridization in a *Heliconius* butterfly. *Evolution* **25:** 471–482.

Turner JRG. 1976. Adaptive radiation and convergence in subdivisions of the butterfly genus *Heliconius* (Lepidoptera: Nymphalidae). *Zoological Journal of the Linnean Society* **58:** 297–308.

Turner JRG, Johnson MS, Eanes WF. 1979. Contrasted modes of evolution in the same genome: allozymes and adaptive change in *Heliconius*. *Proceedings of the National Academy of Science.* USA **76:** 1924–1928.

APPENDIX

Complete list of wild caught hybrids and their phenotypes

Ref. no.	Proposed hybrid class	Sex	Site no.	Collection	Fw band	Yellow hw bar cyrbia	Red hw bar himera	Blue iridescence	White hw edge
Parental phenotypes									
Heliconius erato cyrbia					red	+	–	+	+
Heliconius himera					ye	–	+	–	–
1	F1	m	4	Descimon	red(wh)	–	+	(+)	–
2	F1	m	4	Descimon	red(wh)	–	+	(+)	–
3	F1	m	4	Descimon	red(wh)	–	+	(+)	–
4	BC	m	4	Descimon	red	+	+	+	+
5	BC	m	4	Descimon	red	+	+	(+)	+
6	BH	m	4	Descimon	wh/red	–	+	–	–
7	F1	m	4	Mallet	red(wh)	–	+	(+)	–
8	F1	m	4	Mallet	red	–	+	(+)	–
9	BC	m	4	Mallet	red	+	–	–	+
10	BH	m	4	Neukirchen	wh/red	–	+	–	–
11	F1	m	4	Neukirchen	red	–	+	(+)	–
12	BC	m	4	Neukirchen	red	–	+	+	(+)
13	BC	m	4	Neukirchen	red	–	+	+	(+)
14	BC	f	4	Neukirchen	red	(+)	–	+	(+)
15	BH	m	4	Neukirchen	ye/red	–	+	–	–
16	F1	m	4	Neukirchen	red(wh)	–	+	(+)	–
17	BC	m	4	Neukirchen	red	+	+	+	+
18	BC	f	4	Neukirchen	red	+	+	–	+
19	F1	m	5	J/M/M	red	(+)	+	–	–
20	F1	m	9	J/M/M	red	(+)	+	–	–
21	F1	m	6	J/M/M	red	(+)	+	–	–
22	F1	m	6	J/M/M	red	(+)	+	–	–
23	BH	m	5	J/M/M	wh/red	–	+	–	–
24	F1	m	4	J/M/M	red(wh)	–	+	(+)	–
25	BH	m	4	J/M/M	none(ye)	–	+	–	–
26	BC	m	4	J/M/M	red	(+)	–	+	(+)
27	BC	f	4	J/M/M	red	(+)	–	+	(+)
28	BC	f	3	J/M/M	red	+	+	+	+
29	BC	m	2	J/M/M	red	(+)	–	+	(+)
30	BC	m	6	J/M/M	red	(+)	–	+	(+)
31	BH	M	6	J/M/M	none(ye)	–	+	–	–
32	F1	M	5	J/M/M	red	–	+	(+)	–
33	F1	M	5	J/M/M	red(wh)	–	+	(+)	–
34	F1	m	5	J/M/M	red(wh)	–	+	(+)	–
35	BC	m	5	J/M/M	red	(+)	–	+	–
36	F1	m	8	J/M/M	red	–	+	(+)	–
37	F1	f	15	J/M/M	red	–	+	(+)	–
38	BC	m	15	J/M/M	red	(+)	–	+	(+)
39	BH	f	4	J/M/M	red(ye)	–	+	–	–
40	F1	m	4	J/M/M	red	–	+	(+)	–
41	BH	m	7	J/M/M	red/ye	–	+	–	–
42	BH	f	5	J/M/M	red/ye	–	+	(+)	–
43	BC	m	7	J/M/M	red	+	+	+	+

Site numbers are as shown in Figure 2. Nos. 1–6 were illustrated by Descimon & Mast de Maeght (1984). Nos. 10–18 are held in the collection of W. Neukirchen. Nos. 19–43 were collected by Jiggens, Mallet and McMillan in 1994–5 and are held by the authors. The 'proposed hybrid class' is based upon our interpretation of the phenotypes where BC = backcross to *H. e. cybria* and BH = backcross to *H. himera*. Where pattern elements are shown in brackets they were present as a trace; for the purposes of our analysis (Table 4) they were considered absent. Forewing band colours are wh = white, ye = yellow.

CHAPTER 2

Maintenance of species differences across a *Heliconius* hybrid zone

Submitted to *Heredity* with:
WO McMillan, P King and J Mallet

Abstract

A contact zone between *Heliconius erato* and *H. himera* in southern Ecuador provides an opportunity to study the transition from races to species in heliconiine butterflies. Genetic differentiation at 30 allozyme loci (D=0.28) is five times greater between allopatric populations of *himera* and *erato* than among races of *erato* (D<0.062). Analysis of restriction fragment length polymorphisms in an 800 base pair region of the mitochondrial genome shows fixed differences between the species. Despite 5-10% hybridisation per generation these mtDNA, allozyme and colour pattern differences are in almost complete linkage disequilibrium throughout the contact zone. In mixed populations there was no consistent evidence for convergence of *himera* and *erato* allozyme allele frequencies, and only four individuals out of 383 examined showed evidence for interspecific mtDNA gene flow. Linkage analysis of backcross broods showed that the eleven allozyme and 2 colour pattern loci which are markedly divergent between the species map to eight of 21 chromosomal linkage groups. Therefore barriers to gene flow are not restricted to just a few strongly selected loci. Although analysis of population structure shows little evidence for interspecific gene flow, strong differences between allozyme loci in levels of divergence suggest that selection and gene flow may affect loci in different ways. Hybrid zones such as this, where divergent genotypes coexist, should provide good model systems for the study of speciation.

Introduction

The claim that hybrid zones have provided 'natural laboratories' for the study of speciation (Barton & Hewitt, 1989) may be overly optimistic. Hybrid zones in organisms as diverse as mice, grasshoppers, crickets, sunfish, toads and butterflies separate highly differentiated taxa, but the morphological, ecological and behavioural differences between hybridising forms are broken down in contact zone populations, which consist mainly of hybrids (Hunt & Selander, 1973; Avise & Smith, 1974; Szymura & Barton, 1986 & 1991; Mallet, 1986; Mallet & Barton, 1989; Moore, 1987; Butlin & Hewitt, 1985; Rand & Harrison, 1989). In other words speciation has not taken place, and given that many of these zones appear to have been stable over thousands of generations, it is not clear that speciation is imminent. Whilst such studies permit the estimation of i) dispersal, ii) the strength and nature of selection and iii) even the number of loci under selection (Szymura & Barton, 1991; Barton & Gale, 1993), they tell us little about the coexistence of divergent genotypes.

Contact zones where multi-locus genotypic differences are maintained in mixed populations may be better natural laboratories for the study of speciation (e.g. Chu, Powers & Howard, 1995). Needless to say, many of the selection pressures identified in traditional hybrid zone studies are likely to be important in speciation. For example, F1 males in crosses between *Chorthippus parallelus* races are completely sterile, i.e.'Haldane's Rule' (Haldane, 1922; Hewitt et al., 1987). It is now important to make the link between the processes operating in hybrid zones and those involved in the coexistence of species.

Heliconius butterflies provide an opportunity to study the changes associated with the transition from randomly mating racial varieties to non-hybridising species (Jiggins et al., 1996; McMillan et al., 1997). *Heliconius* show striking divergence in warning colour both within and between species. For example, there are nearly 30 parapatric colour pattern races of *H. erato* across the New World Tropics, which coexist with races of the Müllerian co-mimic, *H. melpomene* (Brown, 1979). Boundaries between these races are abrupt clines at just a few loci, maintained by strong frequency-dependent selection (in some cases $s \approx 0.5$, Mallet & Barton, 1989; Mallet et al., 1990). This strong selection on colour pattern loci generates only a weak barrier to gene flow across most of the genome. There is no evidence that races differ at

allozyme loci, and geographic differentiation in mitochondrial haplotypes is not concordant with colour pattern boundaries (Turner et al., 1979; Brower, 1996).

H. himera is a sister taxon to *H. erato* which appears to have evolved sufficient barriers to gene flow to allow stable co-existence throughout areas of overlap. *H. himera* replaces *erato* in the dry forests of south western Ecuador and northern Peru. The taxa meet in narrow zones where mixed populations consist of only 5-10% phenotypically distinguishable hybrids (Jiggins et al., 1996). This deficit of hybrids is primarily due to strong mating preferences (McMillan et al., 1997). Colour pattern differences between *himera* and *erato* are defined by only a few major loci which are likely to be under strong stabilizing selection (Benson, 1972; Mallet & Barton, 1989; Chapter 3). It is conceivable therefore that the deficit of colour pattern hybrids observed in the *himera* / *erato* hybrid zone does not reflect a genome wide pattern. Crosses between the two parental types, as well as first and second generation backcrosses and F2s, produce fertile and viable offspring (McMillan et al., 1997). A combination of persistent hybridisation and weak post-mating isolation may permit most of the genome to recombine freely, as occurs across racial boundaries.

Here we use allozyme and mtDNA markers to investigate genetic differentiation between *himera* and *erato*. Although nothing was known about levels of differentiation at allozyme loci, a previous study suggested marked differences (≈1.5% sequence divergence) between mitochondrial cytochrome oxidase sequences of the two species (Brower, 1994). However this conclusion was based on only two *himera* individuals collected from northern Peru, and it is unclear whether the observation reflects fixed species differences. Extensive sampling across the range of both species allows us to address the following questions; 1) Are there genetic differences between *himera* and *erato* in addition to colour pattern differences? 2) If there are genetic differences, is there strong linkage disequilibrium between allozyme, mtDNA and colour pattern loci across the contact zone? 3) From the patterns observed what deductions can be made about the strength and nature of the barrier to gene flow?

Methods

Butterflies were collected in Ecuador between January 1994 and April 1995. Wings were removed and the bodies frozen in LN_2 and transported to the UK. Collection sites correspond to those of Jiggins et al. (1996). Additional collections were made in allopatric populations 390km (*cyrbia*, Alluriquín, Prov. Pichincha) and 60km (*himera*, Vilcabamba, Prov. Loja) from the contact zone centre. Throughout this paper 'allopatric populations' are defined as all populations which contain no individuals with colour pattern markers characteristic of the other species; 'sympatric populations' are all populations which contain colour pattern alleles of both species. The classification of individuals as *erato cyrbia*, *himera* or 'hybrid' is based on colour pattern morphology (Jiggins et. al., 1996).

Protein electrophoresis

A total of 259 individuals from allopatric populations and 411 individuals from mixed populations were analysed for electrophoretic variation. The thorax and abdomen of each individual was homogenised on ice in 80µl of grinding buffer (Mallet et al., 1993; Emelianov et al., 1995). The homogenate was centrifuged to remove cellular debris. Electrophoresis was performed on Helena cellulose acetate plates using two buffer systems: Phosphate, 0.36% NaH_2PO_4, 0.22% Na_2HPO_4, pH 6.3 and TrisGly, 0.3% Trizma, 1.4% glycine, pH 8.6. Gels were run with both species on each plate in order to ensure accurate scoring of alleles between runs. Plates were stained according to recipes described elsewhere (Mallet et al., 1993; Emelianov et al., 1995).

mtDNA RFLP analysis

Total cellular DNA was extracted from either 50µl of protein homogenate in 250µl STE buffer (0.05M Tris buffer; 0.45M NaCl; 0.1M EDTA), or a small amount of frozen tissue (≈ half a thorax) homogenised in 300µl STE buffer. Sodium dodecyl sulphate was added to make a final concentration of 0.5%. The solution was then digested overnight at 55°C with 0.6mg Proteinase K. After three extractions, firstly with phenol, then phenol / chloroform and finally chloroform, the DNA was precipitated with ethanol (Sambrook et. al., 1989).

An 800bp region of mitochondrial DNA spanning the 3' end of the cytochrome oxidase subunit I (COI) gene, the leucine tRNA gene and most of the cytochrome

oxidase subunit II (COII) gene was amplified using primer sequences 5'ccataataaattccaggycggttaa3' and 5'gaaatattatttcctcttttggatcc3'. The PCR protocol used was 40 cycles with a cycling profile of 94°C/30secs; 50°C/30secs; 73°C/1min30secs. In published sequences there were 19 base pair substitutions between *himera* and *erato cyrbia* in this region (Brower, 1994 & 1996). Two of these fell within the recognition sequences of the restriction enzymes *Mun I* and *Rca I* (position 689 and 455 respectively of Appendix Fig. 1, Brower 1996). This allowed the simple diagnosis of individual haplotypes through analysis of differences in restriction fragment lengths (RFLP). The PCR product was digested overnight with the two restriction enzymes (0.1unit/µl) at 37°C. Digestion products were separated by electrophoresis on 1.5% agarose gel and detected by staining with ethidium bromide.

Analysis of data

Calculation of genetic distance and tests for deviation from Hardy-Weinberg equilibrium were performed using BIOSYS (Swofford and Selander, 1989). For sex linked loci χ^2 tests for deviation from Hardy-Weinberg equilibrium were carried out excluding all hemizygous (i.e. female) individuals. Estimates of Fst were calculated using GENEPOP (Raymond and Rousset, 1995) at all loci for which data was available in all populations. This program employs the method of Weir and Cockerham (1984) to calculate θ as an estimate of Fst and tests whether these estimates differ significantly from zero using a Fisher exact test.

Two further races of *H. erato, H. e. etylus* (Zamora, prov. Zamora-Chinchipe) and *H. e. lativitta* (Tena, prov. Napo), were sampled on the eastern slopes of the Andes. These were not included in the population study due to a lack of data at several loci, but were used to generate a neighbour joining tree (Saitou & Nei, 1987) of the three races of *erato,* (*etylus, cyrbia* and *lativitta*) and *H. himera.* The tree was drawn according to Swofford & Olsen (1990) using Nei's genetic distance (1978), calculated for the 19 loci at which data was available (GPI, GOT-s, GOT-f, PGM, MPI, MDH-s, 3-P, G6-s, PK, AK, ENO, ACON-f, HBDH, ME, GPT, α-GPD, 6-PGD, LA and SDH). Genetic distance and allele frequency estimates were calculated between allopatric populations of *H. himera* and *H. erato.*

Linkage relationships between diagnostic allozyme markers were established using hybrid broods. It is relatively simple to produce crude linkage maps in *Heliconius* as

there is no crossing over in females (Turner & Sheppard, 1975). Thus only a small number of progeny are necessary to determine whether two loci are syntenic, provided the female parent is a double heterozygote. In such crosses any recombination implies that markers lie on different chromosomes. Protein electrophoresis for 11 differentiated loci was carried out on eight backcross broods, four F1 x *himera* and four F1 x *erato*, with a minimum of 24 progeny from each. Support for synteny or otherwise is deduced from at least one of these broods. The parents of each brood were run on the same plate as the offspring. The colour patterns of *Heliconius* butterflies are controlled by relatively few genes of large effect (Sheppard et al., 1985; Mallet, 1989). In the case of *himera* and *erato* two major loci have been identified (see Chapter 3). These were also scored in hybrid broods to investigate linkage with allozyme loci.

Species index scores

None of the allozyme loci were completely fixed for different alleles between the species, so introgressed alleles were difficult to identify with any certainty. 'Species index' scores were devised to give populations or individuals a numerical score which measures their genetic similarity with allopatric *himera* or *erato.* This was done by weighting each allele according to the species in which it was most frequent. Only alleles which showed either a greater than 0.1 frequency difference between the two species, or those only found in one species were used. Alleles most common in *himera* were given a score of 0 and those in *erato* 1 (Table 1). Mean values were then calculated over all alleles to give a score between 0 and 1. These mean scores were calculated both for individuals (over all loci) and for each locus (summed over individuals within a population). Correlations between individual locus index scores and distance from the centre of the contact zone were investigated within each species, for all populations within 30km of the contact zone.

Results

A total of 30 enzyme loci were scored (Table 1). Of these 9 loci were monomorphic, and at four more *himera* and *erato* shared a common allele with frequency >0.9 in both species. The remaining 17 polymorphic loci showed some frequency or allelic differences between the species. Six loci were virtually fixed for different alleles in *himera* and *erato* - SOD, PK, GOT-s, G6-s, ACON-s and HBDH, whilst the

remainder showed frequency differences between the species and in most cases rare alleles characteristic of one or other species. Overall levels of heterozygosity were not significantly different in the two species (0.139 ±0.040 SE in *himera* and 0.143 ±0.035 SE in *erato,* Nei's (1978) unbiased estimates). However the mean number of alleles per locus was significantly lower in *himera* (1.9 ±0.2) as compared to *erato* (2.7 ±0.3). The overall genetic distance between allopatric populations of *H. e. cyrbia* and *H. himera* was 0.278 (Nei's (1978) unbiased estimate) over all 30 loci. This estimate was nearly five times greater than the maximum distance (D=0.062) between geographic races of *H. erato* (Fig. 1).

Deviation from Hardy-Weinberg equilibrium were examined within each species, at each of the 14 loci used for population structure analysis (Table 1), excluding colour pattern hybrids. 11 in 153 tests showed significant deviations from Hardy-Weinberg expectations in *H. e. cyrbia*, and 6 in 109 tests in *H. himera.* These deviations were almost invariably due to occasional homozygotes for rare alleles with very low expected values, which may have been mis-scored individuals. When χ^2 values were summed within loci or populations there were no significant deviations from Hardy-Weinberg equilibrium [using the 'pooled genotype test' where appropriate (Swofford & Selander, 1989)]. Most allozyme data sets show a few significant deviations from Hardy-Weinberg equilibrium (e.g. Mallet et al., 1993; Emelianov et al., 1995), but if there is no consistent pattern across loci or populations these deviations are unlikely to be due to pervasive effects such as selection, non-random mating or hybridisation.

There was no evidence for population structuring within either species. Estimates of Fst within each species were, without exception, very low and not significantly different from zero. Overall values of θ were 0.0060 in *H. erato cyrbia* and 0.0003 in *H. himera.* Although most of the samples were from within 30km of the contact zone, this estimate also includes samples 390km (*H. e. cyrbia*), and 60km (*H. himera*) from the contact zone.

In contrast there is a sharp genetic break between *himera* and *erato* with no consistent evidence for convergence of species gene frequencies in the contact zone (Fig. 3). Correlation analysis of species index score against distance from the centre of the zone showed only two significant associations out of 22 tests (11 loci in each

species). These were 3-P in *H. himera* (r=0.698, p=0.025) and ACON-s in *H. e. cyrbia* (r=0.578, p=0.05). Both of these were in the right direction to suggest introgression, i.e. gene frequencies became more similar to the other species in populations near the contact zone. Although the correlation coefficient was significant in ACON-s, the slope of the regression line was very shallow(Fig. 3j). In contrast the pattern at 3-P does suggest that there was consistent introgression at this locus, although it only occurred in one direction (from *erato* into *himera*). There was, therefore, only evidence in one test of 22 that hybridisation has significantly affected the geographic distribution of allele frequencies. Furthermore the combined data showed no significant effects (Fig. 3a; *cyrbia*; r=0.296, NS; *himera*; r=0.366, NS).

Indeed, the distribution of individual species index values in sympatric populations (Fig. 2) forms a bimodal distribution. The phenotypically 'pure species' form two distinct genetic clusters with the colour pattern hybrids lying between the two. If these hybrids were to be removed from the plot there would be little difference between the allopatric and sympatric genotypic distributions of *himera* and *erato* (Fig. 2).

Linkage analysis of *himera x erato* hybrid broods has identified three linkage groups among the 11 loci which show marked species differences [AK - G6-s - GOT-s]; [PK - ACON-f]; [3-P - SOD] (Table 3). The latter group are X-linked. In addition ACON-s is linked to a colour pattern locus, Cr. The lack of recombination in female *Heliconius* (Turner & Sheppard, 1975) means these linkage groups, and other unlinked loci can be confidently allocated to different chromosomes, with the brood sizes analysed here (N>24). Thus the 11 allozyme and two colour pattern loci described represent 8 chromosomal linkage groups of the 21 in *Heliconius erato* (Brown et al., 1992).

The mtDNA data show a similarly abrupt break between *himera* and *erato*. There was a fixed mtDNA haplotype difference between the species in allopatric populations, and only 4 *himera* and no *erato* individuals had introgressed haplotypes in sympatric populations (Table 2). The allozyme genotypes of these introgressed individuals suggest that three of the four could be first generation backcrosses to *himera* (species index scores of the four individuals are 0.143, 0.263, 0.286 and 0.318; for comparison see Fig. 2). The pattern in hybrids was also informative. Of

11 F1 phenotypes, nine had an *erato* haplotype and two a *himera* haplotype, which implies that hybridisation occurs in both directions.

There was no evidence for heteroplasmy in any individuals. Two allopatric *cyrbia* individuals showed patterns which implied the loss or gain of a restriction site, but retained a characteristic fragment length so could be confidently scored as *cyrbia* haplotypes. One allopatric *cyrbia* individual showed a banding pattern which could not be confidently assigned to either haplotype.

Discussion

An important characteristic of a species is the maintenance of genetic integrity when coexisting with related taxa (Templeton, 1989). Colour pattern differentiation between *himera* and *erato* is associated with a geographically concordant break at nuclear (D = 0.28) and cytoplasmic loci. Furthermore, despite persistent hybridisation and no hybrid sterility or inviability (McMillan et al., 1997), *Heliconius himera* and *erato* maintain their genetic differences in sympatry. Throughout the contact zone there is strong linkage disequilibrium between colour pattern, mtDNA and allozyme markers. Linkage analysis shows that these multi-locus genetic differences map to eight of the 21 chromosomes in *H. erato.* This implies that barriers to gene flow are dispersed across the genome and not just associated with a few strongly selected colour pattern loci.

The abrupt genetic break between *himera* and *erato* is far larger than any differentiation acquired solely through isolation by distance. Across western Ecuador we found no evidence for population structure within either species. This is largely in agreement with previous studies of *H. erato,* which showed no large frequency or fixed differences between populations from as far apart as Trinidad, Panama, western Ecuador and Peru (Turner et al., 1979; Mallet, 1988; Silva & Araújo, 1994). The allozyme cluster analysis shows that races of *erato* from both sides of the Andes cluster together with Nei's D ≈ 0.06 (Fig. 1). In contrast the genetic break between *himera* and *erato* is five times greater and occurs over the space of just a few kilometres.

The barrier to gene flow is highlighted by the lack of mtDNA introgression. Because cytoplasmic makers are less likely to be linked to genes involved in reproductive, behavioural or ecological differences, they might be expected to introgress more readily than nuclear markers (Barton and Jones, 1983). This prediction is supported by a number of studies which have shown introgression of mtDNA across strong barriers to nuclear gene flow, such as hybrid zones or even between reproductively isolated sympatric species (Takahata & Slatkin, 1984; Gyllensten et al., 1985). However, there is little evidence for introgression in *himera* and *erato* where we found only 4 introgressed mtDNA haplotypes in 383 individuals sampled from mixed populations.

Despite the abrupt transition at many unlinked loci, there is some evidence for differential effects of selection among allozyme loci and even gene flow across this species boundary. Allozyme loci fall into three distinct groups (Fig. 4), with six showing almost fixed differences between *himera* and *erato* ($\theta > 0.8$), five showing intermediate differentiation ($0.2 < \theta < 0.35$) and the remaining 11 little or no differentiation ($\theta < 0.15$). Under neutral theory the rate of evolution, and hence divergence between populations, is directly proportional to mutation rate (Kimura, 1983). When loci are compared within a population, thus eliminating variation in population size, levels of heterozygosity will reflect mutation rate. If the pattern observed at different loci were solely a result of neutral divergence we would expect those loci with higher levels of heterozygosity to show the greatest differentiation. This is not the case and there is no relationship between divergence (θ) and overall heterozygosity ($r=0.02$; NS) which suggests that the differences between loci cannot be explained by neutral theory alone. Similar patterns have been observed in studies of sibling species, such as *Drosophila pseudoobscura* and *D. persimilis* (Prakash, 1976) and may indicate that loci are subject to different evolutionary pressures (Takahata & Slatkin, 1984). For example, faster rates of divergence might result from disruptive selection on protein differences (Feder et al., 1997), whilst slower rates of divergence could be due to either gene flow across the species barrier or stabilising selection on polymorphisms (Watt, 1983; Watt et al., 1983; Bancroft et al., 1995).

Marked differences between loci are also evident in phylogenetic comparisons of *himera* with the races of *erato*. MtDNA data imply a similar level of divergence

between west and east Andean *erato,* as between *erato* and *himera* (1.5 - 2% sequence divergence in the mitochondrial COI and COII genes) (Brower, 1994). In contrast, allozyme data show *himera* to be strongly differentiated from all the races of *erato* (Fig. 1). The most likely reasons for this discordance are, firstly, stabilising selection on allozymes within *erato* (e.g. Karl & Avise, 1992) , and secondly disruptive selection on protein loci between *himera* and *erato.* Disruptive selection is likely to result from adaptation to novel environments, such as the shift from dry to wet forest which occurs across the *himera / erato* hybrid zone (Chapter 5). In the future gene genealogies of protein coding loci provide a promising means to differentiate the effects of selection and gene flow among loci.

The apparent strength of the barrier to gene flow between *H. himera* and *H. erato* is perhaps surprising in light of the observed level of hybridisation. However, the lack of completely fixed species differences, and the marked differences in rates of divergence between loci probably indicate that some interspecific gene flow occurs. The barrier to gene flow is sufficient to allow the accumulation of neutral or divergently adaptive differences, but may be permeable to the spread of globally advantageous alleles.

The pattern described provides a marked contrast, not only with racial hybrid zones in *H. erato*, but with most other hybrid zones described in the literature where even highly differentiated taxa mate randomly and the genomes recombine freely when in contact. For example when the distribution of allozyme hybrid index scores is plotted for a *Bombina* hybrid zone, there is a unimodal distribution (Szymura & Barton, 1986; Barton & Gale, 1993). Similarly, in the centre of hybrid zones between *Mus musculus musculus* and *M. m. domesticus*, there is no deviation from Hardy-Weinberg equilibrium at allozyme loci (Hunt & Selander, 1973). In contrast, across the *himera / erato* contact zone parental genotypes coexist despite hybridisation, which allows identification of the processes which prevent homogenisation of gene pools in the early stages of species formation. In the case of *himera* and *erato,* the barrier to gene flow is most likely a result of divergence in mate preferences, warning colour and ecology, because there is no evidence for hybrid inviability or sterility (Jiggins et al., 1996; McMillan, et al., 1997). Examples such as this can truly be considered 'natural laboratories' for the study of speciation.

References

AVISE, J.C. AND SMITH, M.H. 1974. Biochemical genetics of sunfish. I. Geographic variation and subspecific intergradation in the bluegill, *Lepomis macrochirus*. *Evolution*, **28,** 42-56.

BANCROFT, D.R., PEMBERTON, J.M., ALBON, S.D., ROBERTSON, A., MACCOLL, A.D.C., SMITH, J.A., STEVENSON, I.R. AND CLUTTON-BROCK, T.H. 1995. Molecular genetic variation and individual survival during population crashes of an unmanaged ungulate population. *Phil. Trans. Roy. Soc. Lond. (B)*, **347,** 263-273.

BARTON, N.H. AND GALE, K.S. 1993. Genetic analysis of hybrid zones, In: Harrison, R.G. (ed), *Hybrid Zones and the Evolutionary Process*. pp. 13-45. Oxford University Press, New York.

BARTON, N.H. AND HEWITT, G.M. 1989. Adaptation, speciation and hybrid zones. *Nature*, **341,** 497-503.

BARTON, N. AND JONES, J.S. 1983. Mitochondrial DNA: new clues about evolution. *Nature*, **306,** 317-318.

BENSON, W.W. 1972. Natural Selection for Müllerian mimicry in *Heliconius erato* in Costa Rica. *Science*, **176,** 936-939.

BROWER, A.V.Z. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc. Natl. Acad. Sci., USA*, **91,** 6491-6495.

BROWER, A.V.Z. 1996. Parallel race formation and the evolution of mimicry in *Heliconius* butterflies: a phylogenetic hypothesis from mitochondrial DNA sequences. *Evolution*, **50,** 195-221.

BROWN, K.S. 1979. *Ecologia Geográfica e Evoluçâo nas Florestas Neotropicais*. 2 vols. (Livre de Docencia) Universidade Estadual de Campinas, Campinas, Brazil.

BROWN, K.S., EMMEL, T.C., ELIAZAR, P.J. AND SUOMALAINEN, E. 1992. Evolutionary patterns in chromosome numbers in neotropical Lepidoptera. I. Chromosomes of the Heliconiini (Family Nymphalidae: Subfamily Nymphalinae). *Hereditas*, **117,** 109-125.

BUTLIN, R.K. AND HEWITT, G.M. 1985. A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): morphological and electrophoretic characters. *Biol. J. Linn. Soc.*, **26,** 269-285.

CHU, J., POWERS, E. AND HOWARD, D.J. 1995. Gene exchange in a ground cricket hybrid zone. *J. Hered.*, **86,** 17-21.

EMELIANOV, I., MALLET, J. AND BALTENSWEILER, W. 1995. Genetic differentiation in *Zeiraphera diniana* (Lepidoptera: Tortricidae, the larch budmoth): polymorphism, host races or sibling species? *Heredity*, **75,** 416-424.

FEDER, J.L., ROETHELE, J.B., WLAZLO, B. AND BERLOCHER, S.H. 1997. Reproductive isolation, fitness tradeoffs and temperature: sympatric race formation in the apple maggot fly. *Proc. Natl. Acad. Sci., USA*, xxx, 000.

GYLLENSTEN, U., WHARTON, D. AND WILSON, A.C. 1985. Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. *J. Hered.*, **76,** 321-324.

HALDANE, J.B.S. 1922. Sex ratio and unisexual sterility in hybrid animals. *J. Genet.*, **12,** 101-109.

HEWITT, G.M., BUTLIN, R.K. AND EAST, T.M. 1987. Testicular dysfunction in hybrids between parapatric subspecies of the grasshopper *Chorthippus parallelus*. *Biol. J. Linn. Soc.*, **31,** 25-34.

HUNT, W.G. AND SELANDER, R.K. 1973. Biochemical genetics of hybridization in European house mice. *Heredity*, **31,** 11-33.

JIGGINS, C., MCMILLAN, W.O., NEUKIRCHEN, W. AND MALLET, J. 1996. What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.*, **59,** 000.

KARL, S.A. AND AVISE, J.C. 1992. Balancing selection at allozyme loci in oysters: Implications from nuclear RFLPs. *Science*, **256,** 100-102.

KIMURA, M. 1983. *The neutral theory of molecular evolution.* Cambridge University Press. Cambridge, UK.

MALLET, J. 1986. Hybrid zones in *Heliconius* butterflies in Panama, and the stability and movement of warning colour clines. *Heredity*, **56,** 191-202.

MALLET, J. 1988. Natural selection and gene flow in *Heliconius* hybrid zones. NERC Postdoctoral Fellowship Report, University College London, London.

MALLET, J. 1989. The genetics of warning colour in Peruvian hybrid zones of *Heliconius erato* and *H. melpomene*. *Proc. Roy. Soc. Lond. B*, **236,** 163-185.

MALLET, J. AND BARTON, N.H. 1989. Strong natural selection in a warning color hybrid zone. *Evolution*, **43,** 421-431.

MALLET, J., BARTON, N., LAMAS, G., SANTISTEBAN, J., MUEDAS, M. AND EELEY, H. 1990. Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *Heliconius* hybrid zones. *Genetics*, **124,** 921-936.

MALLET, J., KORMAN, A., HECKEL, D.G. AND KING, P. 1993. Biochemical genetics of *Heliothis* and *Helicoverpa* (Lepidoptera: Noctuidae) and evidence for a founder event in *Helicoverpa zea*. *Ann. Entomol. Soc. Amer.*, **86,** 189-197.

MCMILLAN, W.O., JIGGINS, C.D. AND MALLET, J. 1997. What initiates speciation in passion vine butterflies? *Proc. Natl. Acad. Sci., USA*, submitted. [see Appendix]

MOORE, W.S. 1987. Random mating in the northern flicker hybrid zone: implications for the evolution of bright and contrasting plumage patterns in birds. *Evolution*, **41,** 539-546.

NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89,** 583-590.

PRAKASH, S. 1976. Genetic divergence in closely related sibling species, *Drosophila pseudoobscura*, *D. persimilis* and *D. miranda*. *Evolution*, **31,** 14-23.

RAND, D.M. AND HARRISON, R.G. 1989. Ecological genetics of a mosaic hybrid zone: mitochondrial, nuclear, and reproductive differentiation of crickets by soil type. *Evolution*, **43,** 432-449.

RAYMOND, M. AND ROUSSET, F. 1995. GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenicism. *J. Hered.*, **86,** 248-249.

SAITOU, N. AND NEI, M. 1987. The neighbour joining-method: A new method for reconstructing phylogenetic trees. *Molec. Biol. Evol.*, **4,** 406-425.

SAMBROOK, J., FRITSCH, E.F. AND MANIATIS, T. 1989. *Molecular cloning: A laboratory manual.* 2nd ed. Cold Spring Harbour Laboratory Press, NY.

SHEPPARD, P.M., TURNER, J.R.G., BROWN, K.S., BENSON, W.W. AND SINGER, M.C. 1985. Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. *Phil. Trans. Roy. Soc. Lond. (B)*, **308,** 433-613.

SILVA, L.M. AND ARAUJO, A.M. 1994. The genetic structure of *Heliconius erato* populations (Lepidoptera; Nymphalidae). *Rev. Bras. Genet.*, **17,** 19-24.

SWOFFORD, D.L. AND OLSEN, G.J. 1990. Phylogeny reconstruction, In: Hillis, D.M. and Moritz, C. (eds), *Molecular systematics*, 1st ed. pp. 411-501. Sinauer, Sunderland, Massachusetts.

SWOFFORD, D.L. AND SELANDER, R.B. 1989. *BIOSYS-1. A Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics*. Release 1.7 Illinois Natural History Survey, Champaign, Illinois.

SZYMURA, J.M. AND BARTON, N.H. 1986. Genetic analysis of hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata* near Cracow in Southern Poland. *Evolution*, **40,** 1141-1159.

SZYMURA, J.M. AND BARTON, N.H. 1991. The genetic structure of the hybrid zone between the fire-bellied toads *Bombina bombina* and *B. variegata*: comparisons between transects and between loci. *Evolution*, **45,** 237-261.

TAKAHATA, N. AND SLATKIN, M. 1984. Mitochondrial gene flow. *Proc. Natl. Acad. Sci., USA*, **81,** 1764-1767.

TEMPLETON, A.R. 1989. The meaning of species and speciation: a genetic perspective, In: Otte, D. and Endler, J.A. (eds), *Speciation and its Consequences.* pp. 3-27. Sinauer Associates, Sunderland, Mass.

TURNER, J.R.G. AND SHEPPARD. 1975. Absence of crossing-over in female butterflies (*Heliconius*). *Heredity*, **34,** 265-269.

TURNER, J.R.G., JOHNSON, M.S. AND EANES, W.F. 1979. Contrasted modes of evolution in the same genome: allozymes and adaptive change in *Heliconius*. *Proc. Natl. Acad. Sci., USA*, **76,** 1924-1928.

WATT, W.B. 1983. Adaptation at specific loci. 2. Demographic and biochemical elements in the maintenance of the *Colias* PGI polymorphism. *Genetics*, **103,** 691-724.

WATT, W.B., CASSIN, R.C. AND SWAN, M.S. 1983. Adaptation at specific loci. III. Field behaviour and survivorship differences among *Colias* PGI genotypes are predictable from *in vitro* biochemistry. *Genetics*, **103,** 725-739.

WEIR, B.S. AND COCKERHAM, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution*, **38,** 1358-1370.

Table 1. Allele frequencies in combined allopatric populations of *H. himera* and *H. erato cyrbia,* and in the two east Andean races *H. e. lativitta* and *H. e. etylus.* Loci in bold are those used to calculate estimated Fst values (i.e. all polymorphic loci with a complete data set) and those in italics were used to calculate species index scores (i.e. all loci with a complete data set and marked species differences - see Methods for details). In addition the following loci were scored but were found to be monomorphic across both species; Fructose 1-6 diphosphatase; Sorbitol dehydrogenase; Isocitrate dehydrogenase (NAD); Leu-Gly-Gly peptidase; Hexokinase; Glyceraldehyde 3-phosphate dehydrogenase; Glucose-6-phosphate dehydrogenase; Acid phosphatase.

Locus	mobility	index score	*H. himera*	*H. e. cyrbia*	*H. e. etylus*	*H. e. lativitta*
Glucose-6-phosphate isomerase						
GPI						
(N)		n/a	128	131	10	8
	255		-	0.061	-	-
	144		0.035	0.137	-	0.063
	120		-	-	-	0.063
	100		0.820	0.737	0.900	0.750
	50		0.145	0.038	-	-
	33		-	0.023	0.100	0.125
	13		-	0.004	-	-
Glutamate oxaloacetate transaminase						
GOT-f						
(N)		n/a	128	131	10	8
	120		-	0.031	-	-
	110		-	-	-	0.188
	100		1.000	0.958	1.000	0.813
	72		-	0.011	-	-
	55		-	-	-	-
Glutamate oxaloacetate transaminase						
GOT-s						
(N)			128	131	10	8
	-220	0	0.961	0.004	-	-
	-200	1	-	0.023	-	0.188
	-180	1	-	0.042	-	0.063
	-160	-	-	-	-	0.063
	-100	1	0.039	0.931	1.000	0.688
Phosphoglucomutase						
PGM						
(N)			127	131	10	8
	138	-	-	-	0.100	-
	129	0	0.161	0.004	-	-

	118	1	0.004	0.118	0.400	0.313
	100	1	0.331	0.634	0.400	0.500
	92	-	-	-	0.050	-
	82	-	0.244	0.229	0.050	0.188
	68	0	0.079	0.015	-	-
	50	0	0.181	-	-	-
Mannose-6-phosphate isomerase						
MPI						
(N)			128	130	10	8
	119	1	-	0.004	-	-
	112	0	0.750	0.035	-	0.063
	108	1	0.016	0.392	0.150	0.125
	100	-	0.234	0.342	0.500	0.500
	96	1	-	0.088	0.050	0.125
	92	1	-	0.046	0.150	0.125
	86	1	-	0.088	0.150	0.063
Malate dehydrogenase						
MDH-s						
(N)		n/a	127	128	10	8
	180		-	0.008	-	-
	157		-	0.063	-	-
	100		1.000	0.926	1.000	1.000
	20		-	0.004	-	-
3-Phosphoglycerate dehydrogenase						
3-P						
(N)			86	93	7	6
	150	0	0.413	0.016	0.286	0.583
	100	1 v	0.587	0.984	0.714	0.417
Glucose-6-phosphate dehydrogenase						
G6-s						
(N)			127	130	7	8
	125	1 x	-	0.019	-	-
	100	1	0.134	0.969	1.000	1.000
	88	0	0.004	-	-	-
	60	0	0.862	0.012	-	-
Pyruvate kinase						
PK						
(N)			128	131	3	8
	155	-	-	-	0.167	0.563
	144	0	1.000	0.050	0.667	-
	100	1	-	0.950	0.167	0.438
Adenylate kinase						
AK						
(N)			126	130	10	8
	115	0	0.381	0.012	-	-
	100	1	0.619	0.973	1.000	1.000

	85	1	-	0.015	-	-
Enolase						
ENO						
(N)		n/a	128	131	10	8
	100		0.996	0.893	0.800	0.688
	85		0.004	0.107	0.200	0.313
Aconitase						
ACON-f						
(N)			127	127	10	7
	113	1	-	0.008	-	-
	108	1	-	0.004	-	-
	104	1	0.012	0.260	-	-
	100	0	0.984	0.661	1.000	0.929
	94	1	0.004	0.054	-	0.071
	88	1	-	0.016	-	-
Aconitase						
ACON-s						
(N)			127	129	-	8
	-150	0	1.000	0.027		0.750
	-100	1	-	0.880		0.250
	-55	1	-	0.093		-
β-Hydroxy-butyrate dehydrogenase						
HBDH						
(N)			124	131	10	8
	200	0	0.044	-	-	-
	143	0	0.956	0.057	-	-
	100	1	-	0.924	0.900	0.750
	24	1	-	0.019	0.100	0.250
Superoxide dismutase						
SOD						
(N)			88	103	-	8
	-100	1	0.006	1.000		-
	-10	0	0.994	-		1.000
Isocitrate dehydrogenase (NADP)						
IDH						
(N)		n/a	64	76	-	8
	128		0.016	0.105		0.188
	117		0.328	0.046		-
	100		0.656	0.783		0.813
	69		-	0.066		-
Malic enzyme						
ME						
(N)		n/a	52	38	10	8
	117		-	0.039	-	-
	104		0.010	0.118	0.050	-

	100	0.981	0.803	0.950	1.000
	89	0.010	0.039	-	-
Malate dehydrogenase					
MDH-f					
(N)	n/a	45	23	-	8
	144	-	0.022		0.188
	113	0.100	0.043		0.188
	100	0.433	0.913		0.625
	91	0.467	0.022		-
Glutamate pyruvate transaminase					
GPT					
(N)	n/a	60	52	10	8
	130	-	0.010	-	-
	100	1.000	0.952	0.950	0.938
	77	-	0.029	-	-
	58	-	0.010	0.050	0.063
α-Glycerophosphate dehydrogenase					
α-GPD					
(N)	n/a	60	52	10	8
	113	0.008	-	-	-
	100	0.992	1.000	1.000	1.000
6-Phosphogluconate dehydrogenase					
6-PGD					
(N)	n/a	45	45	7	8
	120	0.022	-	-	-
	100	0.978	1.000	0.643	0.875
	83	-	-	0.357	0.125
Leu-Ala peptidase					
LA					
(N)	n/a	21	16	-	-
	100	0.929	1.000		
	90	0.071	-		

	Colour pattern phenotype				
mtDNA	*himera*	backcross to *himera*	F1	backcross to *erato*	*erato*
Sympatric populations					
himera haplotype	147	6	2	0	0
erato haplotype	4	0	9	9	206
Allopatric populations					
himera haplotype	125	-	-	-	-
erato haplotype	-	-	-	-	110

Table 2. Concordance of colour pattern and mtDNA markers. All individuals are classified into hybrid classes according to colour pattern phenotype (Jiggins et. al., 1996). The mtDNA haplotype of each individual was determined using an RFLP analysis.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	SOD	x	-	-	-	-	-	-	+	-	-	-	-	-
2	GOT-2		x	-	-	+	-	+	-	-	-	-	-	-
3	PGM			x	-	-	-	-	-	-	-	-	-	-
4	MPI				x		-	(-)	-	-	-	-	(-)	(-)
5	G-6					x	-	+	(-)	(-)	(-)	-	-	-
6	PK						x	-	-	+	-	-	-	-
7	AK							x	(-)	(-)	-	-	-	-
8	3-P								x	-	-	-	-	-
9	ACO-f									x	-	-	-	-
10	ACO-s										x	-	-	+
11	HBDH											x	-	-
12	D												x	-
13	Cr													x

Table 3; Linkage relationships between species diagnostic genetic markers used in this study. += linked markers, - = unlinked. Markers 12 and 13 are colour pattern loci (Jiggins et al., in prep.). Data in brackets were not tested explicitly but are inferred from other linkage relationships. SOD and 3-P are sex linked.

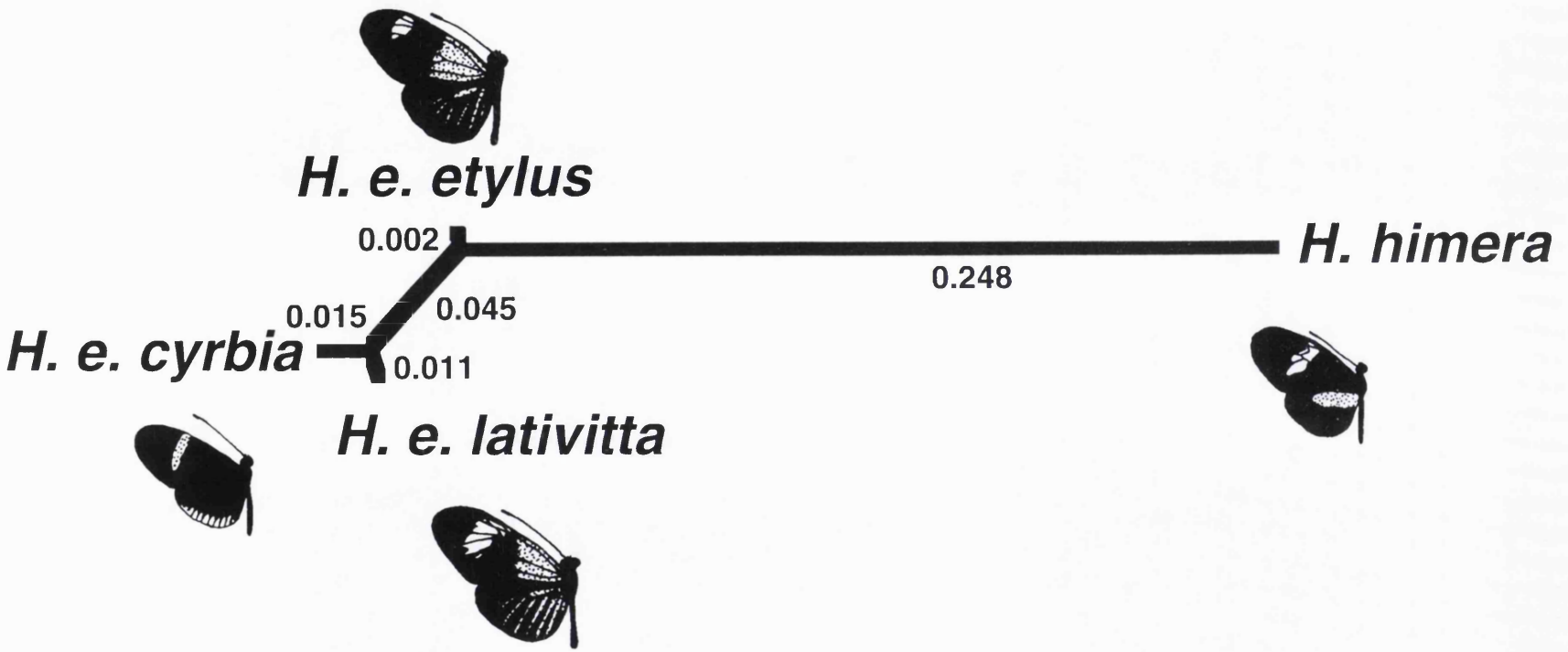


Figure 1 Neighbour joining tree of *H. himera* and three races of *H. erato*. *H. e. cyrbia* is from the western Andes while *H. e. lativitta* and *etylus* are from the eastern slopes. Nei's (1978) genetic distances were calculated over 19 loci (see methods)

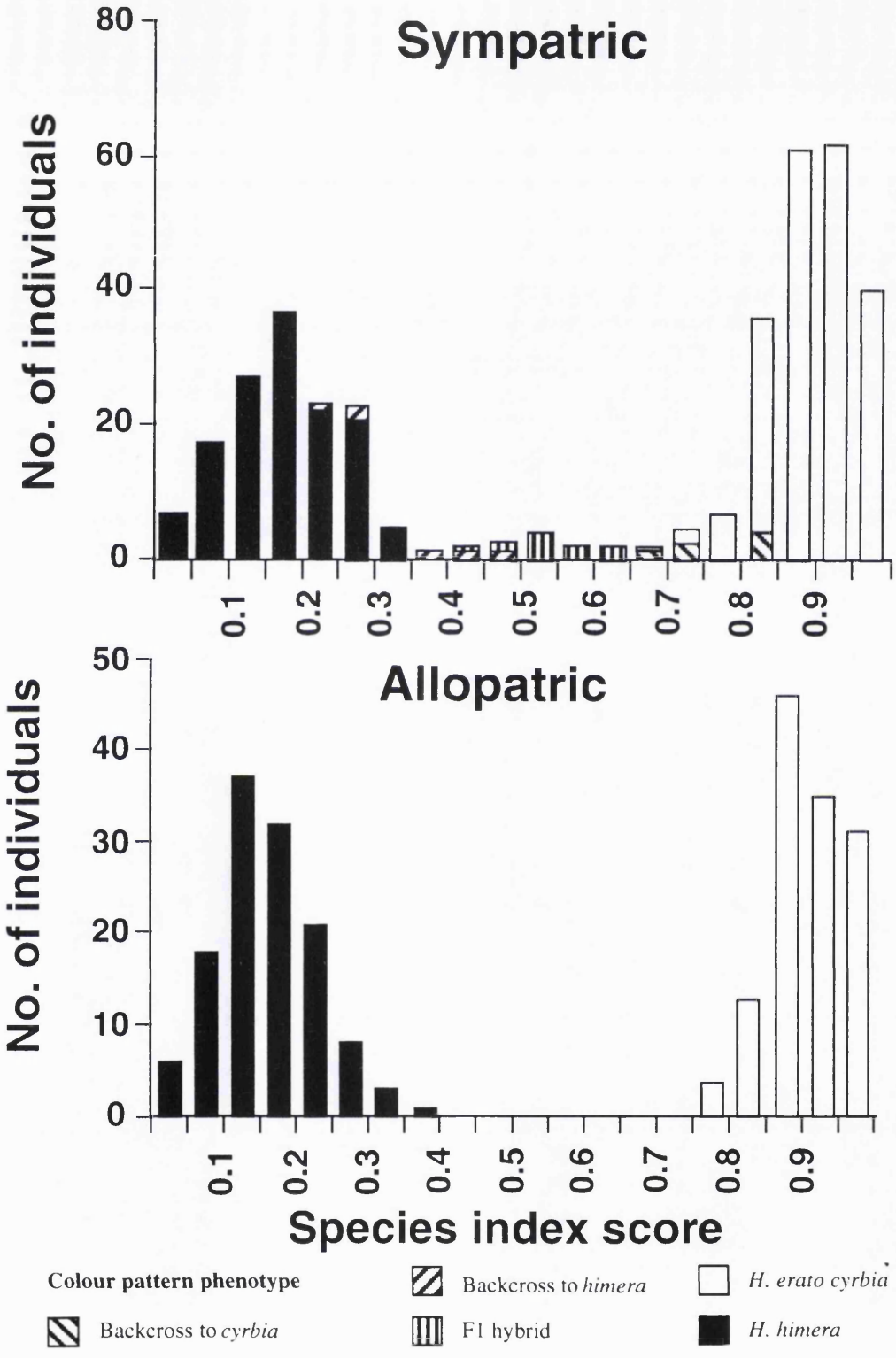


Figure 2. Distribution of individual species index values. All hybrid zone populations combined (sympatry) and non hybrid zone populations (allopatry) are shown. Black bars are *H. himera*, unshaded bars are *H. e. cyrbia* and hatched bars are colour pattern hybrids.

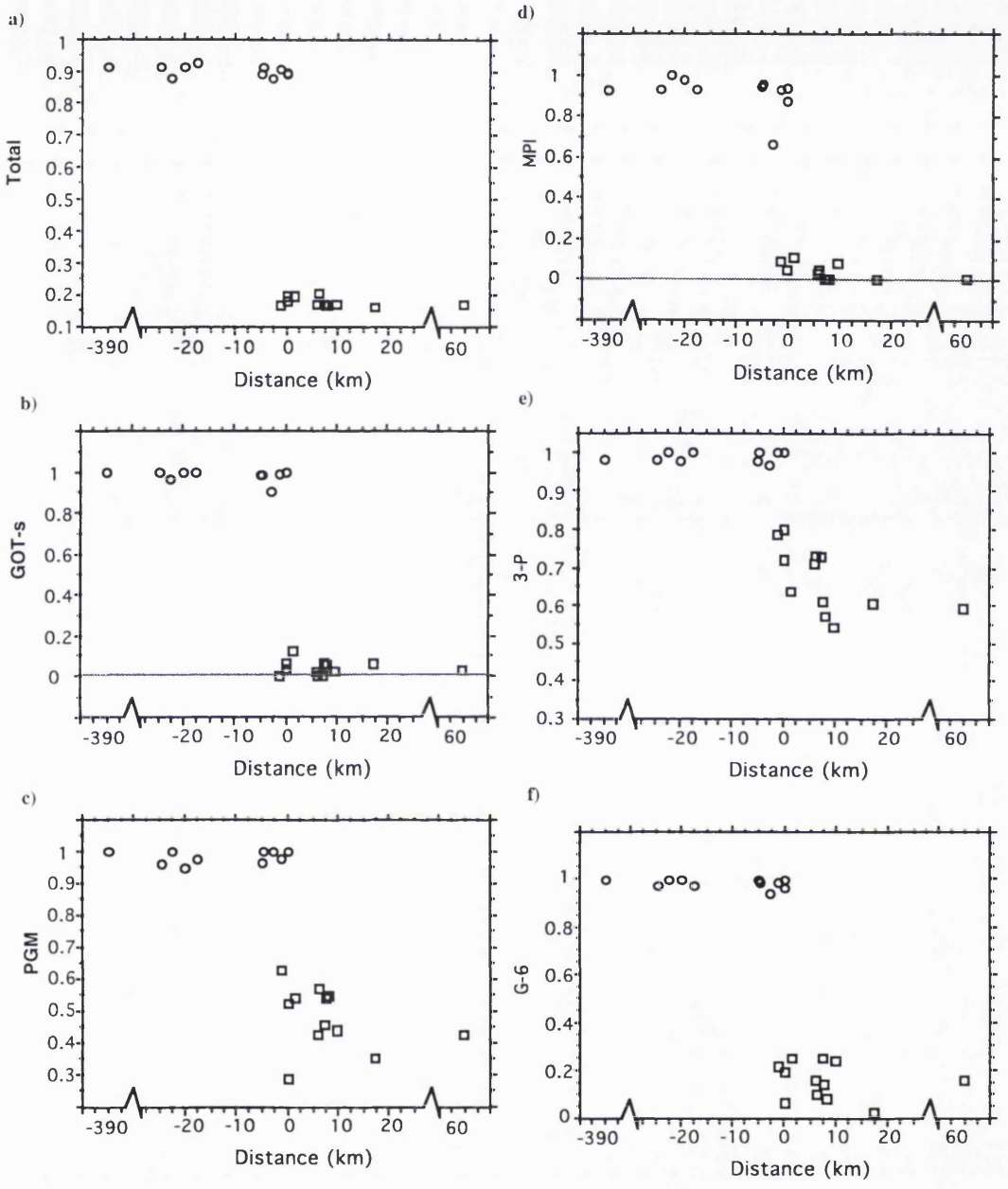


Figure 3. Population species index values plotted against distance from the centre of the hybrid zone. Each point represents a single population sampled. Circles are *H. c. cyrbia* and squares, *H. himera*. 2 a) shows combined values over all loci while the remaining plots show each locus separately. Only two loci, 3-P in *himera* and ACON-s in *cyrbia* show significant correlations of gene frequency with distance, a pattern which may imply introgression.

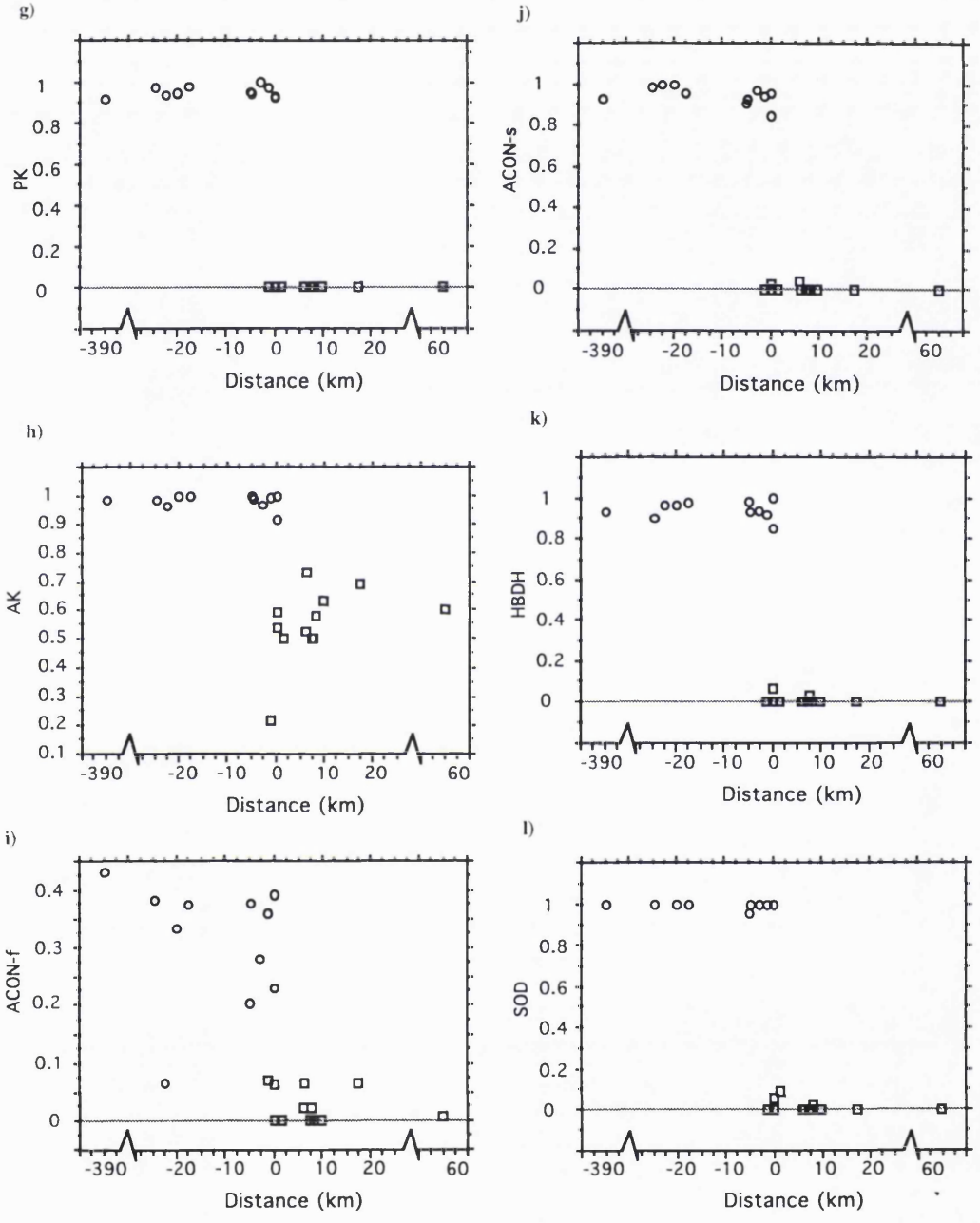


Figure 3 (continued)

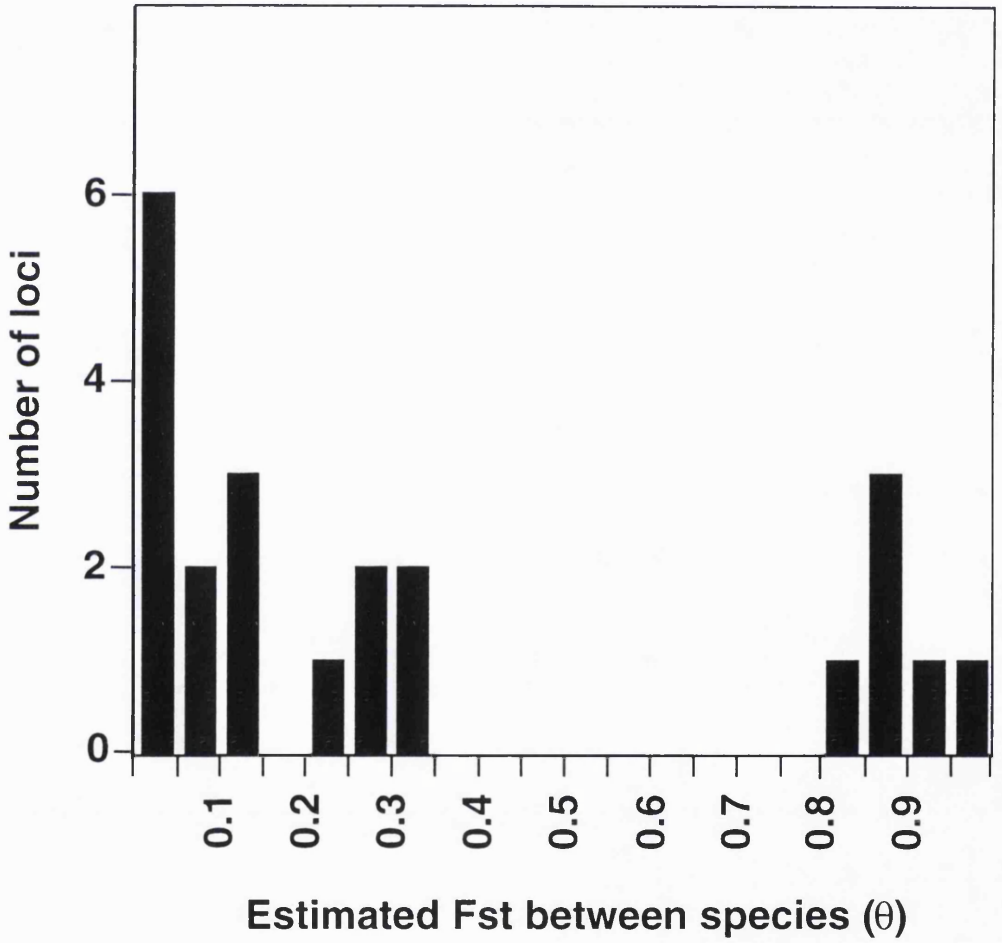


Figure 4. Estimated interspecific Fst values at all polymorphic loci. These were calculated between combined allopatric populations of *H. himera* and *H. erato cyrbia.* The trimodal pattern is not compatible with divergence through random drift (see discussion) and suggests that at least some of these loci must be influenced by selection or gene flow across the species boundary.

APPENDIX TO CHAPTER 2

Further linkage analysis.

This appendix contains linkage data for additional markers not considered in Chapter 2, recombination rates between linked markers and the brood data used in this analysis. Methods for scoring allozyme and colour pattern markers, and identification of linkage groups are described in Chapters 2 & 3. Analysis of eight backcross broods showed evidence for six chromosomal linkage groups involving 14 allozyme and colour pattern markers. A further six markers were shown to be unlinked (Figure 1). Thus, the 20 markers considered in this analysis map to twelve chromosomes of the 21 in *H. erato*.

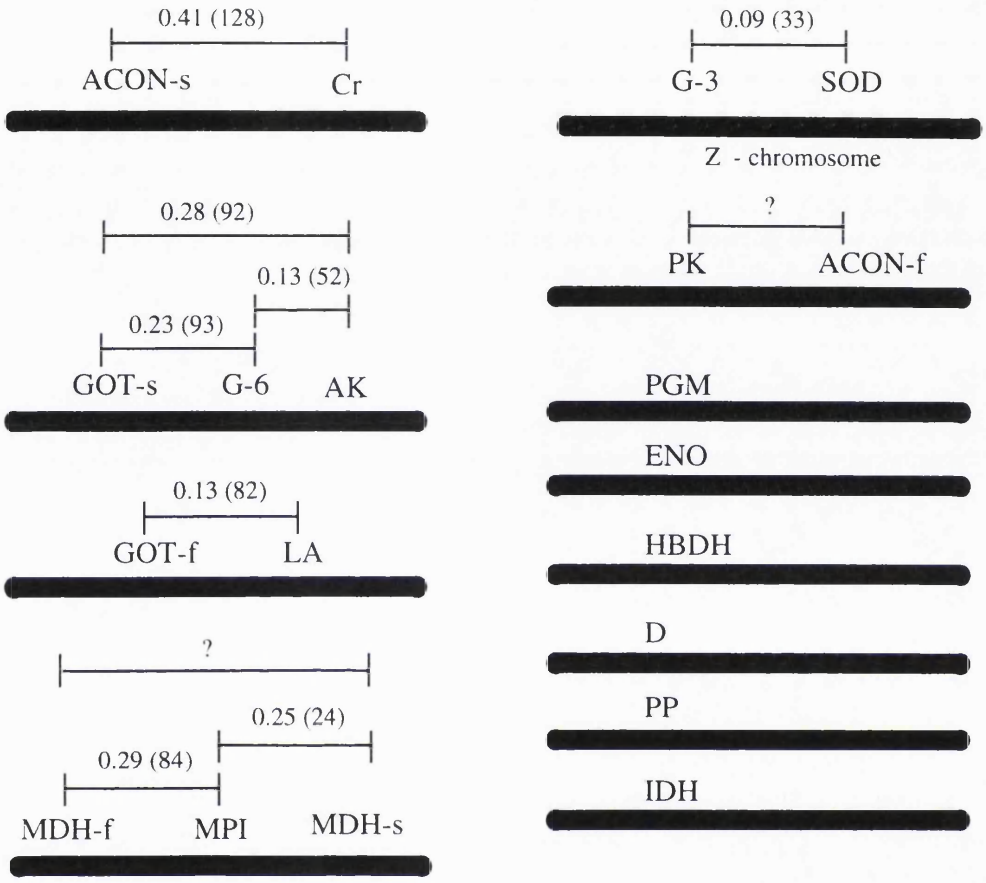


Figure 1. Linkage relationships between allozyme and colour pattern markers. Recombination rates shown are calculated from combined broods with overall sample sizes given in brackets. D and Cr are colour pattern genes, and the remaining loci are allozymes. With the exception of MPI - MDH-s, synteny or otherwise was initially confirmed using broods with no recombination between diagnostic markers. Loci shown as unlinked are based on pairwise comparisons with at least one member of all other linkage groups. Recombination rates between PK - ACON-f, and MDH-f - MDH-s, are not known. All abbreviations are given in Table 1 except LA (Leu-Ala peptidase) and PP (Phe-Pro peptidase).

Brood data used for linkage analysis;

Brood 126. Backcross to *himera*

#	sex	GPI	GOT-s	PGM	MPI	MDHf	G-6	PK	AK	ACO-s	HBDH	D	Cr
parents													
2855	m(H)aa		bb	cd	bb	ab	bb	bb	ab	bb	bb	bb	bb
2458	f(F1)ab		ab	bc	bc	ab	ab	ab	ab	ab	ab	ab	ab
offspring													
2829	f	ab	ab	cc	bc	ab	bb	ab	bb	ab	bb	bb	bb
2870	f	aa	ab	bd	bc	aa	ab	ab	ab	ab	bb	ab	bb
2871	m	aa	ab	bc	bb	ab	ab	ab	ab	bb	bb	bb	bb
2920	m	aa	bb	bd	bc	ab	bb	ab	ab	bb	ab	ab	ab
2947	f	ab	ab	bd	bc	ab	ab	ab	aa	ab	0	ab	ab
2946	f	ab	ab	cc	bb	ab	ab	bb	aa	bb	0	ab	ab
2930	m	ab	ab	bd	bc	ab	ab	ab	bb	ab	0	bb	ab
2929	f	ab	ab	bd	bc	aa	ab	bb	bb	bb	0	ab	bb
2928	f	aa	ab	cd	bb	aa	ab	ab	ab	bb	0	bb	bb
2927	f	ab	ab	bd	bb	ab	ab	ab	bb	bb	0	ab	ab
2998	f	ab	bb	cd	bc	aa	bb	ab	bb	bb	0	0	0
2958	m	aa	ab	bd	bc	ab	bb	ab	bb	bb	0	ab	ab
2996	f	aa	ab	cd	bc	aa	ab	bb	aa	ab	0	ab	bb
3005	m	aa	bb	cd	bc	ab	bb	ab	bb	bb	0	bb	bb
3143	m	ab	bb	cc	bc	ab	ab	bb	aa	bb	0	ab	bb
3161	f	ab	ab	bd	bb	ab	ab	ab	aa	ab	0	ab	ab
3198	m	ab	ab	bd	bb	bb	ab	bb	aa	ab	0	ab	ab
3199	m	aa	bb	bd	bc	aa	bb	ab	bb	ab	0	bb	ab
3226	f	ab	bb	cd	bc	bb	bb	bb	ab	ab	0	bb	bb
3247	f	ab	ab	cc	bc	ab	ab	ab	ab	bb	0	ab	bb
3297	m	aa	bb	cc	bb	bb	bb	ab	ab	bb	0	ab	bb
3406	m	aa	bb	cd	bc	aa	bb	bb	aa	ab	0	bb	bb
3407	f	aa	bb	cc	bb	ab	bb	ab	bb	bb	0	ab	bb
3568	m	aa	bb	bd	bc	ab	bb	bb	ab	bb	0	ab	bb
3619	f	ab	bb	bc	bb	bb	ab	0	ab	bb	0	ab	ab
3667	f	aa	bb	bd	bb	ab	bb	ab	bb	bb	0	bb	ab
3668	m	ab	ab	bc	bc	ab	ab	ab	aa	ab	0	bb	ab

Brood 127; Backcross to *cyrbia*

#	sex	GPI	GOT-2	MDHf	3-P	G-6	PK	AK	ACO-s	HBDH	SOD	IDH	GPT	PP	D	Cr
parents																
2856	f(C)	ab	aa	aa	a0	aa	aa	aa	0	ab	a0	ab	aa	ab	aa	aa
2459	m(F1)ab		ab	ab	ab	ab	ab	ab	0	ab	ab	aa	ab	aa	ab	ab
offspring																
2828	f	aa	aa	aa	b0	aa	aa	aa	0	ab	b0	aa	aa	ab	ab	ab
2954	f	ab	ab	ab	a0	ab	aa	ab	0	ab	a0	aa	ab	aa	aa	ab
2968	m	aa	aa	ab	aa	aa	ab	aa	0	ab	aa	ab	aa	aa	ab	aa
2973	f	aa	aa	aa	b0	aa	aa	ab	0	aa	b0	ab	aa	ab	aa	aa
3006	f	aa	ab	ab	b0	aa	0	aa	ab	0	b0	0	aa	ab	ab	aa
3055	f	ab	ab	ab	b0	ab	0	ab	ab	0	b0	0	ab	aa	ab	aa
3056	f	aa	ab	aa	a0	ab	0	ab	aa	0	a0	0	aa	ab	ab	aa
3057	m	bb	ab	aa	ab	aa	0	ab	ab	0	0	0	aa	aa	ab	aa
3058	f	ab	ab	aa	a0	ab	0	ab	ab	0	a0	0	aa	aa	aa	ab
3110	f	bb	aa	aa	a0	aa	0	aa	aa	0	a0	0	ab	aa	ab	ab
3173	f	bb	aa	aa	a0	aa	0	aa	ab	0	b0	0	aa	aa	ab	ab
3227	m	aa	aa	ab	ab	ab	0	ab	aa	0	ab	0	aa	aa	aa	aa
3245	f	ab	aa	aa	a0	aa	0	aa	ab	0	a0	0	aa	ab	ab	aa
3246	f	bb	aa	aa	b0	aa	0	aa	ab	0	b0	0	aa	ab	aa	ab
3298	f	bb	ab	ab	a0	ab	0	ab	aa	0	a0	0	aa	ab	aa	aa
3299	m	bb	ab	ab	ab	ab	0	ab	aa	0	ab	0	ab	ab	aa	aa
3300	f	ab	ab	aa	a0	aa	0	aa	aa	0	b0	0	aa	aa	ab	aa
3322	f	bb	ab	aa	b0	aa	0	aa	ab	0	b0	0	aa	aa	ab	aa
3323	f	ab	aa	aa	a0	ab	0	ab	aa	0	a0	0	ab	ab	ab	aa
3324	f	aa	aa	aa	a0	aa	0	aa	aa	0	a0	0	ab	aa	ab	ab
3393	f	ab	ab	aa	b0	aa	0	aa	aa	0	b0	0	aa	aa	ab	ab
3394	m	ab	aa	aa	ab	aa	0	aa	ab	0	ab	0	aa	ab	aa	ab
3408	f	ab	aa	ab	a0	ab	0	ab	ab	0	a0	0	aa	ab	ab	aa

3415 f	aa	ab	aa	a0	ab	0	ab	ab	0	a0	0	ab	ab	aa	ab	
3522 f	aa	ab	aa	b0	ab	0	aa	ab	0	b0	0	aa	aa	ab	aa	
3523 m	ab	aa	ab	aa	aa	0	aa	aa	0	aa	0	aa	aa	ab	aa	
3524 m	ab	aa	ab	ab	aa	0	aa	ab	0	ab	0	ab	aa	ab	aa	
3525 f	ab	ab	ab	b0	ab	0	ab	ab	0	b0	0	ab	aa	ab	aa	
3433 f	aa	aa	aa	b0	aa	aa	aa	ab	0	b0	ab	ab	ab	aa	ab	
3434 f	aa	aa	ab	b0	ab	ab	ab	ab	0	b0	aa	aa	ab	ab	ab	
3569 f	aa	ab	aa	b0	aa	ab	aa	aa	0	b0	ab	ab	0	ab	ab	
3586 f	bb	ab	ab	b0	ab	aa	ab	aa	0	b0	ab	aa	aa	aa	ab	
3661 m	ab	ab	aa	aa	ab	aa	ab	aa	0	ab	aa	aa	aa	ab	aa	
3662 f	ab	ab	aa	b0	ab	ab	ab	aa	0	b0	aa	aa	aa	aa	ab	

Brood 51 Backcross to *cyrbia*

#	sex	GOT-f	GOT-s	PGM	MPI	MDHf	MDHs	PK	ENO	ACO-f	ACO-s	HBDH	IDH	LA	PP	D	Cr
parents																	
625	m(C)	ab	aa	ac	ac	ac	ab	aa	ac	aa	aa	aa	ab	ac	bb	ab	ab
No data for female F1 parent																	
offspring																	
892	f	aa	ac	ab	bc	0	aa	ab	aa	ac	aa	ab	bb	ab	ab	aa	aa
902	m	ab	ab	bc	ab	0	aa	aa	aa	aa	ac	ab	ab	aa	ab	aa	ab
945	f	aa	ab	aa	ab	aa	aa	ab	ac	ac	ab	ab	aa	ac	ab	aa	aa
979	m	aa	ab	bc	bc	ac	ab	aa	aa	bc	ac	ab	aa	ac	ab	aa	ab
980	m	ab	ac	aa	ab	ac	ab	aa	ac	ab	ac	ab	bb	ab	bb	ab	ab
1010	f	ab	ac	bc	bc	ac	aa	aa	ac	bc	ac	aa	aa	aa	bb	ab	ab
1032	m	ab	ac	ac	bc	ac	0	ab	aa	ac	ab	ab	ab	ab	ab	aa	aa
1033	f	ab	ab	ac	bc	0	ab	aa	aa	bc	ac	aa	ab	aa	ab	aa	ab
1037	m	aa	ac	ab	bc	aa	ab	aa	ac	bc	ac	ab	ab	bc	ab	aa	ab
1071	f	aa	ac	aa	ab	aa	aa	ab	ac	aa	ac	ab	ab	ac	bb	aa	ab
1171	m	aa	ac	ac	bc	ac	ab	ab	ac	ac	ab	ab	aa	ab	bb	aa	aa
1181	m	ab	ac	ab	bc	ac	aa	ab	ac	ac	ac	ab	aa	aa	ab	ab	ab
1221	f	ab	ac	aa	ab	ac	aa	ab	aa	aa	ab	aa	bb	aa	ab	0	0
1229	f	ab	ac	ab	ab	aa	0	ab	aa	ac	ac	aa	bb	ab	ab	aa	ab
1234	m	ab	ab	ac	ab	aa	aa	aa	ac	aa	ab	aa	ab	ab	ab	ab	aa
1237	m	aa	ab	ac	bc	aa	ab	ab	aa	ac	ab	ab	ab	bc	bb	aa	aa
1343	f	ab	ac	ab	bc	ac	aa	ab	ac	aa	ab	aa	bb	ab	bb	aa	aa
1418	f	aa	ab	bc	bc	aa	ab	aa	aa	ab	ab	aa	ab	ac	bb	aa	aa
1419	f	aa	ac	bc	bc	aa	ab	aa	aa	ab	ab	ab	ab	bc	ab	ab	aa
1433	f	aa	ac	bc	ab	ac	aa	aa	ac	ab	ab	ab	bb	ac	bb	ab	aa
1434	m	aa	ac	ac	ab	aa	aa	ab	aa	ac	ac	aa	aa	ac	bb	ab	ab
1499	f	aa	ac	ac	bc	ac	ab	ab	ac	ac	ac	ab	aa	ac	bb	ab	ab
1569	f	aa	ac	bc	ab	ac	aa	aa	aa	bc	ab	aa	ab	bc	ab	ab	aa
1611	f	ab	ab	ac	bc	aa	ab	aa	ac	bc	ab	ab	bb	ab	bb	aa	aa
1628	m	aa	ac	aa	bc	ac	ab	aa	aa	bc	ac	ab	bb	ac	ab	ab	ab

Brood 63 Backcross to *himera*

#	sex	GPI	GOT-s	PGM	MPI	MDHf	PK	AK	ENO	ACO-s	HBDH	IDH	D	Cr
parents														
1188	f(F1)	ab	ac	dc	ac	ac	ab	ab	ac	ac	ab	ab	ab	ab
698	m(H)	aa	cc	bc	aa	cc	bb	aa	aa	cc	cc	aa	aa	aa
offspring														
1208	f	ab	cc	dc	ac	ac	bb	ab	ac	ac	bc	ab	aa	ab
1214	f	aa	cc	bd	aa	cc	ab	ab	aa	ac	bc	aa	aa	ab
1225	m	aa	cc	dc	aa	cc	bb	ab	aa	cc	bc	aa	ab	aa
1226	m	ab	cc	bd	aa	cc	ab	ab	ac	ac	ac	aa	ab	ab
1272	m	ab	ac	bc	ac	ac	ab	aa	ac	cc	ac	aa	ab	aa
1316	f	aa	ac	bd	aa	cc	bb	aa	aa	cc	ac	aa	ab	aa
1318	f	ab	ac	bd	ac	ac	ab	aa	ac	cc	ac	ab	aa	aa
1368	f	ab	ac	bc	ac	ac	ab	aa	ac	ac	ac	aa	aa	ab
1369	m	ab	cc	bd	aa	cc	bb	ab	ac	ac	ac	ab	aa	ab
1372	f	ab	ac	bc	ac	ac	bb	aa	ac	cc	ac	aa	aa	aa
1475	f	ab	cc	cc	aa	cc	bb	ab	ac	cc	ac	ab	ab	aa
1498	m	aa	cc	cc	ac	ac	ab	ab	aa	ac	ac	aa	ab	ab
1519	m	ab	ac	dc	aa	cc	bb	aa	ac	ac	ac	ab	ab	ab
1520	m	ab	ac	cc	aa	cc	ab	aa	ac	ac	bc	aa	ab	ab
1521	m	aa	ac	bc	aa	cc	ab	aa	aa	ac	ac	ab	ab	ab

1544 f	aa	cc	bd	aa	cc	ab	ab	aa	cc	bc	aa	ab	aa
1545 m	ab	ac	bd	aa	cc	bb	aa	ac	ac	bc	ab	ab	ab
1563 m	ab	cc	bc	ac	ac	bb	aa	aa	cc	ac	ab	aa	aa
1604 f	ab	ac	bd	aa	cc	bb	aa	ac	cc	ac	aa	aa	ab
1632 m	aa	cc	dc	ac	ac	bb	ab	aa	ac	bc	aa	ab	ab
1670 m	ab	ac	bc	aa	cc	ab	aa	ac	ac	ac	aa	aa	ab
1671 f	ab	cc	cc	ac	ac	ab	ab	ac	cc	ac	aa	aa	aa
1970 m	ab	cc	bc	ac	ac	ab	ab	ac	ac	ac	aa	aa	aa
2145 f	aa	ac	bc	ac	ac	bb	aa	aa	ac	bc	ab	aa	ab
2207 f	ab	ac	dc	ac	ac	bb	aa	ac	ac	ac	aa	ab	ab

Brood 67 Backcross to *himera*

# sex	GPI	GOT-f	GOT-s	PGM	MPI	MDHf	G-6	PK	ACO-s	LA	D	Cr
parents												
1388 f(H)	ab	aa	bb	bc	ab	aa	bb	bb	bb	ab	bb	bb
740 m(F1)	ab	ab	ab	ab	bc	ab	ab	ab	ab	ac	ab	ab
offspring												
1268 m	ab	aa	ab	ab	ac	aa	0	bb	ab	ab	ab	bb
1271 m	ab	ab	ab	ab	ac	aa	bb	ab	bb	bc	bb	bb
1319 f	bb	ab	ab	bc	bc	aa	0	ab	bb	bc	bb	bb
1338 f	bb	ab	ab	ac	ab	ab	bb	ab	bb	aa	ab	bb
1371 f	aa	aa	ab	ab	ab	ab	ab	bb	bb	ab	ab	ab
1427 f	aa	aa	ab	bc	ab	ab	ab	ab	bb	aa	ab	ab
1428 f	ab	ab	bb	ac	ac	aa	bb	bb	ab	ab	ab	ab
1490 m	ab	aa	ab	bb	bc	ab	ab	bb	ab	bc	ab	ab
1565 f	ab	aa	bb	ab	bc	aa	bb	bb	bb	ab	ab	bb
1566 f	aa	aa	ab	ac	bb	ab	ab	ab	bb	aa	bb	ab
1629 m	aa	aa	bb	ac	ab	ab	ab	ab	ab	ab	ab	ab
1713 m	aa	aa	bb	ab	bc	ab	bb	ab	ab	bc	ab	bb
1731 f	aa	ab	ab	bc	bc	aa	ab	bb	ab	bc	bb	ab
1732 f	bb	aa	bb	bc	ab	ab	bb	ab	bb	aa	bb	bb
1797 m	aa	aa	bb	bc	bb	ab	ab	bb	bb	ac	ab	bb
1895 m	ab	aa	ab	bb	ab	aa	ab	bb	bb	ab	ab	bb
1896 f	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab
1897 m	ab	aa	ab	ac	bb	aa	ab	bb	ab	aa	ab	ab
1898 f	bb	aa	bb	ab	bc	ab	bb	bb	ab	ab	ab	ab
1899 f	bb	ab	bb	ac	bc	aa	0	bb	ab	ab	ab	ab
1908 m	aa	ab	ab	bc	ab	ab	ab	ab	ab	bc	ab	ab
1909 f	bb	ab	ab	bc	bb	ab	bb	bb	bb	bc	bb	bb
1960 f	ab	aa	bb	ac	bb	ab	bb	ab	bb	aa	ab	bb
1961 m	aa	aa	bb	bc	bb	aa	bb	bb	bb	ab	bb	ab
1962 f	ab	ab	ab	ac	bb	ab	ab	ab	ab	bc	ab	bb
2005 f	aa	ab	ab	ab	ab	aa	ab	bb	ab	ac	bb	bb
2006 m	ab	ab	bb	ac	bc	ab	bb	bb	ab	bc	bb	ab
2007 f	ab	aa	ab	bb	ac	aa	ab	ab	bb	aa	bb	bb
2032 f	ab	aa	ab	bb	bc	aa	ab	bb	ab	aa	bb	bb
2048 f	aa	aa	bb	ab	ac	aa	ab	bb	bb	aa	bb	bb
2049 f	aa	ab	bb	bb	ab	aa	ab	bb	bb	bc	ab	bb
2171 m	bb	aa	bb	bc	bc	aa	bb	bb	bb	ab	bb	ab
2172 m	ab	aa	bb	ab	ac	aa	bb	ab	ab	ab	ab	bb
2476 m	ab	ab	bb	bb	ab	ab	bb	bb	ab	bc	ab	bb
2511 m	ab	aa	bb	ab	ac	aa	bb	bb	0	ac	bb	ab

Brood 68 Backcross to *himera*

# sex	GPI	GOT-s	PGM	MDHf	PK	AK	ACO-s	HBDH	IDH	D	Cr
parents											
1507 f(F1)	ab	ac	ad	ac	ab	ab	ac	ab	aa	ab	ab
151 m(H)	aa	cc	dc	ac	bb	bb	cc	bb	ab	aa	aa
offspring											
1414 f	ab	cc	ad	ac	ab	bb	cc	ab	aa	ab	aa
1417 m	aa	ac	ad	aa	bb	ab	cc	ab	ab	ab	aa
1435 f	aa	cc	ac	ac	bb	bb	ac	ab	ab	ab	ab
1449 f	aa	ac	dc	aa	ab	ab	cc	bb	aa	ab	aa
1450 f	ab	cc	dd	cc	bb	bb	cc	ab	aa	aa	aa
1486 f	ab	ac	ad	cc	ab	ab	ac	ab	ab	aa	ab

1487 f	aa	ac	dd	0	ab	ab	cc	ab	ab	aa	aa
1522 m	aa	cc	ac	ac	ab	bb	ac	ab	ab	aa	ab
1605 m	ab	cc	ad	aa	bb	bb	cc	ab	aa	aa	aa
1630 f	aa	cc	ad	ac	ab	bb	ac	bb	ab	aa	ab
1715 m	ab	cc	dd	ac	bb	bb	ac	bb	ab	ab	ab
1757 m	ab	ac	dc	ac	bb	ab	cc	ab	aa	aa	aa
1808 m	aa	cc	dd	cc	ab	bb	ac	bb	aa	ab	ab
1911 f	ab	cc	dd	ac	ab	bb	ac	ab	ab	ab	ab
1912 m	aa	cc	ac	aa	bb	bb	cc	bb	ab	aa	aa
1913 f	aa	ac	ac	ac	bb	ab	cc	bb	ab	ab	aa
1914 f	aa	cc	dd	0	0	bb	cc	bb	aa	ab	aa
1933 m	ab	ac	dd	aa	bb	ab	cc	ab	aa	aa	aa
2047 m	aa	ac	dd	ac	ab	ab	0	ab	ab	ab	ab
2098 f	ab	ac	dd	aa	bb	ab	cc	ab	aa	ab	aa
2134 f	aa	ac	ac	ac	bb	ab	cc	ab	ab	aa	aa
2135 f	ab	cc	ac	aa	bb	bb	cc	ab	ab	aa	aa
2137 m	ab	cc	ac	0	ab	bb	cc	ab	ab	ab	aa
2339 m	ab	ac	dd	aa	ab	ab	ac	ab	ab	aa	ab
2558 m	ab	ac	dc	ac	ab	ab	cc	ab	ab	ab	aa
2851 f	ab	cc	dc	ac	bb	bb	cc	ab	aa	ab	aa

Brood 72 Backcross to *cyrbia*

# sex	GOT-f	GOT-s	PGM	MPI	MDHf	PK	AK	ENO	ACO-s	LA	D	Cr
parents												
1590 f(C)	ab	aa	ab	bc	aa	aa	aa	aa	ac	aa	aa	aa
860 m(F1)	ab	ab	cd	ab	ab	ab	ab	ab	ab	ab	ab	ab
offspring												
1509 f	ab	ab	ac	bc	aa	aa	ab	aa	ac	ab	ab	aa
1543 m	ab	aa	bd	bc	ab	ab	aa	ab	ab	aa	aa	ab
1607 m	aa	aa	bc	bc	ab	aa	aa	aa	aa	aa	ab	aa
1668 f	aa	aa	ad	bc	ab	ab	aa	0	aa	ab	aa	ab
1669 m	aa	ab	bc	bc	ab	aa	ab	0	aa	ab	aa	aa
1727 f	aa	ab	bc	bb	ab	ab	ab	0	bc	ab	ab	ab
1728 m	bb	ab	ac	bb	ab	aa	ab	0	ab	aa	aa	ab
1799 f	bb	ab	ad	ab	aa	aa	ab	0	ac	aa	ab	ab
1830 f	ab	ab	ac	ab	aa	ab	ab	0	ac	ab	ab	aa
1832 m	ab	ab	ad	ac	aa	aa	ab	0	ac	aa	ab	aa
1851 f	aa	aa	bc	bc	ab	ab	aa	0	bc	ab	ab	ab
1905 f	ab	aa	bd	bc	ab	ab	ab	0	bc	aa	aa	ab
1907 m	ab	aa	bc	ac	aa	ab	aa	0	bc	ab	aa	ab
1932 m	aa	aa	ac	bc	ab	aa	ab	0	ab	ab	ab	ab
1964 m	aa	aa	bc	bc	aa	aa	ab	0	ac	ab	aa	aa
1965 f	ab	ab	bc	ab	aa	ab	ab	0	ab	aa	aa	ab
1966 f	aa	ab	ad	ab	aa	aa	ab	0	ac	ab	aa	aa
1967 f	ab	aa	bd	bc	aa	aa	aa	0	ab	aa	ab	aa
1968 m	ab	ab	bd	ab	aa	aa	ab	0	ab	aa	aa	aa
2004 m	bb	ab	bc	bb	aa	ab	ab	0	bc	aa	ab	ab
2028 f	ab	aa	bc	ac	ab	aa	aa	0	bc	ab	aa	ab
2050 f	bb	aa	bd	bb	aa	ab	aa	0	ac	aa	ab	aa
2051 f	ab	aa	bd	ab	ab	aa	aa	0	aa	ab	aa	aa
2052 f	aa	ab	ad	bb	aa	ab	ab	0	bc	ab	ab	ab
2053 f	ab	aa	ad	bc	aa	ab	aa	0	bc	ab	ab	aa
2174 m	aa	aa	bc	bb	ab	aa	aa	0	bc	ab	ab	aa
2197 f	bb	ab	bc	ab	ab	ab	aa	0	aa	aa	0	0
2198 m	ab	ab	ad	bb	aa	aa	aa	0	ac	aa	ab	aa
2199 f	ab	aa	ac	ac	aa	ab	aa	0	ab	ab	ab	aa
2200 m	bb	aa	ad	ac	aa	ab	aa	0	aa	aa	aa	ab
2218 f	aa	aa	ac	ac	aa	aa	aa	0	bc	ab	ab	aa
2236 m	ab	ab	bd	bb	aa	aa	ab	0	ac	aa	ab	aa
2264 m	ab	ab	ad	ab	ab	aa	aa	0	aa	aa	ab	ab
2265 f	aa	ab	bc	ab	aa	ab	aa	0	aa	ab	ab	ab
2300 f	ab	aa	ad	ab	aa	ab	aa	0	ac	ab	aa	ab
2301 m	bb	aa	ad	bb	ab	ab	ab	0	bc	aa	aa	aa
2852 f	aa	aa	bc	ab	aa	aa	aa	0	ab	ab	aa	ab

2858 m	bb	aa	ad	ab	aa	ab	aa	0	ac	aa	ab	ab

Brood 75 Backcross to *cyrbia*

# sex	GOT-s	PGM	MPI	MDHf	G-6	PK	AK	ENO	ACO-s	HBDH	LA	PP	D	Cr
parents														
1364 f(F1)	ac	ac	aa	ac	ac	ab	ab	ac	ac	bc	ad	ab	ab	ab
1007 m(C)	aa	ad	cd	aa	ab	aa	aa	ac	ac	aa	ac	aa	aa	aa
offspring														
1514 f	aa	ad	ac	aa	ab	aa	aa	ac	ac	ab	ad	aa	ab	aa
1550 m	ac	aa	ac	aa	ac	aa	ab	ac	ac	ab	aa	aa	aa	aa
1549 m	aa	ac	ad	ac	ab	ab	aa	aa	cc	ac	ac	aa	aa	ab
1617 m	aa	ad	0	aa	ab	ab	aa	ac	cc	ab	cd	0	aa	ab
1624 f	ac	aa	0	ac	0	aa	ab	ac	0	ac	ac	ab	ab	ab
1625 f	aa	aa	ad	aa	ab	ab	aa	ac	0	ac	ad	aa	ab	ab
1626 m	aa	cd	ad	aa	aa	aa	aa	cc	0	ac	aa	aa	ab	ab
1627 f	ac	ac	ac	aa	bc	ab	ab	aa	0	ab	cd	ab	0	0
1708 f	ac	ac	ac	aa	ac	ab	ab	aa	aa	ac	ac	ab	ab	aa
1711 m	aa	cd	ac	aa	ab	aa	aa	ac	cc	ac	cd	ab	ab	ab
1712 m	aa	cd	ad	aa	aa	aa	aa	ac	ac	ab	cd	aa	aa	aa
1729 m	ac	aa	ad	aa	bc	ab	ab	cc	0	ab	aa	ab	ab	ab
1730 m	ac	ac	ad	aa	ac	ab	ab	ac	cc	ac	aa	aa	ab	ab
1759 m	ac	ad	ac	ac	ac	ab	ab	ac	ac	ab	aa	aa	aa	aa
1760 f	ac	ad	ac	aa	bc	ab	ab	ac	aa	ab	ad	aa	aa	aa
1761 m	ac	cd	ac	ac	bc	ab	ab	cc	ac	ac	ac	ab	aa	aa
1804 f	ac	aa	ac	ac	ac	ab	ab	aa	cc	ac	ad	ab	aa	ab
1825 m	aa	cd	ac	aa	aa	ab	aa	ac	cc	ac	aa	aa	ab	ab
1826 m	ac	ac	ad	aa	bc	ab	ab	ac	aa	ab	ac	aa	aa	aa
1859 f	ac	aa	ad	ac	ac	aa	ab	ac	aa	ac	cd	aa	ab	aa
1893 m	ac	ac	ad	aa	ac	aa	ab	aa	ac	ab	cd	ab	ab	aa
1894 f	0	ac	ad	0	0	ab	0	0	0	ab	cd	ab	ab	ab
1900 f	ac	cd	ac	aa	ac	aa	ab	aa	ac	ab	ac	aa	ab	aa
1901 f	aa	ac	ac	aa	aa	ab	aa	aa	aa	ab	aa	ab	aa	aa
1902 m	ac	ac	ac	aa	bc	ab	ab	ac	ac	ab	ac	ab	ab	aa
1903 m	aa	ad	ac	ac	ab	ab	aa	aa	aa	ac	aa	aa	ab	aa
1904 m	ac	cd	ac	ac	ac	aa	ab	ac	cc	ab	ad	ab	aa	ab

CHAPTER 3

The genetic basis of an adaptive radiation: Mimicry in two *Heliconius* species.

Abstract

Divergent mimetic races of *Heliconius erato* and *H. melpomene* provide a striking example of adaptive radiation. This radiation has occurred through divergence at just a handful of genetic loci. Previous authors (Turner, 1983; Sheppard et al., 1985) have used allelic changes at these loci to construct evolutionary networks connecting colour pattern races, based on the assumption that similar phenotypes are uniquely derived. However, phylogenies of mitochondrial DNA variation in *Heliconius erato* and *H. melpomene* show that mtDNA relatedness is determined largely by geography (Brower, 1996). In *H. erato* there is a genetic break between west and east Andean populations, which occurs within a widespread colour pattern race, *hydara*. Brower interpreted this as evidence that apparently homologous colour patterns have multiple origins. Here new data on the likely ancestor to the *erato* radiation are presented which allow a more complete analysis of colour pattern evolution. *H. himera* is a sister species to *H. erato* which is non-mimetic and monomorphic across its range. Hence analysis of the genetic basis of colour pattern differentiation between *himera* and *erato* provides information on the most probable ancestral genotype to the *erato* radiation. Just two major loci control most of the colour pattern variation within *himera x erato* hybrid broods. These loci are almost certainly homologous with others previously identified in *erato x erato* broods. Hence the morphological divergence of races and species involves similar genetic processes. Using these data it is possible to reconstruct a tree of colour pattern evolution within *erato* which is largely compatible with both the assumption that colour patterns are uniquely derived and the 1.5-2 million year old break in the mtDNA phylogeny. This resolution is primarily a result of placing the race *hydara*, which occurs across this mtDNA break, as an ancestral form, based on the results of the crossing experiments described. Hence the mtDNA phylogeny does not necessarily imply that apparently homologous colour patterns have multiple origins.

Introduction

The colour patterns of aposematic heliconiine butterflies are probably best known as an example of adaptive convergence, in the form of Müllerian mimicry, between species. At any one site in the neotropical rainforest there are five or more easily distinguished mimetic assemblages. These groups of species, known as mimicry rings, consist of heliconiine, ithomiine and other lepidopteran species which share a common aposematic colour pattern. Furthermore the common pattern in some of these mimicry rings changes abruptly every few hundred kilometres across the neotropics, producing concordant changes in the appearance of many species. Thus there are two dimensions to the diversity observed; distinct coexisting mimicry rings and abrupt changes in the dominant pattern across geographic space.

Perhaps the most extreme adaptive radiation of colour pattern has occurred in the Müllerian co-mimics, *Heliconius erato* and *H. melpomene*. These two species occur across the neotropics, where they have evolved into nearly 30 parapatric colour pattern races. Colour pattern radiation in both species is largely the result of divergence at just a few genetic loci. For example, although ten unlinked genes (or gene complexes) have been described in previous analysis of interracial crosses of *H. erato*, most of the phenotypic variation observed in this species can be explained by changes at only four major loci. A similar number of loci are involved in colour pattern change in *H. melpomene*. This pattern in which major phenotypic transitions involve changes at a few loci is predicted by mimicry theory (Turner, 1984) but could also result from genetic constraints, if just a few conserved loci underly the placement of colour pattern elements (Carroll, 1992; Nijhout, 1994).

Information on the evolutionary history of wing colour pattern within *H. erato* and *H. melpomene* is crucial to understanding the biogeographical and population genetic processes driving mimicry. Previous attempts to unravel the history of colour pattern change in these groups have been based either on allelic changes at wing pattern loci or the pattern of variation within mitochondrial DNA (Turner, 1983, 1984; Brower, 1994b, 1996). For example, based on a theoretical prediction that dominant alleles are more likely to become fixed and, therefore, be the derived state, Turner (1983, 1984) reconstructed the radiation of colour pattern in *erato* and *melpomene*. Clearly implicit in Turner's ideas is that alleles coding for the same colour pattern elements are uniquely derived. Brower has recently challenged this assumption using phylogenies

derived from mtDNA variation. He found that geography, rather than colour pattern phenotype, was a better predictor of mitochondrial relatedness (Brower, 1994b). Indeed, the only major break in the mtDNA phylogeny occurred within a widespread colour pattern race, *hydara*, which occurs from Panama to Guyana. Brower used this discordance as evidence that colour pattern radiation has involved repeated parallel evolution of identical patterns.

Evaluating these competing hypothesis is made difficult because of the lack of any information on the genetics of wing colour pattern change in closely related outgroup taxa. For *H. erato* at least, molecular and morphological trees show *H. himera* to be the nearest outgroup (Brown, 1979; Brower, 1994a; Jiggins et al., 1996). Furthermore it is likely that the ancestor to both species resembled *himera*. The yellow forewing and red hindwing band pattern characteristic of *himera* also occurs in another closely related species to *H. erato*, *H. clysonymus*. In addition, unlike *H. erato*, neither *H. himera* nor *H. clysonymus* are involved in mimicry or exhibit much geographic variation in colour pattern. Thus it is less likely that these species are subject to convergent evolutionary pressures that might lead to rapid colour pattern change.

H. himera and *H. erato* are completely inter-fertile which allows detailed hybridisation experiments to examine the genetic basis of colour pattern differences. Here I re-evaluate colour pattern evolution within *Heliconius erato,* using these crosses. I use these data, in the light of Brower's mitochondrial DNA phylogeny and past inter-racial crosses, to answer two specific questions about colour pattern evolution in this group. First, are the genetic loci responsible for colour pattern differences between *himera* and *erato* similar to those already observed within *erato*? Second, using the assumption that the *H. himera* pattern is ancestral, is it possible to reconcile the mtDNA phylogeny with a hypothesis of parsmonious colour pattern evolution?

Methods

Crosses were carried out between stocks collected in Vilcabamba, (*H. himera*), Piñas and Balsas (*H. e. cyrbia*) in southern Ecuador. Rearing methods are described

elsewhere (Chapter 4; McMillan et. al., 1997). After eclosion wings were removed and stored in glassine envelopes, whilst the bodies were immediately frozen in LN_2 for genetic analysis.

Virgin females were obtained by rearing the offspring of wild caught females. These were then mated in an outdoor cage with either wild caught males or healthy reared males. The latter would only mate readily when over a week old and supplied with ample pollen and nectar. In total 20 F1, 17 backcross, 7 F2 and 21 further crosses were carried out. Four F1 broods and all other crosses were scored for wing colour pattern phenotype. A total of 1243 offspring (not including F1 broods) are included in the analysis. In the description of crosses, 'F1' and 'F2' are used according to strict definition, i.e. *himera* x *cyrbia* and F1 x F1 crosses respectively. 'BH' shall refer to a backcross to *himera*, i.e a cross between an F1 and a pure *himera*; 'BC' a backcross to *cyrbia*; 'BHH', a cross between offspring of a BH brood and a pure *himera*; 'BHF2', a cross between a BH offspring and an F2, etc.

Variation in the position and width of the forewing band was scored by measuring the shortest distance from the distal and proximal edges of the band, to the point at which the third medial vein joins the cell. The proportion of the band lying distal to this point was then calculated as an estimate of band position and the sum of the two measurements was used as a measure of band width.

The cathodal Aconitase enzyme shows a virtually fixed difference between *himera* and *erato* (Chapter 2) and therefore segregates in phase with colour pattern markers ($Acon^{100}$ is the *erato* allele, $Acon^{150}$ the *himera* allele). Eight backcross broods were analysed to investigate linkage with colour pattern genes. As there is no crossing over in female *Heliconius* (Turner et al., 1975; pers. obs.), backcross broods with female F1 parents were used to detect linkage: Any recombination implies that markers are on different chromosomes.

Hypotheses for the inheritance of colour pattern were developed and then tested in all broods. Goodness of fit and homogeneity G-tests were used and probabilities were determined using the χ^2 distribution (G approximates to χ^2). All broods with the same expected pattern of segregation were combined for each test. Where significant heterogeneity was found between broods, this is discussed in the text.

Results

H. himera and *H. erato cyrbia* are very different in appearance (Fig. 1). The forewing band is yellow in *himera* and red in *cyrbia.* On the hindwing *himera* has a broad red bar on the upperside, whilst *cyrbia* has a narrow yellow underside bar and a white margin visible on both upper- and undersides. Finally, the upperside background colour of *cyrbia* is iridescent blue, whilst that of *himera* is black. Details of all crosses are given in the Appendix.

The red locus

The red *cyrbia* forewing band and red *himera* hindwing bar were controlled by alternate alleles at a single locus, D, here named D^{hi} in *himera* and D^{c} in *cyrbia* (Fig. 1). F1 hybrids exhibited both elements, whilst backcrosses to *himera* all exhibited the red hindwing bar, and backcrosses to *cyrbia* all had the red forewing band, which implies that the alleles are co-dominant. In backcrosses to *cyrbia* there was segregation of individuals with and without red hindwing bars in the expected 1:1 ratio (162:150; G_1=0.46, NS). In backcrosses to *himera* there was 1:1 segregation of individuals with red (which can vary from a full red band to only 5% red scales on a yellow band) and without red in the forewing band (127:101; G_1=2.97, NS). Similarly as expected for a dominant gene, both elements showed 3:1 segregation in F2 broods (red forewing, 135:51, G_1=0.568, NS; red hindwing, 139:47, G_1=0.007, NS).

There was no evidence for recombination between the red forewing and red hindwing alleles. In these and previous crosses between races of *H. erato* no individuals are known with neither red in the forewing nor hindwing, which would imply recombination within the D locus (Sheppard et al., 1985; Mallet, 1989). In F2 crosses 9:3:3:1 segregation would be expected under independent assortment of D^{hi} and D^{c} as separate loci. In fact there was a ratio of 86:48:52:0 summed over 7 broods which did not deviate from the 2:1:1 ratio expected for alternate co-dominant alleles at the same locus (G_2=0.70, NS). All 21 further crosses involving backcross or F2 parents were consistent with the proposed hypothesis (Appendix).

Certain phenotypes were only seen in hybrids suggesting additional interactions between *himera* and *erato* alleles. The red *himera* hindwing bar was expressed on the underside of the wing in many hybrids. This occured in all F1 individuals although there was considerable variation in the level of expression. In BH and further backcross broods there was stronger expression of red on the hindwing underside in those individuals which do not express red in the forewing. For example in Brood 147 (F2H) 3 / 16 $D^{c}D^{hi}$, and 28 / 28 $D^{hi}D^{hi}$ genotypes showed expression of red on the hindwing underside. This was somewhat counterintuitive, as the homozygous *himera* genotype was more likely to express the hybrid phenotype. Other loci must therefore interact with D in the underside expression of red bar.

The yellow locus

The yellow and white colour pattern elements, i.e. the *cyrbia* underside bar, the *cyrbia* white margin and the *himera* forewing band, were controlled by alternate alleles at a locus, Cr (Fig. 1). The alleles are here named Cr^{c} in *cyrbia* and Cr^{hi} in *himera*. None of these yellow or white pattern elements were strongly expressed in F1 broods which implies that the alleles were largely recessive.

The *cyrbia* yellow bar and white margin segregated together in backcrosses to *cyrbia* and further crosses in the expected 1:1 ratio (205:209; G_{1}=0.04, NS). $Cr^{hi}Cr^{c}$ genotypes occasionally showed a black shadow in the position of the yellow bar, and a scattering of white scales in the position of the white margin whilst $Cr^{c}Cr^{c}$ homozygotes always showed full expression of both elements. In 8 F2 broods there was segregation of phenotypes with : without yellow bar + white margin in the expected 1:3 ratio (36:150, G_{1}=3.34, NS). In two BC broods with female F1 parents tested for allozyme linkage (#51 & 62; n=48), there was a complete association between presence of the *himera* $Acon^{150}$ allele and absence of the yellow bar and white margin. There was no significant association between the loci in broods with male F1 parents (#72 & 127; n=67; G_{1}=1.83, NS).

The yellow forewing also segregated in a simple Mendelian fashion. This was best illustrated in three broods (#108, 120 & 122) which showed segregation of the yellow forewing in the absence of red (i.e. parental genotypes are $Cr^{hi}Cr^{c}$, $D^{hi}D^{hi}$ and $Cr^{hi}Cr^{hi}$, $D^{hi}D^{hi}$). The offspring of these broods fell into two classes; one with an entirely yellow forewing band (full yellow) and the other a smudgy yellow / black forewing,

which resulted from a mixing of black and yellow scales (yellow / black). Full yellow bands may have fuzzy edges, but the centre of the band always consisted of pure yellow scales. These phenotypes did not deviate from the 1:1 ratio expected under a single gene hypothesis (28:26; G_1=0.511, NS). In the absence of red, Cr^{hi} is therefore partially recessive, although in some genetic backgrounds Cr^{hi} may be completely recessive to Cr^{c}; e.g. male parent of Brood 136 (Appendix).

The BH broods illustrate the interaction of Cr^{hi} with D^{c} in the forewing. Those offspring with $D^{hi}D^{c}$ genotypes showed variation in expression of red in the forewing from 100% to 5% red scales in an otherwise yellow band. Analysis of linkage with the Aconitase locus implies that the relative expression of yellow and red reflects segregation of $Cr^{hi}Cr^{hi}$ $D^{c}D^{hi}$ and $Cr^{c}Cr^{hi}$ $D^{c}D^{hi}$ genotypes (Table 1). The latter all showed yellow with 50-100% red scales in the forewing band, whilst the former exhibited yellow with <50% red scales. However when the overall ratios were tested for the four phenotypes found in BH broods, (full yellow: yellow / black: yellow with <50% red: yellow with >50% red) this gave 67:72:86:68, deviating from the expected ratio of 1:1:1:1 (G_3=13.17, $p<0.01$), and showing significant between brood heterogeneity (G_{39}=49.74, $p<0.05$). This heterogeneity was due to variation in the proportions of yellow with <50% red : yellow with >50% red phenotypes. Therefore, although Aconitase linkage analysis implies that within brood variation in the expression of red and yellow in the forewing band can be mostly explained by segregation of Cr and D, other modifier loci must be involved which give rise to the observed between brood variation

Similar heterogeneity was seen in F1 broods, where it is easier to interpret as all offspring have $D^{hi}D^{c}$ $Cr^{hi}Cr^{c}$ genotypes. In most F1 broods all individuals had full red bands, but others showed variation from pure red to 60% yellow scales in the band. Females were more likely to have a high proportion of yellow scales. For example in brood 49; 16 males all showed >80% red scales in the forewing, whilst of 15 females, only 7 had >80% red scales (the test for heterogeneity on this data gives G_1=14.7, $p<0.0005$). Therefore some of the modifier genes which affect the interaction of Cr^{hi} and D^{c} have sex-linked or sex-limited expression.

There was further evidence for interaction between production of red and yellow pigment. Hybrid $D^{hi}D^{c}$ genotypes commonly exhibit white scales in the forewing

band, a trait never seen in any $D^{hi}D^{hi}$ or $D^{c}D^{c}$ genotypes. For example, in F2 broods 21 / 23 $Cr^{hi}Cr^{hi}$ $D^{hi}D^{c}$ genotypes, and 34 / 46 $Cr^{hi}Cr^{c}$ $D^{hi}D^{c}$ genotypes expressed some white scales in the yellow and red forewing band. In contrast none of the 42 $Cr^{hi}Cr^{hi}$ $D^{hi}D^{hi}$ and $Cr^{hi}Cr^{c}$ $D^{hi}D^{hi}$ genotypes expressed any white scales. Therefore the white colour seemed to result from disruption of yellow pigment production by the D^{c} allele.

The Cr locus also controlled some of the variation in forewing band shape. In *H. himera* the forewing band was broader and more proximal than in *erato*. F1 broods were approximately intermediate in position and shape. BH broods show little variation and were similar to *himera*, suggesting that *himera* allele(s) controlling this effect are largely dominant. BC broods showed considerable variation in phenotype, with $Cr^{hi}Cr^{c}$ genotypes having significantly broader forewing bands than $Cr^{c}Cr^{c}$ genotypes (Table 2). No significant association was found with any of the other pattern elements tested or with band position (Table 2). Therefore band width is controlled at least in part by the Cr locus.

There was limited evidence that the Cr locus may consist of two closely linked genes. Of 186 F2 offspring, one individual has a phenotype which implies recombination between *himera* and *cyrbia* alleles within Cr. This individual had a 30% yellow / 70% black forewing band, and a hindwing white margin and yellow bar, a phenotype most easily interpreted as a heterozygote for the *himera* Cr^{hi} allele but a recessive homozygote for the *cyrbia* Cr^{c} allele. This genotype would have an expected frequency of 1/32 under random assortment of *himera* and *cyrbia* alleles, giving an expected 5.2 individuals against the one observed. Only one brood (#136) showed segregation of both yellow elements in the absence of the red forewing, allowing all Cr genotypes to be scored with confidence (Appendix). This showed no evidence for recombination in 26 offspring. Unfortunately phenotypic overlap between genotypes ($Cr^{hi}Cr^{c}$ $D^{hi}D^{hi}$ with $Cr^{c}Cr^{c}$ $D^{hi}D^{hi}$, and $Cr^{hi}Cr^{hi}$ $D^{hi}D^{c}$ with $Cr^{hi}Cr^{c}$ $D^{hi}D^{c}$) precluded an accurate estimation of recombination rates across all broods.

The difficulty of identifying recombinant phenotypes was further highlighted by analysis of the *cyrbia* yellow bar and white margin traits. Six individuals with apparent recombinant phenotypes were seen (Appendix), which expressed only one or other element. However three of these individuals occurred in first generation

backcross broods with a female F1 parent. As there is no recombination in females, these phenotypes could not have been generated by recombination and must be due instead to hybrid combinations of modifier genes. This must cast some doubt on the interpretation of these unusual phenotypes as evidence for recombination within major loci.

Blue iridescence

Although not inherited as a simple Mendelian trait, there was some evidence that the blue iridescence is affected by at least one gene of major effect. F1 broods showed intermediate levels of blue and backcrosses to *himera* mostly exhibited faint blue. Backcrosses to *cyrbia* segregated approximately into individuals with half vs. full iridescence in a 1:1 ratio (143:167; G_1=1.86, NS) which would imply that one partially recessive major gene is involved. However there was significant heterogeneity between broods (G_{10}=38.63, $p<0.0005$), suggesting differences between broods in genetic background. There was no evidence for any association between expression of iridesence and the other colour pattern traits, implying that the gene(s) is not linked to Cr or D. Levels of iridescence were more continuously variable than might be expected if they reflected segregation of a single gene and it seems most likely that iridescence and blue colour are controlled by more than one locus.

A comparison with previous crosses between H. erato races

The D locus is very similar in action, and presumably homologous to the D, R, Y linkage group of Sheppard et al. (1985). The D, R and Y loci were identified by Sheppard et al. (1985) as being responsible for the positioning of different red elements in a number of different crosses. They are very closely linked and possible recombinant phenotypes are extremely rare between D and R and not known between Y and D or R (Mallet, 1989). D, R and Y are therefore considered here as if they were a single locus. The D^c allele is probably homologous to the d^{rY} allele, which produces a red forewing band in other races of *H. erato*. Alleles at the Cr locus control the yellow hindwing bar in all crosses of *H. erato*. In addition Cr was linked to Aconitase in crosses between *H. e. lativitta* and *H. e. favorinus* in a manner similar to that described above (Mallet, 1989). Despite this, the phenotypic effects of Cr are different in the *himera x erato* crosses. Most notably, expression of both red and yellow forewing band phenotypes reflected segregation of alleles at the D locus in

previous *erato* crosses, with all hybrids showing either a red or yellow band. This contrasts with the situation described here where the red and yellow result from segregation of two unlinked loci. Nonetheless, there is good evidence for homology between the loci described here and those previously identified within *H. erato*, even though patterns of expression differ.

Discussion

The genetics of mimicry

The mimetic differences between *Heliconius himera* and *H. erato cyrbia* are controlled by a remarkably simple genetic system. Virtually all the variation observed in hybrid broods can be explained by the segregation of alleles at just two major loci. The only major trait not influenced by these loci is the blue iridescent background colour of *cyrbia.* Genetic studies of a number of races of both *H. erato* and *H. melpomene* have also shown major locus inheritance (Sheppard et. al., 1985). For example hybrid zones near Tarapoto in Peru separate races differing at only three colour pattern loci in *H. erato* and four in *H. melpomene* (Mallet, 1989). On the basis of linkage analysis and very similar patterns of segregation, the genetic loci described here are almost certainly homologous with those already identified within *H. erato* (Sheppard et al., 1985; Mallet, 1989). The divergence of colour pattern morphology between species and races therefore appears to involve the same genetic machinery.

It has been suggested that these major colour pattern loci are gene complexes, consisting of several tightly linked loci. Field caught and laboratory reared recombinant phenotypes provide evidence for two genes, or perhaps two distinct mutation sites within the D locus (Mallet, 1989). Similarly in the crosses described here there is limited evidence for recombination within the Cr locus. However all of the recombinant phenotypes described here might also be explicable as a result of hybrid combinations of modifier genes.

The pattern described broadly supports the work of Nijhout (1991, 1994) who has proposed a model of *Heliconius* wing pattern development, based on inferred homologies with the so called 'nymphalid ground plan'. His work implies that small changes in developmental pathways can lead to large visual changes in pattern

elements, which provides a developmental rationale for the genetic system observed. Nonetheless some of the details of Nijhout's model do not seem to be supported by the crosses described here. In particular he suggests that red elements are 'pattern' whilst yellow is 'background' (Nijhout, 1994). The mixed expression of red and yellow within the clearly defined forewing band of *himera* x *erato* hybrids seems to suggest that these pigments are switched on (or off) in response to the same signal. Red and yellow seem to be produced in a homologous manner, in contradiction to Nijhout's model. In general it may be impossible to infer the developmental pathways of species with rapidly evolving colour patterns, such as *Heliconius,* from highly conserved model systems, such as those used to derive the 'nymphalid ground plan'.

The history of the erato colour pattern radiation

Previous attempts to understand the evolutionary history of wing colour radiation have used two apparently conflicting approaches. Turner (1983, 1984) constructed a colour pattern network connecting races based on two assumptions, firstly that wing pattern phenotypes are uniquely derived, and secondly that dominant alleles are the derived state. Recently Brower (1994b, 1996) has criticised this approach on the basis that wing colour genes are known to be under strong mimetic selection and therefore liable to convergent evolution (Mallet & Barton, 1989). He suggested that it would be better to use presumed neutral genetic markers, which are not subject to such evolutionary pressures to infer the phylogeny of geographic races. Based on a phylogeny of mitochondrial sequence variation he showed that there was no association between mtDNA haplotypes and colour pattern, and concluded that independent evolution of identical colour patterns has occurred a number of times within mitochondrial clades of both species.

Although in general neutral genetic variation is likely to be a better indicator of relatedness between species than genes which are under stabilising or convergent selection, this may not be the case for races within a species. If gene flow leads to a dissociation of neutral variation from the selected traits which identify races, then that variation will not be informative as to the evolutionary history of the races. In this case decoupling of markers is likely to occur in two ways. Firstly colour pattern hybrid zones are likely to move, as they are not associated with any extrinsic habitat features. If one phenotype is favoured by natural selection or if the alleles are

dominant, then that pattern will spread at the expense of inferior or recessive phenotypes. Secondly, in hybrid zones between races, mating occurs at random and there is no evidence that such boundaries would form a barrier to mtDNA gene flow (Mallet et al., 1996). Therefore advantageous or even neutral mtDNA variation will spread across colour pattern boundaries. Given the general lack of concordance between mtDNA haplotype and colour pattern within the major mtDNA clades, it seems likely that both these processes have occurred.

It follows that the best way to understand the relationships between colour pattern phenotypes is to study the genes underlying those traits. The mtDNA tree might also provide some information with respect to the history of races. The existence of a deep genetic break in the mtDNA phylogeny between west and east Andean clades (approx 1.5 - 2 million years old) suggests a vicariance event (Brower 1994b). When the position of that break is concordant in two species (*erato* and *melpomene*) and occurs in an area characterised by faunal transition then the evidence is compelling that the genetic pattern is the result of a past or current barrier to gene flow (the eastern and western Andean fauna are very distinct, both in the Lepidoptera and other taxa, Brown, 1979; Haffer, 1967). This vicariance event needs to be taken into account when attempting to reconstruct the history of colour pattern evolution within *erato*. Here the relationships between races are re-evaluated in the light of the *himera* x *erato* crosses, previous inter-racial crosses, and the mtDNA evidence.

Assuming only that *himera* is the ancestral genotype, it is possible to reconstruct an evolutionary network which is consistent firstly with the hypothesis that similar colour patterns are uniquely derived, and secondly with the mtDNA clades (Fig. 2). Under this hypothesis the red forewing band race, *hydara*, which occurs across the mtDNA break, is the most ancestral pattern within *erato* and is derived by a single gene substitution from the *himera* ancestor. This genotype is actually produced in our F2 broods, where $D^{c}D^{c}Cr^{hi}Cr^{hi}$ genotypes are very similar in appearance to *hydara* (Fig. 1). This change must have occurred before the mtDNA split. All subsequent colour pattern evolution occurs within the two mtDNA clades. At this level the mitochondrial phylogeny is uninformative, as the mtDNA tree shows no resolution within western and eastern clades (Brower, 1994b & 1996). This lack of resolution could be due either to a very recent origin of colour patterns within these areas, such

that coalesence of haplotypes has not yet occurred, or to gene flow of haplotypes between colour pattern races.

The allelic changes shown (Fig. 2) depend on a revised interpretation of previous crosses, in the light of our *himera* x *erato* broods. The hypothesis shows the amazonian races as retaining the ancestral Cr^{hi} allele, giving rise to a yellow forewing phenotype. This is a slightly different interpretation than that of Sheppard et al. (1985) and implies that all races previously crossed are fixed for a homolog of Cr^{hi}, which gives a yellow forewing as the background phenotype, expressed in the absence of the d^{rY} (red forewing) genotype. The alternative hypothesis, that the yellow forewing has arisen in the Amazon by a change at the D locus, would add another two steps to the tree (loss of Cr^{hi} followed by gain of D^{ry}) but would not alter the branching pattern. Crosses between *himera* and amazonian races would be necessary to test whether the yellow forewing is homologous in the two forms.

The only apparently non-parsimonious feature of the hypothesis is that the hindwing yellow bar has arisen twice, once on either side of the Andes, which somewhat contradicts previous interpretation of inter-racial crosses (Sheppard et. al., 1985). However there is good genetic evidence that this phenotype is produced by independently derived alleles on either side of the Andes. Crosses between butterflies from Panama and east Brazil produce F1 offspring with a strongly disrupted yellow bar. Indeed the yellow bar in the east results from two independent loci, cr and yl, in two races investigated (*phyllis* and *favorinus*) whilst a yellow bar phenotype in the west is produced by a single allele also at the Cr locus in both *cyrbia* and *petiverana*. The yellow bar has evolved independently at least once more in the genus in *H. telesiphe* which supports the plausibility of this hypothesis (*melpomene* cannot be considered an independent test because of mimicry with *erato*). Therefore it may be possible to identify convergent evolution of colour pattern phenotypes from the results of crossing experiments.

It is therefore possible to reconstruct a pathway for the evolution of colour patterns in *H. erato* which is consistent with both the mtDNA phylogeny and what is known about the genetics underlying colour pattern. This is primarily a result of placing *hydara* as an ancestral pattern that evolved prior to the vicariance event between east and west Andean populations, and secondly the dual origin of the yellow bar in the

east and western clades. There are a number of outstanding questions which remain to be answered about the evolution of colour pattern in *Heliconius erato*. In particular the race *H. e. chestertonii* has a distinct mtDNA haplotype, suggesting that it may be an ancestral form. However, in the absence of any data on the colour pattern genetics of this race, it is placed in a relatively derived position on the tree with the other west Andean yellow bar races. Crossing experiments on this, and other races not yet investigated, would be necessary to provide a more complete analysis of the system. Nonetheless, it is clear that, in contrast to the conclusions of Brower (1994b & 1996), there is little evidence for multiple origins of apparently homologous pattern genes.

References

Brower, A.V.Z. 1994a Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Molec. Phylog. Evol.* **3**, 159-174.

Brower, A.V.Z. 1994b Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc. Natl. Acad. Sci., USA* **91**, 6491-6495.

Brower, A.V.Z. 1996 Parallel race formation and the evolution of mimicry in *Heliconius* butterflies: a phylogenetic hypothesis from mitochondrial DNA sequences. *Evolution* **50**, 195-221.

Brown, K.S. 1979 *Ecologia Geográfica e Evoluçâo nas Florestas Neotropicais.* (Livre de Docencia) Campinas, Brazil: Universidade Estadual de Campinas.

Carroll, S.B., Gates, J., Keys, D.N., Paddock, S.W., Panganiban, G.E.F., Selegue, J.E. & Williams, J.A. 1994 Pattern formation and eyespot determination in butterfly wings. *Science* **265**, 109-114.

Haffer, J. 1967 Speciation in Colombian forest birds west of the Andes. *Am. Mus. Novitat.* **2294**, 1-57.

Jiggins, C., McMillan, W.O., Neukirchen, W. & Mallet, J. 1996 What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* **59**, 221-242.

Mallet, J. 1989 The genetics of warning colour in Peruvian hybrid zones of *Heliconius erato* and *H. melpomene*. *Proc. Roy. Soc. Lond. B* **236**, 163-185.

Mallet, J. & Barton, N.H. 1989 Strong natural selection in a warning color hybrid zone. *Evolution* **43**, 421-431.

Mallet, J., Jiggins, C.D. & McMillan, W.O. 1996 Mimicry meets the mitochondrion. *Current Biology* **6**, 937-940.

McMillan, W.O., Jiggins, C.D. and Mallet, J. 1997. What initiates speciation in passion vine butterflies? *Proc. Natl. Acad. Sci., USA*, submitted.[see Appendix]

Nijhout, H.F. 1991 *The Development and Evolution of Butterfly Wing Patterns.* Washington, DC: Smithsonian Institution Press.

Nijhout, H.F. 1994 Developmental perspectives on evolution of butterfly mimicry. *BioScience* **44**, 148-157.

Sheppard, P.M., Turner, J.R.G., Brown, K.S., Benson, W.W. & Singer, M.C. 1985 Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. *Phil. Trans. Roy. Soc. Lond. (B)* **308**, 433-613.

Turner, J.R.G. 1983 Mimetic butterflies and punctuated equilibria: some old light on a new paradigm. *Biol. J. Linn. Soc.* **20**, 277-300.

Turner, J.R.G. 1984 Mimicry: the palatability spectrum and its consequences. In *The Biology of Butterflies* . (ed. R.I. Vane-Wright & P.R. Ackery) (Symposia of the Royal Entomological Society of London, 11.), pp. 141-161. London: Academic Press.

Turner, J.R.G. & Sheppard. 1975 Absence of crossing-over in female butterflies (*Heliconius*). *Heredity* **34**, 265-269.

	forewing band colour			
	full yellow	yellow / black	yellow with <50% red	yellow with >50% red
Genotype	$D^{hi}D^{hi}$ $Cr^{hi}Cr^{hi}$	$D^{hi}D^{hi}$ $Cr^{hi}Cr^{c}$	$D^{hi}D^{c}$ $Cr^{hi}Cr^{hi}$	$D^{hi}D^{c}$ $Cr^{hi}Cr^{c}$
$Acon^{100}Acon^{150}$	0	11	0	12
$Acon^{150}Acon^{150}$	13	0	12	0

Table 1; Linkage of Aconitase-s with yellow forewing. Combined results from BH broods 63 & 68. Both broods have female F1 parents thus excluding the possibility of recombination. In broods with male F1 parents there is no significant association between these markers which implies that linkage is not tight. This locus also segregates with the hindwing yellow bar + white margin phenotype in BC broods (data not shown).

			n	Band width	U	p	Band position	U	p
himera red bar	$D^{hi}D^{c}$	+	88	3.08 ±0.09			0.67 ±0.016		
	$D^{c}D^{c}$	-	112	3.28 ±0.10	4337	NS	0.67 ±0.013	4925	NS
cyrbia yellow bar	$Cr^{c}Cr^{c}$	+	98	3.02 ±0.09			0.66 ±0.015		
and white margin	$Cr^{c}Cr^{hi}$	-	102	3.35 ±0.09	3966	**	0.68 ±0.014	5377	NS
Iridescence		++	81	3.33 ±0.10			0.65 ±0.015		
		+	119	3.10 ±0.09	4158	NS	0.68 ±0.014	4234	NS

Table 2; Band position in backcross to *cyrbia* broods. The mean forewing band width and position are shown for different Cr and D genotypes in BC broods. Mean values and standard errors are shown for each genotype and tested by a Mann-Whitney U test. Values of U and p are shown, where NS signifies $p>0.05$ and ** $p=0.01$.

Figure 1; Summary of *himera* x *erato cyrbia* broods.

The top left and bottom right phenotypes are *Heliconius himera* and *H. erato cyrbia* respectively. The central phenotype is an F1. The two boxes show the BH (dotted line) and BC (solid line) phenotypes. The D locus controls position of the red elements and is co-dominant. The Cr locus controls the yellow and white elements and both alleles are largely recessive. There is considerable variation in expression of certain genotypes. Overlap occurs between the expression of $Cr^{hi}Cr^{c}$ $D^{hi}D^{hi}$ and $Cr^{c}Cr^{c}$ $D^{hi}D^{hi}$ genotypes, which implies variation in the dominance of Cr^{hi}. In addition $Cr^{hi}Cr^{hi}$ $D^{hi}D^{c}$ and $Cr^{hi}Cr^{c}$ $D^{hi}D^{c}$ overlap in phenotype due to modifier loci affecting the interaction between red and yellow in the forewing (see text for details).

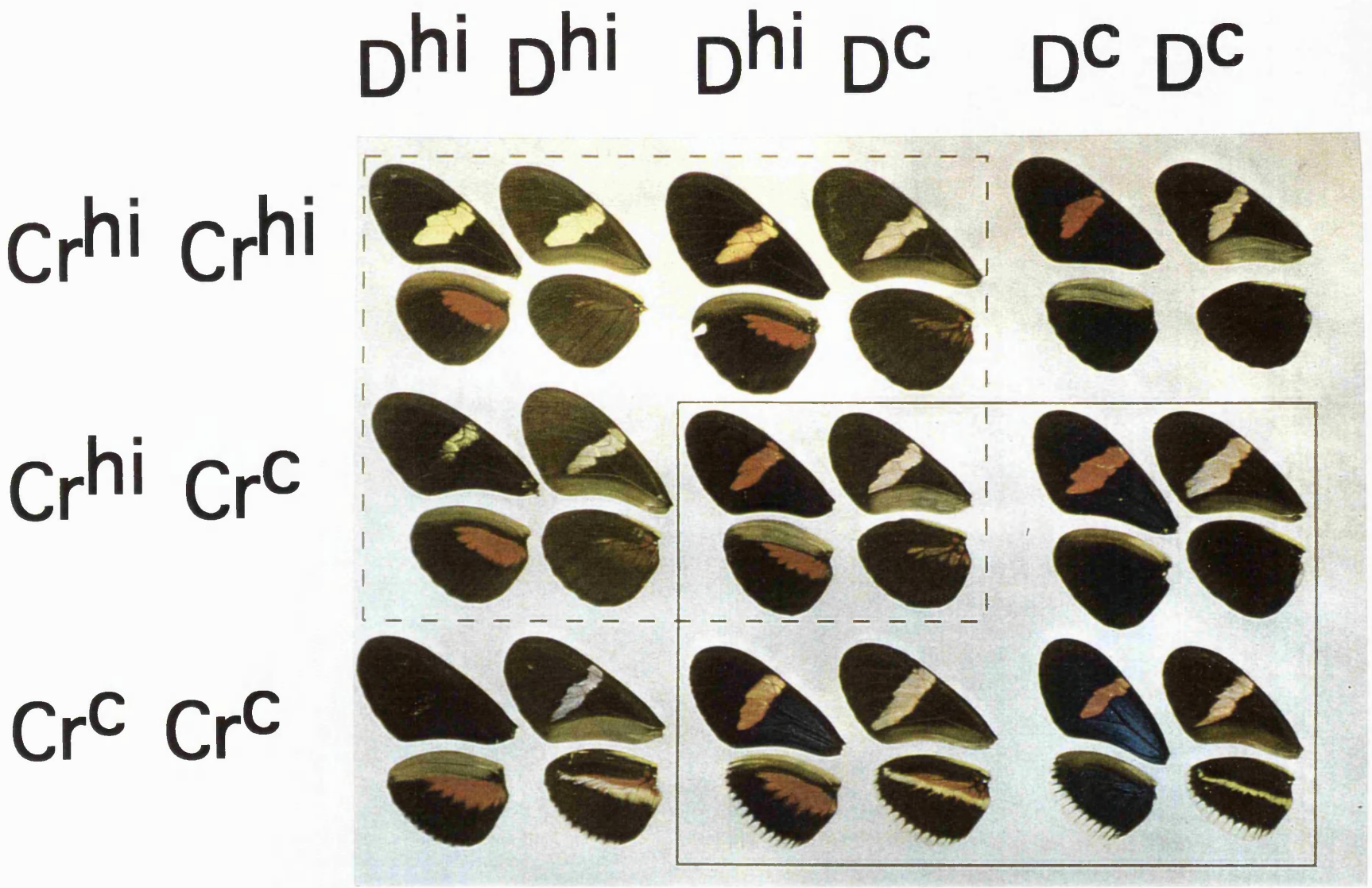
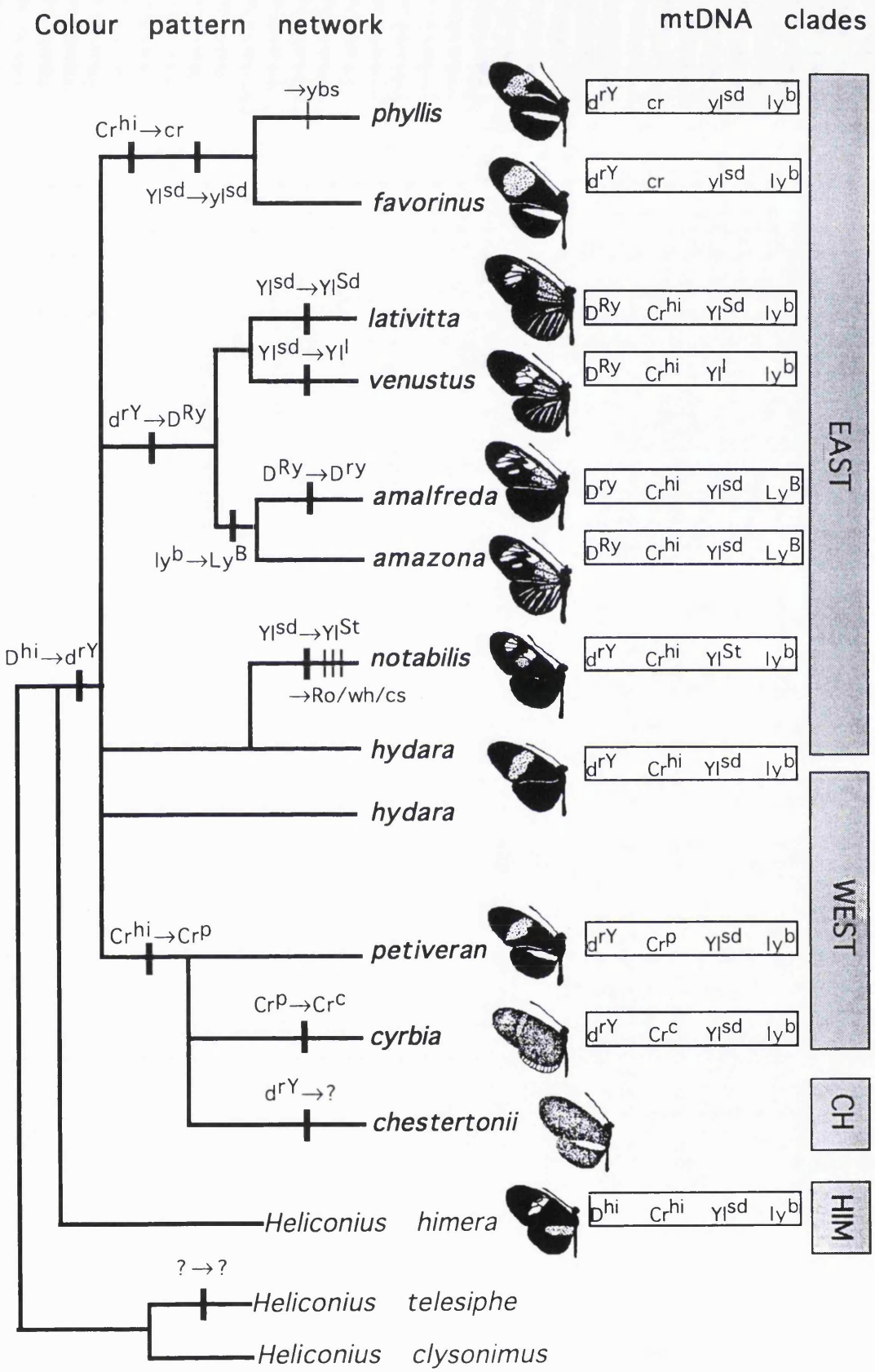

D^{hi} D^{hi}
D^{hi} D^{c}
D^{c} D^{c}
Cr^{hi} Cr^{hi}
Cr^{hi} Cr^{c}
Cr^{c} Cr^{c}

Figure 2; Parsimony network for the evolution of mimetic patterns in *H. erato*. On the right are shown the four major clades within the mtDNA tree of Brower (1996). The mtDNA data provides no further resolution within these clades. Positioning of the outgroups, *H. clysonimus, H. telesiphe* and *H. himera* is also determined from mtDNA sequence data. All races which have been studied by means of crossing experiments are included. Allelic changes at the four major colour pattern linkage groups are shown in bold (|) and genotypes listed, whilst phylogenetically uninformative changes at a further four loci are also shown (|) but genotypes for all races are not listed. Genotypes shown are deduced from Sheppard et al., 1985, Mallet, 1989 and data from the present study. Ur and Or loci (Sheppard et al., 1985) are not included following Mallet (1989). The race *venustus* is listed as having the Yl^{I} allele, although there is some evidence for homology with the yl^{t} allele. *H. e. chestertonii* is also included as this form has a distinct mitochondrial haplotype, although the exact position of this race is necessarily speculative as nothing is known about the genetic basis of the phenotype. This is not the only most parsimonious tree based on colour pattern data. For example deriving the rayed races from the ancestral *himera* pattern is equally parsimonious given only the colour pattern data but less likely in the light of evidence from colour pattern biogeography and mtDNA phylogeography.


Colour pattern network
mtDNA clades
→ybs
phyllis
Cr^hi→cr
favorinus
Yl^sd→yl^sd
d^rY cr yl^sd ly^b
d^rY cr yl^sd ly^b
Yl^sd→Yl^Sd
lativitta
Yl^sd→Yl^l
venustus
D^Ry Cr^hi Yl^Sd ly^b
D^Ry Cr^hi Yl^l ly^b
d^rY→D^Ry
D^Ry→D^ry
amalfreda
ly^b→Ly^B
amazona
D^ry Cr^hi Yl^sd Ly^B
D^Ry Cr^hi Yl^sd Ly^B
Yl^sd→Yl^St
notabilis
→Ro/wh/cs
D^hi→d^rY
d^rY Cr^hi Yl^St ly^b
hydara
d^rY Cr^hi Yl^sd ly^b
hydara
Cr^hi→Cr^p
petiveran
Cr^p→Cr^c
cyrbia
d^rY Cr^p Yl^sd ly^b
d^rY Cr^c Yl^sd ly^b
d^rY→?
chestertonii
Heliconius himera
D^hi Cr^hi Yl^sd ly^b
?→?
Heliconius telesiphe
Heliconius clysonimus
EAST
WEST
CH
HIM

Appendix; Colour pattern phenotypes of all brood offspring. Blue iridescence is scored separately from the other pattern elements, as no association was found between blue and any other element. (M) and (P) are the maternal and paternal phenotypes respectively. Numbers in brackets refer to the brood # of parental stock. Apparent recombinants within Cr are marked with a * in the notes below.

			Colour pattern phenotypes										
Hindwing *himera* bar			red	red	red	red	red	red	-	-	%		
Hindwing *cyrbia* yellow bar + white margin			-	-	-	-	+	+	-	+	Iridescence		
Forewing band colour			full yellow	yellow / black	yellow with <50% red	yellow with >50% red	black	red	red	red	0	+	++
Proposed genotypes			$Cr^{hi}Cr^{hi}$ $D^{hi}D^{hi}$	$Cr^{hi}Cr^{c}$ $D^{hi}D^{hi}$	$Cr^{hi}Cr^{hi}$ $D^{hi}D^{c}$	$Cr^{hi}Cr^{c}$ $D^{hi}D^{c}$	$Cr^{c}Cr^{c}$ $D^{hi}D^{hi}$	$Cr^{c}Cr^{c}$ $D^{hi}D^{c}$	$Cr^{hi}\_$ $D^{c}D^{c}$	$Cr^{c}Cr^{c}$ $D^{c}D^{c}$			
Brood #	mother	father											
F2 crosses													
58	F1	F1	3	4[7]	2	8	3	4	7	2	9	21	3
69	F1	F1	3	1	5	2	0	0	3	1	8	6	1
83	F1	F1	1	4[9]	2	6	0	2	6[10]	0	6	9	6
87	F1	F1	3	1	2	6	0	2[11]	5	2	5	9	6
109	F1	F1	3	4	5	7	1	6[12]	9	1	11	16	9
117	F1	F1	4[13]	3[14]	4	5	1	2[15]	2	4	3	15	7
137	F1	F1	5	3	3	12	1[16]	1	7	3	13	13	9

Notes

1. Trace of blue in most individuals.
2. No hindwing margin in one individual. *
3. 1 individual with weak white margin and yellow bar.
4. 3 individuals with a weak hindwing yellow bar.
5. Male parent and 3 offspring have an all black forewing
6. 1 individual with 10% black scales in forewing
7. 2 individuals with all black forewing, trace of red and yellow on veins.
8. 1 individual with no yellow bar. *
9. 2 individuals with all black fw band.
10. 1 individual with a 60% yellow and 40% red forewing band.
11. 1 individual with no yellow bar. *
12. 1 individual with weak hw white margin and yellow bar.
13. 1 individual with half length fw band.
14. 1 individual with all black fw band.
15. 1 individual with no yellow bar. *
16. 1 individual with a 30% yellow/70% black forewing band. *

			Colour pattern phenotypes										
Hindwing *himera* bar			red	red	red	red	red	red	-	-	%		
Hindwing *cyrbia* yellow bar + white margin			-	-	-	-	+	+	-	+	Iridescence		
Forewing band colour			yellow	yellow / black	yellow with <50% red	yellow with >50% red	black	red	red	red	0	+	++
Proposed genotypes			$Cr^{hi}Cr^{hi}$ $D^{hi}D^{hi}$	$Cr^{hi}Cr^{c}$ $D^{hi}D^{hi}$	$Cr^{hi}Cr^{hi}$ $D^{hi}D^{c}$	$Cr^{hi}Cr^{c}$ $D^{hi}D^{c}$	$Cr^{c}Cr^{c}$ $D^{hi}D^{hi}$	$Cr^{c}Cr^{c}$ $D^{hi}D^{c}$	$Cr^{hi}\_$ $D^{c}D^{c}$	$Cr^{c}Cr^{c}$ $D^{c}D^{c}$			
Brood #	mother	father											
First generation backcrosses													
52	F1	HIM	6	10	18	2					36[1]		
53	F1	HIM	3	3	4	1					11[1]		
63	F1	HIM	6	9	5	9					29[1]		
67	HIM	F1	7	8	11	11					34[1]		
68	F1	HIM	8	4	9	5					23[1]		
71	HIM	F1	4	3	7	5					19[1]		
126	F1	HIM	7	4	9	8					27[1]		
131	F1	HIM	0	1	0	0					1		
144	HIM	F1	7	2	4	5					18[1]		
149	HIM	F1	0	3	4	4					11[1]		
151	HIM	F1	1	5	1	5					12[1]		
51	F1	CYR				9		4	6[2]	8[2]		17	10
54	F1	CYR				4		2	8	3		10	7
59	F1	CYR				0		2	1	0		2	1
60	CYR	F1				7		6	6	7		12	14
66	F1	CYR				1		3	1	0		0	5
72	CYR	F1				9		13	11	7		14	25
75	F1	CYR				16		13	8	13		22	27
127	CYR	F1				7		16	8	4		24	11
129	F1	CYR				12		15[2]	14	20		31	30
140	F1	CYR				8		10	12	7		5	32
150	CYR	F1				2		3	5	1		6	5

			Colour pattern phenotypes										
Hindwing *himera* bar			red	red	red	red	red	red	-	-	%		
Hindwing *cyrbia* yellow bar + white margin			-	-	-	-	+	+	-	+	Iridescence		
Forewing band colour			yellow	yellow / black	yellow with <50% red	yellow with >50% red	black	red	red	red	0	+	++
Proposed genotypes			$Cr^{hi}Cr^{hi}$ $D^{hi}D^{hi}$	$Cr^{hi}Cr^{c}$ $D^{hi}D^{hi}$	$Cr^{hi}Cr^{hi}$ $D^{hi}D^{c}$	$Cr^{hi}Cr^{c}$ $D^{hi}D^{c}$	$Cr^{c}Cr^{c}$ $D^{hi}D^{hi}$	$Cr^{c}Cr^{c}$ $D^{hi}D^{c}$	Cr^{hi}_ $D^{c}D^{c}$	$Cr^{c}Cr^{c}$ $D^{c}D^{c}$			
Brood #	mother	father											
	Further backcrosses												
85	BH(63)	HIM	3	8	2	4(M)					17		
89	BH(63)	CYR		(M)		14		12				26	
91	CYR	BH(63)		(P)		9^{3}		11^{4}				4	15
97	BC(60)	CYR						3(M)		1			4
98	CYR	BC(60)								7(P)			7
99	HIM	BH(63)	5	3	3	1(P)					12		
107	BC	CYR							16(M)	12			28
108	HIM	BH(73)	1	4(P)							5		
112	BH(68)	HIM	23		16(M)						39		
113	BC(72)	HIM			6	6			(M)		6	6	
114	CYR	BC(72)						2					2
116	HIM	BC(75)		28		23	(P)					52	
120	HIM	BH(68)	16	13(P)									29
122	HIM	F2(83)	11	9(P)							12	8	
123	HIM	BH(71)	10	9	12	9(P)					29	11	
136	BHH(85)	F2(83)	9	10^{5}(M,P)			7				21	5	
141	HIM	BH(68)	18		23(P)						32	9	
142	F2(109)	HIM			13	18			(M)		31^{1}		
145	F2(109)	CYR							15(M)	13			28
147	HIM	F2(109)	28^{6}		16(P)						40	4	

CHAPTER 4

Host plant adaptation has not played a role in the recent speciation of *Heliconius himera* and *Heliconius erato* (Lepidoptera: Nymphalidae).

Submitted to *Ecological Entomology* with
W. Owen McMillan and James Mallet.

Introduction

Herbivorous insects are often highly specialised in their utilisation of host plants. This strong host specificity has been implicated as a driving force in speciation (Tauber & Tauber, 1989; Bush, 1994) and may be responsible for the huge species diversity present among herbivorous taxa (Bush, 1993). The association between *Heliconius* and their Passifloraceae host plants is one of the most widely cited examples of host-herbivore coevolution, in which there is clear evidence for the evolution of host plant defences on one hand, and their subsequent defeat by herbivores on the other (Gilbert, 1971; Smiley, 1978; Brown, 1981). Previous authors have used phylogenetic associations between *Heliconius* and *Passiflora* to support the hypothesis that adaptive radiation has occurred as a result of host-herbivore coevolution (Ehrlich & Raven, 1965; Benson et al., 1975; but see Mitter & Brooks, 1983). Furthermore, detailed studies of heliconiine communities demonstrate that coexisting species of *Heliconius* partition host plant resources (Gilbert, 1991), as expected if speciation is driven by host plant divergence. However, despite the important evolutionary interactions which undoubtedly take place between *Heliconius* and *Passiflora*, there is little direct evidence for host plant mediated speciation.

There are essentially two ways in which host plant adaptation can directly precipitate speciation. Firstly co-speciation, whereby a speciation event in the host directly results in speciation of an obligate herbivore. Secondly, and perhaps more common, are host plant shifts whereby new herbivore races arise which are adapted to alternative host species. In the extreme case this can lead to divergent evolutionary pathways between sympatric forms, as has occurred in *Rhagoletis pomonella* (Feder et al., 1988;

Mcpheron et al., 1988; Smith, 1988). The observed result of both processes will be strong differences in host plant use between closely related sympatric species, as is the case in *Heliconius*. However, such a pattern might also be observed if speciation were unrelated to host plant ecology, but subsequent divergence in host plant use is necessary to allow coexistence of daughter species. To test these possibilities we carried out an experiment to investigate the causes of speciation in a pair of parapatric sister species of *Heliconius*.

Heliconius himera and *Heliconius erato* are a closely related species pair which hybridise occasionally in narrow zones of parapatric contact (Mallet, 1993; Brower, 1994; Jiggins et al. 1996). *H. himera* occurs in the dry thorn scrub woodlands of south western Ecuador, north western Peru and the Marañon valley, whilst the various colour pattern races of *H. erato* are widespread in wet forest across south and central America. Although they have previously been considered as races (Eltringham, 1916; Lamas, 1976; Brown, 1979; Sheppard et al., 1985; but see Kaye, 1916 and Emsley, 1965), we consider them to be separate species as their genetic identity is maintained in the contact zones. In the centre of the contact zone intermediate forms occur at a frequency of 5-10% in the population, a pattern which is concordant at both colour pattern and presumed neutral genetic markers (Jiggins et al., 1997). The reduced frequency of hybrids is primarily a result of strong assortative mating behaviour, with no evidence of reduced fertility or viability in hybrids (McMillan et al., 1997). These species therefore provide an ideal opportunity to investigate the importance of host plant adaptation in the early stages of speciation.

Here we present the results of an experiment to investigate whether *Heliconius erato* and *H. himera* differ in their host plant ecology. Differences in host plant use between these sister species could suggest speciation by host plant shifts. On the other hand, if the species do not differ then we can confidently exclude the possibility that speciation was driven by host plant adaptation.

Methods

Heliconius larvae feed exclusively on the family Passifloraceae (Brown, 1981). Field investigations (Jiggins et al., 1996) have shown that both *H. himera* and *H. erato*

cyrbia utilise two species, *Passiflora rubra* and *P. punctata*, in the contact zone region. Previous studies have shown that *H. erato* also feeds on other species, especially *P. auriculata* (Brown, 1981) which is abundant in wet forest throughout western Ecuador, but does not occur in the dry forest habitat of *H. himera*. Although *P. auriculata* has not been recorded as a host plant in this area (Jiggins et al. 1996), it is potentially interesting as it only occurs on the *erato* side of the contact zone, so is a possible host of *H. erato* but not *H. himera*. *Passiflora rubra*, *P. punctata* and *P. auriculata* are in the subgenus *Plectostemma* (Killip, 1938; Holm-Nielsen et al., 1988).

Host choice experiments were carried out as part of a series of breeding experiments to investigate hybridisation between *himera* and *erato*. All experiments were carried out in Vilcabamba, Loja province, Ecuador, which lies at 1600m in a central valley of the Andes, in a dry forest region. Data from 93 females is presented, representing a total of 3,948 eggs laid. Females used were either captured in the wild already mated, or were reared in laboratory conditions and mated with males in our cages. Laboratory reared butterflies were fed a mixture of *P. rubra* and *P. punctata*. All the females tested in this paper are 'pure types', i.e. non-hybrids, although in some instances they may have been mated with a male hybrid as part of the breeding experiments. Experimental females were kept individually in outdoor, wire mesh cages with dimensions 2x1x1m. Each cage was kept supplied with artificial nectar (≈10% sugar solution) in plastic feeders and fresh cut *Lantana* flowers. These provide a source of pollen which is essential for egg production in *Heliconius* (Boggs, 1981). *Passiflora* were grown in pots in each insectary cage. All cages contained both *Passiflora rubra* and *P. punctata* and a few also contained *P. auriculata*.

Every day the location of each egg was recorded and it was then collected by removing a small portion of the leaf or shoot on which it was laid. Data for all females which laid 10 or more eggs are included in the analysis. The data are divided into two separate experiments, involving a choice of either two or three host plant species, and each is analysed separately. The number of eggs laid by each female on each species was recorded. A nested G-test was then used to test for heterogeneity between individuals and species. Comparisons were also made between wild caught and laboratory reared females.

Results and Discussion

H. himera and *H. erato* showed virtually identical patterns of oviposition behaviour. Both species laid almost equal numbers of eggs on *P. rubra* and *P. punctata* (Fig. 1A). However both *H. himera* and *H. erato* laid fewer eggs on *P. auriculata* than on the other two species (Fig. 1B). This result suggests that the lack of field observations of either butterfly species utilising *P. auriculata* in the contact zone area (Jiggins et al., 1996) was at least partially due to preferences against *P. auriculata*. However, the fact that both species laid on *P. auriculata* confirms field records from other areas which have shown that this is a host of *H. erato* (Benson et al., 1975; Brown, 1981). It seems probable that *P. auriculata* is an occasional host in the field, and the lack of records is simply due to a paucity of data. It is interesting that *H. himera* also laid on *P. auriculata,* which is not known to occur in the dry forest habitat of *himera.*

There was strong heterogeneity between individual females within both butterfly species (Tables 1A &1B), with some females showing strong preferences for one or other host plant (Appendix). To some extent this could have been the result of differences in shoot availability between individual plants. Eggs were almost invariably laid singly on the newest shoots, so differences in the growth form of plants would affect the choices made by particular females. Whilst not affecting the overall comparisons between species, this could have been partly responsible for the large heterogeneity between individuals. However, in general females did have access to healthy plants of both *P. punctata* and *P. rubra,* and it seems likely, therefore, that there was actual variation in individual preferences. Further experiments would be needed to test this hypothesis explicitly. If they exist, individual preferences are almost never absolute, as only two individual females showed complete rejection of *P. rubra* and two of *P. auriculata* (Appendix). In a natural situation host plant use among the two primary *Passiflora* species may be controlled more by the availability of new shoots than by slight individual preferences.

There were no significant differences between wild caught and laboratory reared females in either species (Table 1A), indicating that choice was not affected by the plant on which any individual was reared. Laboratory individuals were raised on a mixture of *P. punctata* and *P. rubra*, whilst any individual caught in the field could have fed only on a single host plant species. *Passiflora* are generally very widely spaced in the habitat and it is therefore extremely unlikely that larvae migrate from one

plant to another. In particular wild *H. himera* females were all, with one exception, caught in the Vilcabamba area where the only host plant available is *P. rubra.* Therefore all of these wild females almost certainly fed on *P. rubra* as larvae, but the adults showed no significant preferences in any direction. In our experiments there was therefore no evidence for any effects of larval conditioning on the host plant preferences of adults, as has been postulated for other taxa (Corbet, 1985).

The analysis of heterogeneity within and between species shows that there were no significant differences in host plant choice between the two butterfly species, in any of the experiments (Table 1A & 1B). In laboratory conditions larvae of *H. himera* and *H. erato* were successfully reared to adulthood on each of the three *Passiflora* species, *P. rubra*, *P. punctata* and *P. auriculata*, although relative success rates on different host species were not explicitly examined. Therefore it seems reasonable to conclude that there are no strong biochemical or behavioural differences between *H. himera* and *H. erato*, with respect to these three *Passiflora* species. This conclusion is supported by our field data (Jiggins et al., 1996), showing that both species use *P. rubra* and *P. punctata* in the contact zone. These experiments provide no evidence that host plant adaptation has played a role in the speciation of *himera* and *erato.* In this case the strong association of all three known *himera / erato* hybrid zones with transitions between dry and wet forest (Mallet, 1993; Jiggins et al., 1996) suggests that ecological divergence has played a role in speciation, but that a broadly different biotope, rather than different host plants has played the major part.

This is not to say that there is no evidence for host plant induced speciation in *Heliconius.* There are many examples, particularly within the *H. melpomene* group, of closely related species with divergent patterns of host plant use (Smiley, 1978; Thomas, 1990b; Gilbert, 1991) which would seem to support a model of host shift mediated diversification. For example *H. cydno* is, by *Heliconius* standards, a generalist which feeds on five *Passiflora* species at La Selva, Costa Rica. Its sympatric sister species, *H. melpomene* (Brown, 1981; Gilbert, 1991; Brower, 1994), is extremely specialised and feeds only on *Passiflora oerstedii.* Other species in the *melpomene* group include *H. hecale* which feeds on *P. vitifolia* and *H. ismenius* on *P. alata* and *P. ambigua.* It has been shown, in the case of *H. melpomene*, that host plant specialisation is due to female oviposition behaviour and is not associated with biochemical adaptations in the larvae, which survive equally well on all the hosts of *H.*

cydno (Smiley, 1978). Similarly *Heliconius erato* and *charitonia* are closely related species which live sympatrically in secondary forest. In this case *H. charitonia* has overcome the defences of *Passiflora adenopoda*, a species with hooked trichomes which kill other *Heliconius* larvae (Gilbert, 1971, 1991). It is therefore protected from competition with other *Heliconius* by the defences of its host. In any community there is not a one-on-one relationship between *Heliconius* and *Passiflora* species, but rather a diversity of different strategies - a few *Heliconius* are generalists (within the family Passifloraceae), some are micro-habitat specialists and use the species found in that habitat, whilst most others are highly specialised on one or a few hosts (Benson, 1978; Thomas, 1990b; Gilbert, 1991).

However, on the basis of this study we caution against interpreting these interspecific comparisons as evidence that host plant shifts lead to speciation in *Heliconius*. The overall diversity of *Heliconius* species in any area is highly correlated with the number of host plant species (Gilbert and Smiley, 1978; Thomas, 1990a) and this implies that the coexistence of species is dependent on divergent patterns of host plant use. *H. himera* and *H. erato* provide a complementary example to this, where an inability to coexist is associated with shared host plants. What is most striking about these species is the extremely abrupt transition between the two. The contact zone between *H. himera* and *H. e. cyrbia* in southern Ecuador is very narrow (approx. 5km) and cannot be explained solely in terms of hybrid inviability or disruptive selection on colour pattern (Jiggins et al., 1996). It seems likely that competitive exclusion on larval hosts is in part preventing these species from becoming sympatric. Competitive interactions almost certainly explain patterns of divergent host plant use between sympatric *Heliconius* species, and parapatric contact between other closely related species which share host plants (Benson, 1978; Gilbert, 1991).

There exists a perennial problem in the study of speciation; adaptations acquired subsequent to the speciation event will become confounded with those which actually led to the initial divergence. In spite of the strong division of the host plant niche among *Heliconius* species, the evidence presented here shows that speciation is not necessarily driven by host plant adaptation. Instead, divergence in host plant use after speciation may be necessary to permit coexistence and persistence of the daughter species. Whilst our results certainly do not preclude host shift speciation in other

Heliconius species, they do suggest an alternative explanation for the observed patterns of host plant use.

Acknowledgments

Many thanks to Martin Brookes, Chris Thomas and an anonymous reviewer for comments on the manuscript. José Carpio, Roberto Carpio, Maria del Carmen Avila, Ashleigh Griffin and Angus Davison who collected the majority of these eggs. Also to INEFAN for granting permission to carry out research in Ecuador; the Museo de Ciencias Naturales in Quito and Fundación Arcoiris in Loja for their support. This project is funded by a Biotechnology and Biological Sciences Research Council research grant and studentship.

References

Benson, W.W. 1978. Resource partitioning in passion vine butterflies. Evolution 32:493-518.

Benson, W.W., K.S. Brown, and L.E. Gilbert. 1975. Coevolution of plants and herbivores: passion flower butterflies. Evolution 29:659-680.

Boggs, C.L. 1981. Nutritional and life-history determinants of resource allocation in holometabolous insects. American Naturalist 117:692-709.

Brower, A.V.Z. 1994. Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalinae). Molecular Phylogenetics and Evolution 3:159-174.

Brown, K.S. 1979. Ecologia Geográfica e Evolução nas Florestas Neotropicais. 2 vols. (Livre de Docencia) Universidade Estadual de Campinas, Campinas, Brazil.

---. 1981. The biology of *Heliconius* and related genera. Annual Review of Entomology 26:427-456.

Bush, G.L. 1993. A reaffirmation of Santa Rosalia, or why are there so many kinds of small animals, pp. 229-249. In: D.R. Lees and D. Edwards (eds.), Evolutionary Patterns and Processes. Linnean Society of London, London.

---. 1994. Sympatric speciation in animals: new wine in old bottles. Trends in Ecology and Evolution 9:285-288.

Corbet, S.A. 1985. Insect chemosensory responses: a chemical legacy hypothesis. Ecological Entomology 10:143-153.

Ehrlich, P.R. and P.H. Raven. 1965. Butterflies and plants: a study in coevolution. Evolution 18:586-608.

Eltringham, H. 1916. On specific and mimetic relationships in the genus *Heliconius*. Transactions of the Entomological Society of London 1916:101-148.

Emsley, M.G. 1965. Speciation in *Heliconius* (Lep., Nymphalidae): morphology and geographic distribution. Zoologica, New York 50:191-254.

Feder, J.L., C.A. Chilcote, and G.L. Bush. 1988. Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. Nature (London) 336:61-64.

Gilbert, L.E. 1971. Butterfly-plant coevolution: has *Passiflora adenopoda* won the selectional race with heliconiine butterflies? Science 172:585-586.

---. 1991. Biodiversity of a Central American *Heliconius* community: pattern, process, and problems, pp. 403-427. In: P.W. Price, T.M. Lewinsohn, T.W.

Fernandes, and W.W. Benson (eds.), Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. John Wiley & Sons, New York.

Gilbert, L.E. and J.T. Smiley. 1978. Determinants of local diversity in phytophagous insects: host specialists in tropical environments, pp. 89-104. In: L.A. Mound and N. Waloff (eds.), Diversity of Insect Faunas (Symposia of the Royal Entomological Society of London, 9.). Blackwell Scientific, Oxford.

Holm-Nielsen, L.B., P.M. Jørgensen, and J.E. Lawesson. 1988. Passifloraceae, pp. 1-129. In: G. Harling and L. Andersson (eds.), Flora of Ecuador, Vol. 31 . Department of Botany, University of Göteborg, Sweden.

Jiggins, C., W.O. McMillan, W. Neukirchen, and J. Mallet. 1996. What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). Biological Journal of the Linnean Society 59:221-242.

Jiggins, C., W.O. McMillan, and J. Mallet. 1996. The maintenance of species differences in a *Heliconius* hybrid zone. Heredity submitted.

Kaye, W.J. 1916. A reply to Dr. Eltringham's paper on the genus *Heliconius*. Transactions of the Entomological Society of London 1916:149-155.

Killip, E.P. 1938. The American Species of Passifloraceae. Publications of the Field Museum of Natural History, Botanical Series 19(1):1-163.

Lamas, G. 1976. Notes on Peruvian butterflies (Lepidoptera). II. New *Heliconius* from Cusco and Madre de Dios. Revista Peruana de Entomologia 19:1-7.

Mallet, J. 1993. Speciation, raciation, and color pattern evolution in *Heliconius* butterflies: evidence from hybrid zones, pp. 226-260. In: R.G. Harrison (ed.), Hybrid Zones and the Evolutionary Process . Oxford University Press, New York.

McMillan, W.O., Jiggins, C.D. and Mallet, J. 1997. What initiates speciation in passion vine butterflies? Proc. Natl. Acad. Sci., USA, submitted.[see Appendix]

McPheron, B.A., D.C. Smith, and S.H. Berlocher. 1988. Genetic differences between host races of *Rhagoletis pomonella*. Nature (London) 336:64-66.

Mitter, C. and D.R. Brooks. 1983. Phylogenetic aspects of coevolution. In: D.J. Futuyma and M. Slatkin (eds.), Coevolution. Sinauer, Massachusetts.

Sheppard, P.M., J.R.G. Turner, K.S. Brown, W.W. Benson, and M.C. Singer. 1985. Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. Philosophical Transactions of the Royal Society of London (B) 308:433-613.

Smiley, J.T. 1978. Plant chemistry and the evolution of host specificity: new evidence from *Heliconius* and *Passiflora*. Science 201:745-747.

Smith, D.C. 1988. Heritable divergence of *Rhagoletis pomonella* host races by seasonal asynchrony. Nature (London) 336:66-67.

Tauber, C.A. and M.J. Tauber. 1989. Sympatric speciation in insects: perception and perspective, pp. 307-344. In: D. Otte and J.A. Endler (eds.), Speciation and its consequences . Sinauer, Sunderland, Massachusetts.

Thomas, C.D. 1990a. Fewer species. Nature (London) 347:237.

---. 1990b. Herbivore diets, herbivore colonization, and the escape hypothesis. Ecology 71:610-615.

rubra* and *punctata	**G**	**df**	**P**
comparison among *cyrbia* females			
within laboratory reared	288.71	24	<0.001
within wild caught	45.84	9	<0.001
wild vs. lab.	0.57	1	ns
within *cyrbia*	335.12	34	<0.001
comparison among *himera* females			
within laboratory reared	294.25	33	<0.001
within wild caught	22.99	8	<0.01
wild vs. lab.	0.03	1	ns
within *himera*	317.27	42	<0.001
cyrbia* vs. *himera	**0.48**	**1**	**ns**
Total heterogeneity	652.87	77	<0.001

Table 1A; Nested G-test to investigate heterogeneity of host plant use between individuals, female type and species in the experiment comparing *rubra* and *punctata*. The test indicates that there are highly significant differences between individual females within all classes, but no significant differences between species or female type.

rubra*, *punctata* and *auriculata	**G**	**df**	**P**
comparison among all females			
within *cyrbia*	57.04	18	<0.001
within *himera*	55.72	8	<0.001
cyrbia* vs. *himera	**0.51**	**1**	**ns**
Total heterogeneity	113.27	27	<0.001

Table 1B; Nested G-test to investigate heterogeneity of host plant use between individuals, female type and species in the experiment comparing *rubra*, *punctata* and *auriculata*. The wild vs lab comparison was not carried out for this experiment due to the small sample sizes involved (only two *cyrbia* females and one *himera* were raised in the laboratory, the remainder all being wild caught).

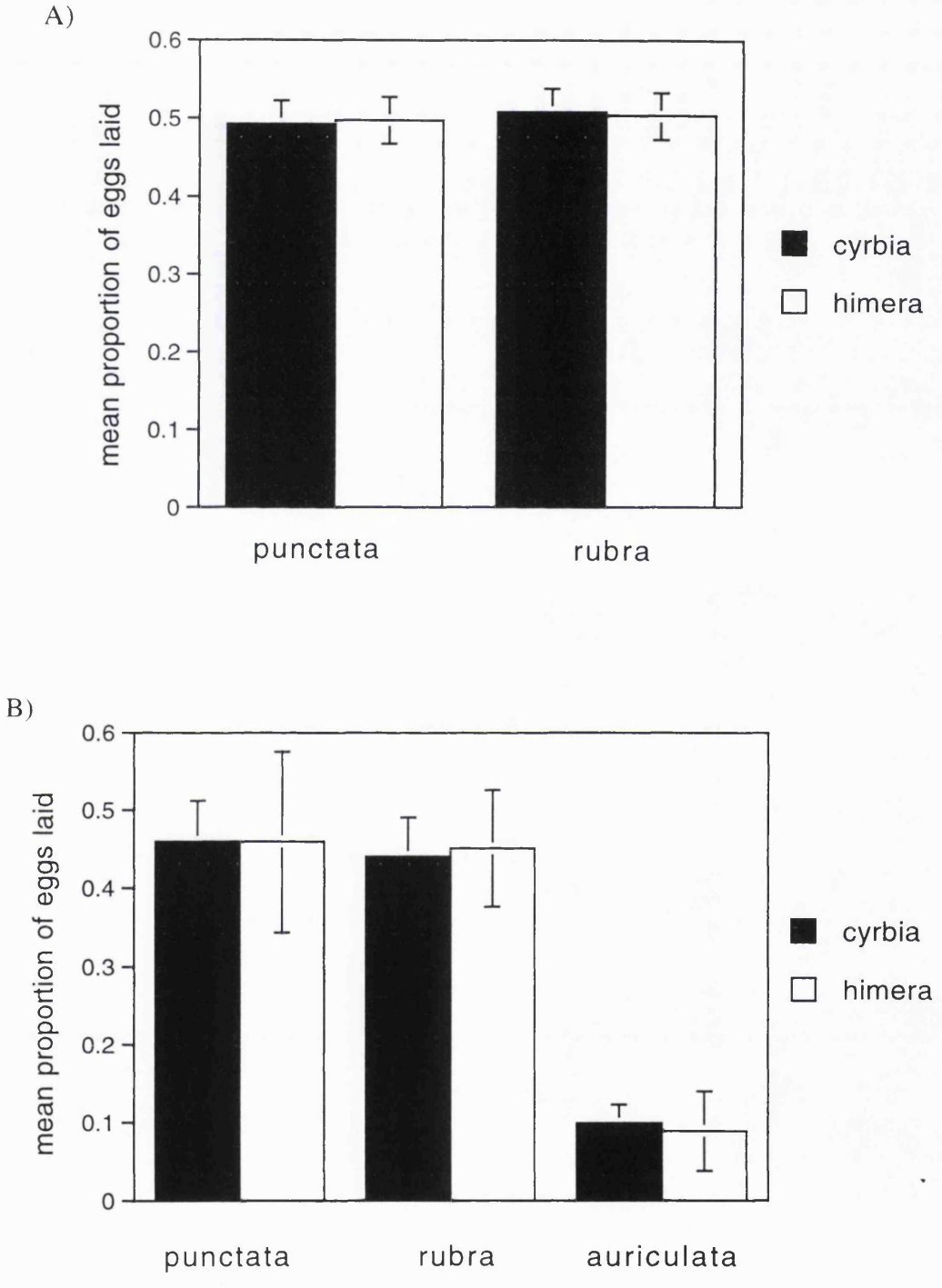


Figure 1; Proportion of eggs laid on each host plant species averaged over all females. Data from A) *rubra* vs. *punctata* experiments and B) *rubra* vs. *punctata* vs. *auriculata* experiments. Standard error bars are shown.

Appendix Table 1. Number of eggs laid by females on each host plant species. Two species experiments (laboratory reared females).

Two species choice - laboratory reared females					
	cyrbia			*himera*	
brood no.	*rubra*	*punctata*	brood no.	*rubra*	*punctata*
91	*15*	*45*	103	*26*	*8*
86	*24*	*3*	116	*65*	*16*
95	*14*	*4*	141	*4*	*61*
102	*15*	*6*	108	*21*	*16*
104	*10*	*6*	99	*29*	*6*
115	*30*	*9*	120	*18*	*24*
119	*50*	*20*	123	*34*	*28*
40	*18*	*22*	139	*26*	*22*
57	*36*	*38*	147	*30*	*38*
62	*40*	*9*	94	*20*	*15*
73	*33*	*8*	100	*26*	*29*
76	*14*	*35*	23	*17*	*13*
72	*56*	*18*	38	*35*	*33*
60	*41*	*13*	44	*15*	*33*
121	*0*	*40*	64	*40*	*37*
101	*4*	*14*	70	*38*	*44*
41	*42*	*51*	78	*15*	*32*
45	*32*	*16*	81	*52*	*68*
49	*26*	*31*	134	*0*	*29*
74	*8*	*58*	144	*10*	*20*
92	*5*	*28*	149	*16*	*5*
80	*16*	*7*	151	*7*	*10*
124	*7*	*11*	67	*54*	*25*
18	*4*	*11*	71	*23*	*32*
19	*28*	*23*	31	*7*	*27*
			96	*22*	*55*
			84	*78*	*46*
			105	*7*	*29*
			118	*41*	*23*
			79	*23*	*27*
			146	*27*	*29*
			82	*59*	*14*
			132	*19*	*20*
			143	*29*	*12*
Total	**568**	**526**	**Total**	**933**	**926**

Appendix Table 2. Number of eggs laid by females on each host plant species. Two species experiments (wild caught females).

Two species choice - wild caught females					
	cyrbia			*himera*	
brood no.	*rubra*	*punctata*	brood no.	*rubra*	*punctata*
11	12	12	34	10	7
12	*6*	*15*	4	*13*	*3*
15	*15*	*8*	6	*19*	*39*
14	*4*	*9*	8	*20*	*23*
17	*10*	*12*	9	*10*	*19*
27	*6*	*6*	20	*20*	*12*
35	*9*	*7*	21	*15*	*9*
36	*4*	*12*	Z-9	*16*	*9*
AB	*24*	*1*	N	*13*	*11*
V	*5*	*17*			
Total	**95**	**99**	**Total**	**136**	**132**

Appendix Table 3. Number of eggs laid by females on each host plant species. Three species experiments.

Three species choice - wild and laboratory females							
	cyrbia				*himera*		
Brood no.	*rubra*	*punctata*	*auriculata*	Brood no.	*rubra*	*punctata*	*auriculata*
AE	*4*	*33*	*1*	47	*44*	*27*	*18*
X	*13*	*9*	*1*	Z-5	*9*	*26*	*0*
48	*18*	*17*	*5*	B	*30*	*27*	*0*
24	*24*	*16*	*13*	A	*14*	*30*	*1*
U	*9*	*11*	*4*	K	*6*	*1*	*2*
M	*5*	*8*	*3*				
I	*19*	*13*	*1*				
H	*19*	*7*	*1*				
E	*8*	*10*	*1*				
D	*11*	*12*	*2*				
Total	**130**	**136**	**32**	**Total**	**103**	**111**	**21**

CHAPTER 5

Ecological correlates of a *Heliconius* hybrid zone

Introduction

Hybrid zones are boundaries between distinct genotypes, which in most cases are maintained by a stable balance of selection and gene flow. Theoretical models of hybrid zones fall into two distinct classes, distinguished by their emphasis on either *endogenous* or *exogenous* selection pressures. The tension-zone model (Barton, 1983; Barton & Gale, 1993) invokes endogenous selection against hybrid genotypes which is independent of the environment. In contrast the ecological selection-gradient model postulates that exogenous selection pressures vary across geographical space, such that each parental type is favoured in its own environment (Moore & Price, 1993). Field studies show that these models are not mutually exclusive, and indeed most hybrid zones show evidence for both endogenous selection against hybrids, and are associated with habitat or climatic features suggesting exogenous selection (Rand & Harrison, 1989; Harrison, 1990; Moore & Price, 1993; Bert & Arnold, 1995). An understanding of the relative importance of these models in different situations will be important in determining the role of adaptation in divergence and speciation.

Hybrid zones between *Heliconius* races are unusual for two reasons. Firstly they are not necessarily associated with any habitat or climatic discontinuity, which suggests that exogenous selection is often not important (Mallet, 1993, but see Benson, 1982). Secondly they are maintained by frequency dependent predation on wing colour patterns. Therefore, although selection is mediated by predators, it is also a kind of endogenous selection pressure because levels of selection are dependent on local population frequencies of colour pattern phenotypes. Indeed this type of hybrid zone is described very well by tension zone models (Mallet & Barton, 1989).

A hybrid zone between the sister species, *Heliconius himera* and *H. erato* provides a contrast with these inter-racial hybrid zones in *Heliconius*. The zone is only 5 km in width, much narrower than any previously described between races of *erato* (≥10 km). This suggests that selection pressures are much stronger in the *himera / erato*

zone. Furthermore unlike races of *H. erato*, the two taxa are genetically distinct at allozyme and mitochondrial DNA markers and these differences are maintained throughout the hybrid zone, where hybrids form only 5-10% of the population (Chapter 2). *H. himera* and *erato* have therefore speciated, but still hybridise at low frequency and an understanding of how selection acts in this hybrid zone will be crucial in determining the causes of speciation.

Within the zone of overlap there is no evidence that *H. himera* and *erato* are ecologically divergent. They utilise the same larval host plants both in the field and in laboratory preference experiments (Chapter 4) and adults fly together and visit the same adult food plants (pers. obs.). However on a continental scale the two species are associated with broadly different habitats. *H. himera* is found in south western Ecuador, northern Peru and the upper Marañon valley. This region is strongly influenced by the cold Humboldt current and thus has a dry climate (Best & Kessler, 1995), in marked contrast to the very wet surrounding areas occupied by *erato*. Here I present a detailed study of the habitat on a local scale in the area of contact between *himera* and *erato*, in order to investigate ecological differences between the species.

Methods

A comprehensive sampling of forest composition was carried out across the hybrid zone between *H. erato* and *H. himera,* and in nearby regions characterised by the parental species. Vegetation was surveyed by means of 23 1000m^2 (0.1ha) plots, which were either 100 x 10m or 50 x 20m, whichever best fitted the parcel of forest being studied. In each plot every tree >10cm diameter at breast height (d.b.h.) was identified on site or a sample collected in triplicate for later herbarium identification. Virtually all samples were identified to genus level. Within genera, where identification to species was not possible, distinct morpho-species were separated for the purposes of data analysis. However this process erred on the side of caution, in that samples were combined where defining morphological characteristics were slight, thus potentially losing resolution but avoiding spurious results. In total 144 morpho-species were included in the analysis.

A single plot was sampled at most of the collection sites across the *himera / erato* hybrid zone (Jiggins et al., 1996). In addition further 'pure' sites were chosen in order to characterise the habitat of each species on either side of the hybrid zone. Two regions were chosen where each *Heliconius* species was known to occur at relatively high population densities. In each of these 'pure' areas, three transects were surveyed at or near sites where the butterflies where found to be common, or had been collected previously. The 'pure' sites selected were Balsas and Piñas (*H.e.cyrbia*), and Catamayo and Vilcabamba (*H.himera*). Sites are shown in Figure 2.

The forest surveyed here could not be considered 'primary' in any sense, however sites with a minimum of apparent human disturbance were chosen. Therefore areas where coffee or other crops were being cultivated under the canopy were not used, but in a number of the plots it was evident that cattle occasionally grazed the area. Within the hybrid zone all transects were sited alongside permanent rivers, so the vegetation studied was of a consistently riverine nature. In each of the four 'pure' areas, three transects were surveyed, one of which ran along a river and the other two were in parcels of forest further up the valley slopes. This gave a more representative sample of the vegetation and to some extent controlled for variation due to localised site differences. This more comprehensive sampling method was impractical within the hybrid zone where the only remaining natural vegetation lies along riverbeds.

Voucher specimens were deposited in the Herbario Nacional and Universidad Catolica in Quito and the Herbario Reinaldo Espinosa in Loja. Identification was carried out by comparison with herbarium material.

Tree species abundance data was analysed using detrended correspondence analysis (DECORANA; Hill , 1979; Hill & Gauch, 1980), an ordination technique. Ordination methods allow the variation in a multi-dimensional data set, such as a site-by-species matrix, to be summarised into a few independent axes. Correspondence analysis is a type of ordination, which uses an iterative procedure to produce simultaneous ordinations of sites and species. Species are initially weighted along an approximate gradient and these weightings used to calculate site scores. These site scores are then used to produce an improved set of species weightings. This procedure is repeated until a stable optimum of site and species scores is reached. The advantage of this method over many other ordination techniques is that it does

not use arbitrarily chosen sites as reference points. The results of this ordination analysis were then compared to *Heliconius* distribution data (Jiggins et. al., 1996), altitude and precipitation (Munday & Munday, 1991) to investigate any correlations.

Results

The *erato* sites at Balsas and Piñas are characteristic of humid mid-elevation forest, albeit heavily disturbed. The commonest species in the Balsas plots were *Cecropia* (Moraceae), *Guarea cartaguenya* (Meliaceae), *Virola sp.* (Myristicaceae) and a Rubiaceae *sp.*. In the Piñas plots *Cyathea sp.* (Cyatheaceae), *Canostegia sp.* (Melastomataceae), *Miconia sp.* (Melastomataceae), *Cecropia* (Moraceae) and *Triplaris sp.* (Polygonaceae) were dominant (the complete species-by-site data matrix is given in the Appendix). The overall species diversity in these areas is high (17.5spp/plot).

In contrast the *himera* habitat is far less diverse (8spp/plot) and is dominated by deciduous species. In the three Vilcabamba plots the dominant species were *Anadenanthera columbrina* (Leguminoseae), *Acacia macracantha* (Leguminoseae) and *Ceiba sp.* (Bombacaceae), whilst the Catamayo plots were dominated by *Acacia macracantha* (Leguminoseae), *Schinus molle* (Anacardiaceae) and *Styrax sp.* (Styracaeae). The transitional forest in the hybrid zone area has species derived from either forest type, but also some characteristic species, such as *Mauria heterophylla* (Anacardiaceae), *Cochlospermum vitifolium* (Cochlospermaceae) and *Erythrina sp.* (Leguminoseae).

Ordination analysis of the tree species data clearly separated the sites into three distinct regions, which correspond to the distribution of *himera, erato* and the hybrid zone (Fig. 1 and Fig. 2). Indeed, no tree species are shared between the six pure *himera* and the six pure *erato* plots. The second axis of the analysis further separated groups of sites within the three geographic regions (Fig. 1).

Statistical analysis of associations between the vegetation and butterfly data is difficult because of spatial auto-correlation between variables due to geographic proximity between sites. There is a significant correlation between butterfly species index and

the first ordination axis of the tree species data ($r = 0.897$; $p = 0.0001$) and the difference between the mean first axis scores for *himera* and *cyrbia* habitat is highly significant (Table 1; $t=39.41$, $p<0.01$; correcting for differences in variance between samples). However this alone is not convincing evidence that the hybrid zone is an area of greater habitat change than surrounding areas. To investigate this, the study area was divided into three regions of similar geographic extent; *cyrbia* habitat, *himera* habitat and hybrid zone. The spread of ordination scores along both the first and second axes was much greater for the hybrid zone region (Fig. 1). When the variance in ordination scores was calculated for each region (Table 1), the hybrid zone has a higher variance than the other two areas (First axis: *cyrbia* vs hybrid zone; $F_{10,5}=28.1$; $p<0.001$, *himera* vs hybrid zone; $F_{10,5}=3.85$; $0.1 > p > 0.05$. Second axis: *cyrbia* vs hybrid zone; $F_{10,5}=1.14$; NS, *himera* vs hybrid zone; $F_{10,5}=29.6$; $p < 0.001$). This analysis confirms that the hybrid zone is a transitional region where tree species turnover between sites is much higher than in surrounding areas.

Discussion

There is a highly significant association of the hybrid zone between *himera* and *erato* with an abrupt change in the floristic composition of the forest. Such associations are commonly observed in studies of hybrid zones (Rand & Harrison, 1989; Moore & Price, 1993) and imply that adaptive ecological differences between hybridising taxa are playing the major role in determining the position of the zone. This is supported, in the case of *himera* and *erato*, by the observation that two other contact zones known between the taxa also occur at wet / dry forest boundaries (Mallet, 1993).

The tree and butterfly species distributions can be partly explained by changes in precipitation across the study area. The *himera* sites receive lower annual rainfall than the *erato* habitat (Fig. 3). Statistical analysis of rainfall against habitat variables was not possible as the sites for which meterological data is available did not correspond well with those studied here. However the importance of precipitation is supported by previous analysis of forest composition in the neotropics which has shown that, below 1700m, species diversity is strongly correlated with precipitation but not greatly influenced by altitude (Gentry, 1988; Josse & Baslev, 1994). In this study there is a significant decline in tree species diversity from *erato* to *himera* sites ($t_5=6.59$, $p<0.005$), as would be predicted if the habitat change were primarily driven by a change in precipitation levels. There is also likely to be a component of habitat

variation due to altitude, as the *himera* sites sampled are all above 1500m whilst *erato* sites are below 1200m. Within this data set, it is impossible to separately test the effects of altitude and precipitation as the two are clearly not independent.

Collections within western Ecuador confirm that *H. himera* is generally found from 600-2000m in deciduous or semi-deciduous woodland, which is indicative of a highly seasonal climate (pers. obs.). In contrast *erato* is found in evergreen forest over its altitudinal range from 0-1500m, although it should be noted that in other parts of the neotropics, such as Venezuela and south east Brazil, *erato* can occur in relatively dry savanna regions (Benson, 1982). Hence *himera* and *erato* are associated with distinct biotopes, which are probably a combined result of changes in precipitation and altitude across the region.

Further evidence for adaptive ecological differences between *himera* and *erato* comes from observations on the diurnal activity and life history traits of butterflies reared in the dry forest habitat, at 1600m (Vilcabamba). This is an area where *himera* occurs naturally, and we might therefore predict that *himera* would be better adapted to local conditions than *erato*. There is good evidence that this is the case, as caged adult *himera* forage for longer every day than *erato,* implying thermal adaptation of *himera* adults to the dry habitat (Davison et al., in prep.). This behavioural difference is associated with differences in fecundity, as caged *himera* females laid significantly more eggs per day than *erato* (*himera*; 2.6±0.8 vs. *erato* 1.9±0.4 eggs per day; McMillan et. al, 1997). There was also evidence for differences in larval physiology between the species. The development time of *himera* was, on average, a day shorter than that of *erato* (*himera*; 28.5±0.9 vs. *erato*; 29.8±1.0). In hybrid broods, development time was strongly correlated with proportion of *erato* genes in the brood (McMillan et. al, 1997). There is therefore good evidence for physiological differences between the two species, possibly due to thermal adaptation. It seems likely that these large differences in life history traits observed in the laboratory would translate into strong selection pressures in the hybrid zone. Such adaptation might explain both the association of the hybrid zone with environmental features and the very narrow width of this cline.

Nonetheless, although exogenous selection almost certainly determines the position of the hybrid zone, it remains a possibility that the cline width is largely a result of

endogenous selection on warning colour. The cline width of around 5km between the species *H. himera* and *H. erato cyrbia* contrasts with that of 10 km between the races *H.e. favorinus* and *H.e. lativitta* (Mallet & Barton, 1989b). There is good evidence that the three colour pattern loci which differentiate the races *H.e. favorinus* and *H.e. lativitta* are acting more or less independently (Mallet & Barton, 1989b), with selection pressures on each locus of about 0.1 - 0.2 ($s \approx 0.11$ from field estimates and $s \approx 0.23$ from genetic estimates Mallet & Barton, 1989b; Mallet et. al., 1990). In contrast, within the *himera* x *erato* hybrid zone there are very few hybrids and therefore extremely strong linkage disequilibria between genetic markers (Chapter 2), largely as a result of assortative mating. This means that most selection on colour pattern must act on the whole genome as a single unit. If the overall selection pressure is similar to that observed in inter-racial hybrid zones ($s \approx 0.5$), then this could produce a hybrid zone width approximately half that observed in the inter-racial case. A reduction in cline width due to reduced coupling between selected loci is predicted by multi-locus cline theory (Barton, 1983). Hence, subject to the assumption that dispersal and overall selection pressures are similar in the inter-racial and inter-species hybrid zones, it is possible to explain the observed cline width without invoking strong exogenous selection. Selection on ecological traits must occur, but could be relatively weak.

There is therefore an apparent contradiction, in that the association of the three known *himera x erato* hybrid zones with habitat discontinuities, and the marked life history differences between the species suggest strong exogenous selection. However the cline width can also be described by a tension-zone model which invokes only endogenous selection. There are at least two possible explanations. It could be that exogenous selection pressures are relatively weak compared to selection on warning colour. Thus ecological selection explains the position but not the narrowness of the hybrid zone. Alternatively, the assumptions of the tension zone model may be flawed. In particular the apparent absence of Jacamars (Galbullidae) from the *himera x erato* hybrid zone (Jiggins et. al., 1996) suggests that selection on warning colour could be weaker here than in many inter-racial hybrid zones. If this were the case then strong ecological selection could be responsible for the narrowness of the *himera x erato* cline. To test these possibilities would require extensive analysis of genotype - genotype and genotype - environment interactions across the zone, to determine the relative strength of endogenous and exogenous selection. This is possible through

cohort analysis of hybrid zone populations, which can be used to measure levels of selection against hybrid genotypes *per se* (Dowling & Moore, 1985; Kocher & Sage, 1986), and even differentiate between endogenous and exogenous selection (Bert & Arnold, 1995), but may be impractical in this case.

In conclusion, the data presented here and elsewhere show that there are marked ecological, colour pattern and presumed neutral genetic differences between *himera* and *erato*. Concordant clines at many different loci are common, and have been studied in taxa such as flickers, grasshoppers, crickets and toads (Moore & Price, 1993; Butlin & Hewitt, 1985; Rand & Harrison, 1989; Szymura and Barton, 1986). Such an association of differences at many loci is usually ascribed to allopatric divergence followed by secondary contact (e.g. Hewitt, 1993), but in some cases might also be explained as a result of *in situ* coalescence of clines for different characters (Barton, 1983; Mallet, 1993). The origin of such boundaries between taxa remains an important outstanding question in our understanding of speciation.

References

Barton, N.H. 1983 Multilocus clines. *Evolution* **37**, 454-471.

Barton, N.H. & Gale, K.S. 1993 Genetic analysis of hybrid zones. In *Hybrid Zones and the Evolutionary Process* . (ed. R.G. Harrison), pp. 13-45. New York: Oxford University Press.

Benson, W.W. 1982 Alternative models for infrageneric diversification in the humid tropics: tests with passion vine butterflies. In *Biological Diversification in the Tropics* . (ed. G.T. Prance), pp. 608-640. New York, NY: Columbia Univ. Press.

Bert, T.M. & Arnold, W.S. 1995 An empirical test of predictions of two competing models for the maintenance and fate of hybrid zones: both models are supported in a hard-clam hybrid zone. *Evolution* **49**, 276-289.

Best, B.J. and Kessler, M. 1995. *Biodiversity and conservation in Tumbesian Ecuador and Peru.* Birdlife International, Cambridge, U.K.

Butlin, R.K. & Hewitt, G.M. 1985 A hybrid zone between *Chorthippus parallelus parallelus* and *Chorthippus parallelus erythropus* (Orthoptera: Acrididae): morphological and electrophoretic characters. *Biol. J. Linn. Soc.* **26**, 269-285.

Davison, A., McMillan, W.O., Griffin, A.S., Jiggins, C.D. & Mallet, J. 1997 Adaptive differences across a parapatric species boundary. In prep.

Dowling, T.E. & Moore, W.S. 1985 Evidence for selection against hybrids in the family Cyprinidae (genus *Notropis*). *Evolution* **39**, 152-158.

Gentry, A.H. 1988 Changes in plant community diversity and floristic composition on environmental and geographical gradients. *Ann. Missouri Bot. Gard.* **75**, 1-34.

Harrison, R.G. 1990 Hybrid zones: windows on evolutionary process. In *Oxford Surveys in Evolutionary Biology*, Vol. 7. (ed. D. Futuyma & J. Antonovics), pp. 69-128. Oxford: Oxford University Press.

Hewitt, G.M. 1993 After the ice: *parallelus* meets *erythropus* in the Pyrenees. In *Hybrid Zones and the Evolutionary Process* . (ed. R.G. Harrison), pp. 140-164. New York: Oxford University Press.

Hill, M.O. 1979. DECORANA-A FORTRAN program for detrended correspondence analysis and reciprocal averaging. Ecology and Systematics, Cornell University, Ithaca, New York 14850.

Hill, M.O. & Gauch, H.G. 1980. Detrended correspondence analysis: an improved ordination technique. *Vegetatio* **42**, 47-58.

Jiggins, C., McMillan, W.O., Neukirchen, W. & Mallet, J. 1996 What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* **59**, 221-242.

Josse, C. & Baslev, H. 1994 The composition and structure of a dry, semideciduous forest in western Ecuador. *Nord. J. Bot.* **14**, 425-434.

Kocher, T.D. & Sage, R.D. 1986 Further genetic analyses of a hybrid zone between leopard frogs (*Rana pipiens* complex) in central Texas. *Evolution* **40**, 21-33.

Mallet, J. 1993 Speciation, raciation, and color pattern evolution in *Heliconius* butterflies: evidence from hybrid zones. In *Hybrid Zones and the Evolutionary Process* . (ed. R.G. Harrison), pp. 226-260. New York: Oxford University Press.

Mallet, J. & Barton, N. 1989a Inference from clines stabilized by frequency-dependent selection. *Genetics* **122**, 967-976.

Mallet, J. & Barton, N.H. 1989b Strong natural selection in a warning color hybrid zone. *Evolution* **43**, 421-431.

McMillan, W.O., Jiggins, C.D. & Mallet, J. 1996 What initiates speciation in passion vine butterflies? *Proc. Natl. Acad. Sci., USA* submitted. [see Appendix]

Moore, W.S. & Price, J.T. 1993 Nature of selection in the northern flicker hybrid zone and its implications for speciation theory. In *Hybrid Zones and the Evolutionary Process* . (ed. R.G. Harrison), pp. 196-225. New York: Oxford University Press.

Munday, G. & Munday, M. 1992 The climate of south west Ecuador. In *The threatened forests of south-west Ecuador* . (ed. B.J. Best), pp. 7-79. Leeds, UK: Biosphere publications.

Rand, D.M. & Harrison, R.G. 1989 Ecological genetics of a mosaic hybrid zone: mitochondrial, nuclear, and reproductive differentiation of crickets by soil type. *Evolution* **43**, 432-449.

Szymura, J.M. & Barton, N.H. 1986 Genetic analysis of hybrid zone between the fire-bellied toads, *Bombina bombina* and *B. variegata* near Cracow in Southern Poland. *Evolution* **40**, 1141-1159.

		Axis 1		Axis 2	
Geographic area	n	mean	variance	mean	variance
cyrbia	6	28.3	298.7	246.0	14502.4
Hybrid zone	11	410.7	8394.2	186.5	16591.1
himera	6	826.2	2181.0	207.5	560.3

Table 1. Analysis of first and second axis ordination scores by geographic area. The hybrid zone has a higher variance, suggesting that this is an area of more rapid habitat change (see text for details).

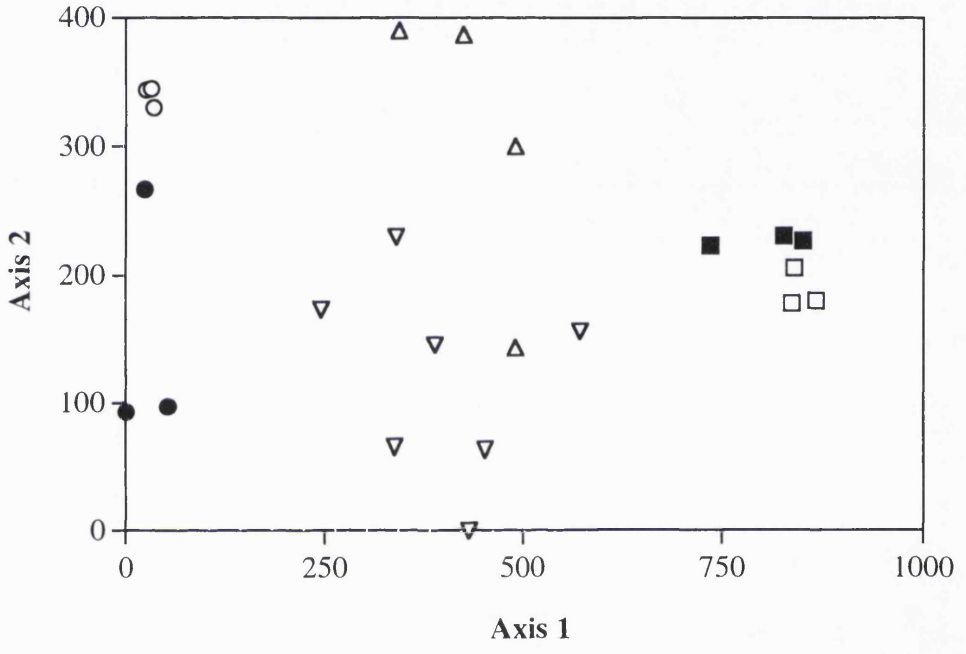


Figure 1. Results of correspondence analysis of tree species abundance data. Each point represents a single site. The sites are divided into geographic areas according to the distribution of *himera* and *erato cyrbia*.

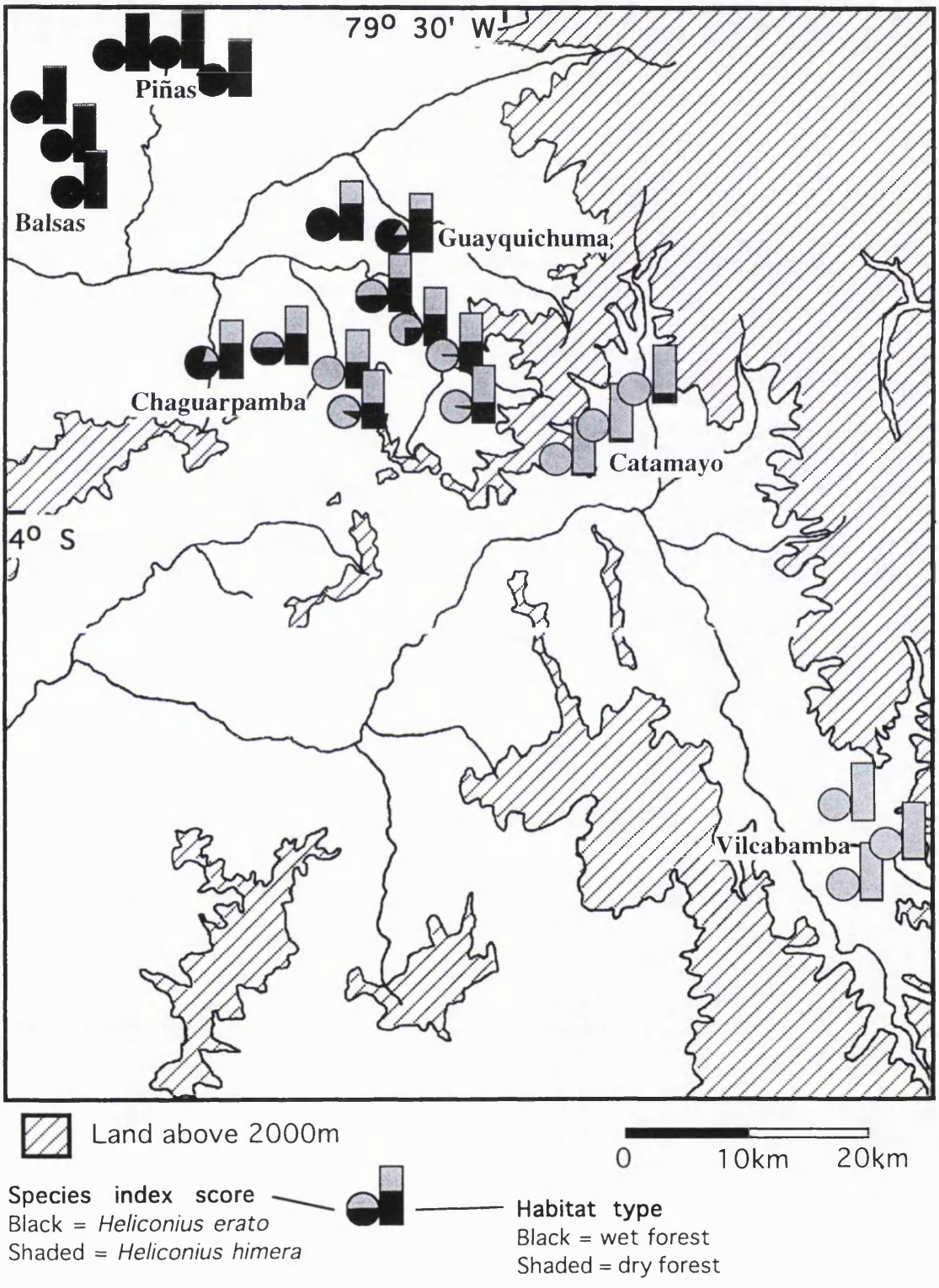


Figure 2. Habitat analysis across the hybrid zone. Species index scores are based on morphology (Jiggins et al., 1996). Site names are intended only as a guide to the descriptions of sites given in the text and Figure 1 - exact locations of towns are not shown. The habitat data shown is the first axis of an ordination analysis of tree species abundance data, shown as a proportion of the highest value.

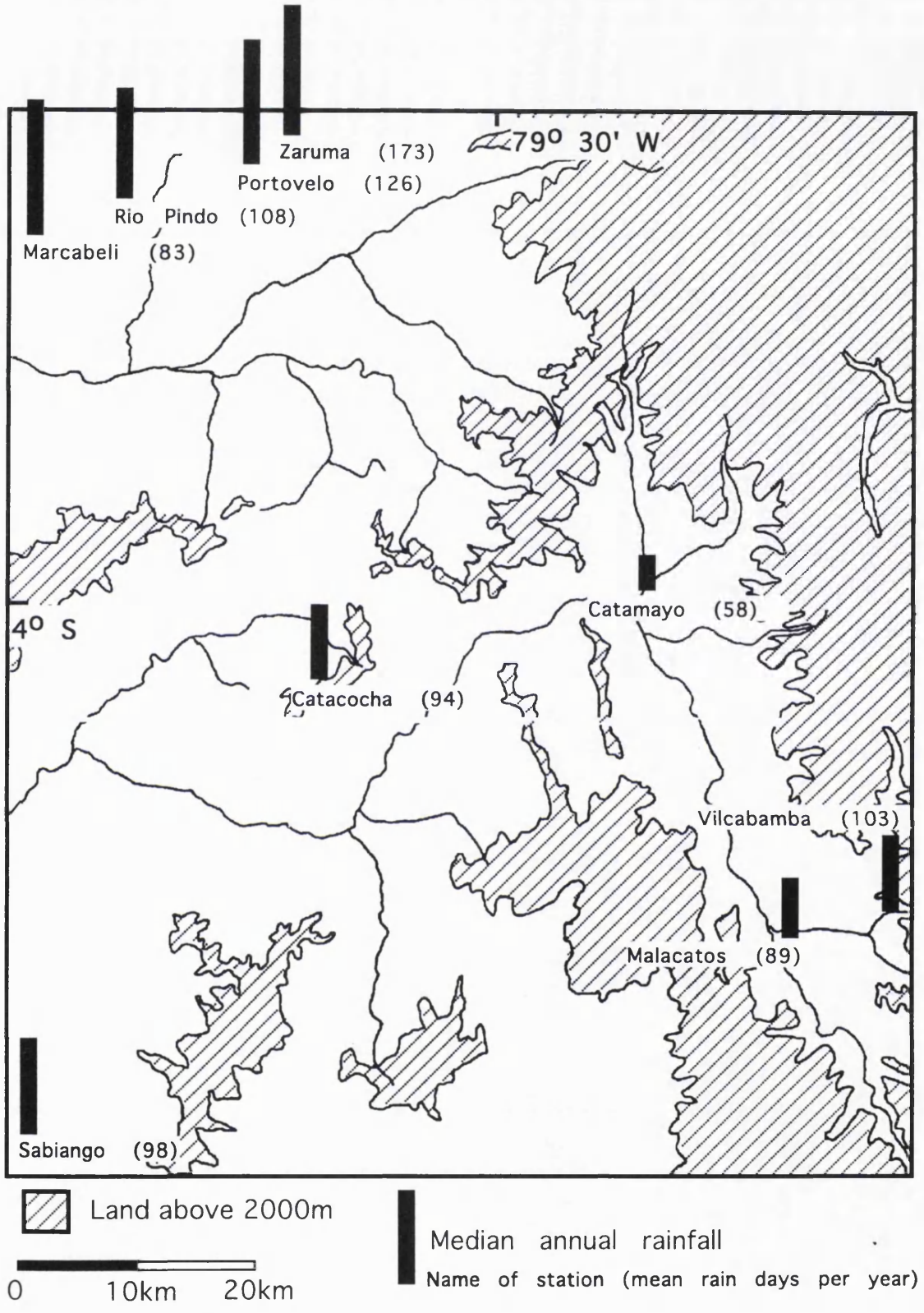


Figure 3. Rainfall records in the hybrid zone area. Data from Munday & Munday (1992). The rainfall bars are drawn to scale, relative to that in the legend which represents 1000mm/year. All stations below 1900m with >10 year records are shown.

Appendix. Tree species abundance data. Hybrid zone site numbers correspond to those in Jiggins et. al., 1996. Vilca. = Vilcabamba and Catam. = Catamayo. Specimens without collection numbers were identified in situ.

Plant species	Coll. No.	Sites																						
		himera							Hybrid zone										*cyrbia*					
		Vilca			Catam														Piñas			Balsas		
		1	2	3	1	2	3	10	9	8	5	4	4	3	18	17	16	15	1	2	3	1	2	3
Incognito (excluded from analysis)		9					1	1		1	2	2	9	2	1				4	2	1		3	3
Cyatheaceae																								
Cyathea									3			3							8		18			
Arecaceae																								
Phytelephas																			4			1		
Ceroxylon																	1							
Bactris																		10						
Aiphanes																				1			7	
Anacardiaceae																								
Mangifera										2				7		5								
Mauria heterophylla	128, 114						3	1	4	4	11	11		9	7	7	2	3						
Schinus molle	3		1		11	36																		
Annonaceae																								
Annona (chirimoya)		1					14																	
Araliaceae																								
Sp. 1	50														4									
Dendropanax	146																							2
Schefflera																			1			1		
Betulaceae																								
Alnus	123				2																			
Bignoniaceae																								
Tabebuia	63								4															
Bixaceae																								
Bixa	159															1								
Bombacaceae																								
Quararibea																4	1							
Ochroma sp.												1	2					1	2	2	3	2		
Ceiba	64								3	2														
Sp. 1	16							30	3	9														
Ceiba		9	4																					
Burseraceae																								
Protium	103, 167																2			4	1	2		1
Caricaceae																								
Jacaratia																	3							
Celastraceae																								
Maytenus	69									2														
Cochlospermaceae																								
Cochlospermum vitifolium									3	9	9			1										
Compositae																								
Sp. 1																			1	1				2
Barnadesia	73								4	3														
Barnadesia	109																				5			
Pollalesta	127						17																	
Pollalesta	92												3		1									
Verbesina	51													2				1						
Cunoniaceae																								
Weinmannia	113																				1			

Plant species	Coll. No.	Sites																					
		himera							Hybrid zone									*cyrbia*					
		Vilca			Catam													Piñas			Balsas		
		1	2	3	1	2	3	10	9	8	5	4	4	3	18	17	16	15	1	2	3	1	2	3
Euphorbiaceae																								
Alchornea	104, 143																			4	2	1	1	7
Euphorbia sp.																1								
Ricinus						1																		
Croton	86, 98											7							5					
Croton	71									1					3	2								
Croton	96																		8					
Sapium																		7						
Flacourtaceae																								
Casearia	54										2													
Guttiferaceae																								
Clusia sp.		1							3															
Vismia	95, 163																5		2			2	1	
Lauraceae																								
Sp. 1													1											
Sp. 2	135																					4		
Aniba	139																					3	4	
Beilschmiedia	81,164											7				1	1							
Cordia	91												1											
Sp. 3	174																2							
Persea																6								
(Avocado)																								
Persea	177																	5						
Persea	140																						6	
Ocotea	182																	1						
Ocotea																								
Ocotea cernua	48,50											3	8	8				4				1	1	3
Sp. 4	48													6										
Ocotea	173																2							
Ocotea	p86																							
Sp. 5	156														1	7								
Sp. 6	179																	3						
Lecythidaceae																								
Sp. 1												2	6											
Leguminoseae																								
Sp. 1	178																	1						
Caesalpinea	122				1		1					1												
Jacaranda															5									
Acacia		5	41	12	29	33	20	4								5								
macracantha																								
Inga sp.										1	4	1		6		2		3	6	7		2	5	1
Leucania	24								2	3														
cf.canescens																								
Pithecellobium	161															1								
Anadenanthera		23	14	39																				
columbrina																								
Erythrina sp.			1	1					2	2	5				5	8		2						
Liliaceae																								
Yucca					2																			
Melastomataceae																								
Sp. 1																							1	
Canostegia	110																		5		16			
Miconia sp.	111																		3	4	14	3	4	1
Meliaceae																								
Tuagea glabra	94																							

Plant species	Coll. No.	Sites																						
		himera							Hybrid zone										*cyrbia*					
		Vilca			Catam														Piñas			Balsas		
		1	2	3	1	2	3	10	9	8	5	4	4	3	18	17	16	15	1	2	3	1	2	3
Carapa	87											4	2											
Cedrela	171																1							
Cedrela	58								8		2	2												
Melea						3																		
Guarea	138																					3	9	3
cartaguenya																								
Guarea	100																		1	8				
Trichilia	134																					4	1	2
pallida																								
Trichilia	121				1																			
tomentosa																								
Moraceae																								
Cecropia										2	7	4	1	5		3	1	3		2	8	9	11	10
Pouroma																						4		
Ficus																				1		1		2
Ficus	77,66,41	4				3	3	14	2	2	3	2			6	1	10							
Ficus	59								1															
Sp. 1																						6	2	
Nacleopsis	165																13					2		
Sorocea	102, 148																1			1				3
Pseudolmedia	76, 119									1	1													
Pseudolmedia	170																7							
Myristicaceae																								
Dialyanthera	101																		1					
Virola	144																						4	9
Myrsinaceae																								
Sp. 1																			3					
Myrsine	158															14								
Sp. 2	61,120								5		2													
Myrtaceae																								
Pomarosa																2	3	2						
Myrcia	166																3	4						
Psidium	180																1	2						
Psidium	60								4	3	1			1										
Myrcianthes	49								4					2										
Calyptranthes	133, 68						1			1														
Sp. 1	74									4														
Sp. 2																							1	
Papaveraceae																								
Bocoonia													9											
Piperaceae																								
Piper	20													1										
Polygonaceae																								
Triplaris	55,62								6	6		1			1			1						
Triplaris	93																		5	5			2	3
Triplaris	99																		6					
Coccoloba	169																5							
Coccoloba	125, 117					2					5	3												
Sp. 1	65								1															
Rubiaceae																								
Sp. 1																								1
Sp. 2	89												3						1					
Coutarea	70									6														
Sp. 3	78											4												
Palicourea	97																		2					
Psychotria	142																							2

Plant species	Coll. No.	Sites																						
		himera						Hybrid zone										*cyrbia*						
		Vilca			Catam													Piñas			Balsas			
		1	2	3	1	2	3	10	9	8	5	4	4	3	18	17	16	15	1	2	3	1	2	3
Simira sp1	112																				3	1		
Simira sp2	105																			1			1	
Sp. 4																					5	12	1	
Rutaceae																								
Zanthoxylum	56								1		2			1				1						
Salicaceae																								
Salix humbolthei					11																			
Sapindaceae																								
Sp. 1	153														2									
Sp. 2	149														1									
Allophylus																	1							
Matayba	80											8												
Sapindus saponaria		1	1																					
Cupania	52,47												6	11		2	10		2					
Sapotaceae																								
Sp. 1	85											3												
Solanaceae																								
Cestrum	107																			3				
Solanum albidum	126						1																	
Solanum	82											4												
Solanum	131,132						2																	
Staphylaceae																								
Turpina	110,108																		1	1				
Styracaceae																								
Styrax	124					1	24																	
Tiliaceae																								
Heliocarpus	183																	3						
Ulmaceae																								
Celtis	129						1																	
Urticaceae																								
Sp. 1											3	3	29						1					
Sp. 2	115										5	2												
Urera	116,130						9				1					5	6	1						
Sp. 3	42							1																
Verbenaceae																								
Sp. 1	154														1									
Vitex	152														16	1								
Unidentified																								
Unidentified	43							1	1															
Unidentified	44							2																
Unidentified	51													1										
Unidentified	53													4										

CHAPTER 6

Genetic evidence for a sibling species of *Heliconius* (Lepidoptera; Nymphalidae).

Abstract

Heliconius charitonia is a widespread species which, unlike many *Heliconius*, is non-mimetic and shows little racial differentiation. Only one form, *peruviana*, which occurs in the dry forest habitats of western Ecuador and Peru, has a distinct and mimetic colour pattern. Here it is shown that *peruviana* is distinct from *charitonia* at allozyme loci (D=0.25 over 22 loci). This differentiation is ten times greater than that between *charitonia* sampled from Ecuador and the Caribbean (D=0.027) and is consistent with analysis of mitochondrial sequence data (3.4-4% sequence divergence between *peruviana* and *charitonia*). One individual with a *peruviana* colour pattern and allozyme genotype was collected in an area where *charitonia* is known to be common, demonstrating that contact between the taxa occurs in western Ecuador. Furthermore the allozyme genotype of another individual is heterozygous for four of five diagnostic loci and is most likely an F1 hybrid between *charitonia* and *peruviana*. These data imply that *charitonia* and *peruviana* should be considered distinct species. This species pair show many similarities with *H. erato* and *H. himera,* which are similarly differentiated genetically and also show ecological and colour pattern differences. These species fulfil some of the predictions of both allopatric refugium, and parapatric adaptationist models of speciation in the neotropics, suggesting that elements of both hypotheses may be true.

Introduction

Neotropical dry forests contain an exceptionally high diversity of locally endemic species, but the mechanisms which give rise to this remain unclear (Dodson & Gentry, 1991; Best & Kessler, 1995). Two competing modes of speciation have been proposed to explain the clustering of taxa into 'centres of endemism' across the neotropics. These are firstly, allopatric isolation in Pleistocene forest refuges (Haffer, 1969), and secondly, parapatric habitat adaptation (Endler, 1977; Benson, 1982). Genetic analysis of recently diverged species might allow an explicit comparison of the two hypotheses. The 'pleistocene refuge hypothesis' would predict concordant genetic divergence between groups of recently evolved species and races, indicating vicariance. The 'parapatric adaptation hypothesis' would predict that genetic breaks and reproductive isolation should primarily coincide with habitat boundaries.

Heliconius butterflies in the dry forests of south western Ecuador and northern Peru offer an opportunity to test these predictions. *Heliconius himera*, a sister species to the widespread *H. erato,* is endemic to this area and has already been studied in detail (Jiggins et al., 1996). Here I present a comparative study of another species *Heliconius charitonia* (Linnaeus). *H. charitonia* is not involved in mimicry with any other species and shows little racial variation in colour pattern across its range from Texas through central America and on both west and east Andean slopes (Comstock & Brown, 1950). *H. charitonia* is replaced by the form *peruviana* (Felder & Felder) in the dry forests of Peru and Ecuador (Comstock & Brown, 1950; Lamas, 1975; Brown, 1979). This race has a distinct colour pattern, with the two distal yellow forewing bands broken up into white spots and the hindwing spots white as opposed to yellow in *charitonia* (Brown and Comstock, 1952). The difference is adaptive, as *peruviana* is a mimic of the ithomiine species, *Elzunia pavonii* (Butler) which is also a dry forest endemic (Poulton, 1931).

H. peruviana was originally described as a separate species by Felder & Felder (1859), but subsequent authors have considered it a race of *charitonia* on the basis of genitalic characters (Eltringham, 1916; Comstock & Brown, 1950; Emsley, 1965). *H. charitonia* and *peruviana* are therefore parapatric sister taxa which provide an opportunity to investigate how divergence occurs in the early stages of speciation. Here patterns of distribution, host plant utilisation, protein and mtDNA variation are examined to assess differentiation between *charitonia* and *peruviana.*

Methods

Ecuador was visited at various times between 1993 and 1996. The distribution of *Heliconius charitonia* was investigated in the western provinces of Ecuador. For genetic analysis a total of 50 individuals were collected and frozen in LN_2 after removal of wings. They were then transported to the UK on dry ice and stored in the laboratory at -80°C. Collection sites for *peruviana* correspond to those of Jiggins et al. (1996). Collections of *H. charitonia* were made in the Rio Toachi valley, Prov. Pichincha. In addition a few individuals collected in the Caribbean (Jamaica and Cuba) were run on the same gels for comparison.

Each individual was dissected and half of each thorax and abdomen was used for this study. These were homogenised on ice in 80µl of grinding buffer (Mallet et al., 1993) and centrifuged. Electrophoresis was performed on Helena cellulose acetate plates using two buffer systems: Phosphate (PB), 0.36% NaH_2PO_4, 0.22% Na_2HPO_4, pH 6.3) and TrisGly (TGB), 0.3% Trizma, 1.4% glycine, pH 8.6. Gels were run with all taxa on each plate in order to ensure accurate scoring of alleles between runs. Plates were stained according to recipes described elsewhere (Mallet et al., 1993; Emelianov et. al., 1995). Analysis of genetic distance was performed using BIOSYS (Swofford and Selander, 1989) according to the method of Nei (1978).

In order to estimate the probability that individual genotypes were derived from a particular population log likelihood ratios were used. Likelihood = p(obs. result | hypothesis), in this case the observed result is a single individual genotype and the hypothesis is given by allele frequencies in the sample of one or other species. In cases where an allele is not present in the sample being investigated, it is assumed that this is due to sampling error. Hence if an allele is not present in 29 diploid genotypes, it is assumed to have a frequency of 1/58 in the population from which that sample is derived. Hypotheses were then compared using $\Delta\log L = \log_e [L(H_*) / L(H_i)]$, where H_* is the most likely hypothesis. Loci with > 0.5 frequency difference between the most common allele in *peruviana* and *charitonia* were used for this analysis.

Passifloraceae are the sole host plants for *Heliconius* (Brown, 1981). To determine which hosts are utilised, a search was made at all sites visited for *Passiflora* and associated larvae or eggs. These were reared to identify the species and sex.

Results and Discussion

Genetic analysis

The colour pattern differences between *peruviana* and *charitonia* are associated with a concordant genetic break. Differentiation between *peruviana* and *charitonia* over 22 loci (Nei's D = 0.250) was almost ten times more than that between samples of *charitonia* from the Carribean and Ecuador (Nei's D = 0.027). The pattern of differentiation at allozyme loci is mirrored in analysis of mitochondrial sequence data in a 1500bp region spanning the COI and COII genes (Davies & Bermingham, in prep.). This also shows that *peruviana* is distinct from *charitonia* (3.4-4% sequence divergence), whilst *charitonia* haplotypes from west Ecuador were made paraphyletic by those from Panama and Cuba (0.7-2% sequence divergence between Ecuador and Caribbean).

The genetic break observed between *peruviana* and *charitonia* is far greater than that expected through isolation by distance. The maximal genetic distance between populations of *charitonia* sampled from different Carribean islands was D = 0.09, and much less than this between most islands (Davies, 1995). Similarly the related species *H. erato* and *H. himera* showed no evidence for any population subdivision in samples taken from western Ecuador up to 400km apart (Chapter 2).

When the level of differentiation is compared with a survey of allozyme genetic distances for pairwise comparisons of Lepidoptera species and populations (Emelianov et al., 1995), the distance between *charitonia* and *peruviana* is well within the range of differentiation found between species and greater than virtually all the conspecific comparisons. Conversely differentiation between Caribbean and Ecuadorean *charitonia* is similar to distances reported for other conspecific populations. The level of genetic divergence between *peruviana* and *charitonia* therefore cannot be easily explained as a result of normal geographic population differentiation.

The distribution of peruviana and charitonia

Sheppard et al. (1985) provide anecdotal evidence that *peruviana* and *charitonia* are sympatric in the Marañon valley. Here the first evidence for contact between

peruviana and *charitonia* west of the Andes is reported. A single specimen with a *peruviana* wing pattern and genotype was collected at Alluriquín, in the Río Toachi valley (780m), flying alongside an individual of *charitonia* (Table 2; Fig. 1). Unfortunately no more individuals were collected at this site. In the same valley *charitonia* is found at 1700m (Hacienda Hesperia) and 750m (Tinalandia; Willmott, pers. comm.). No other individuals of *peruviana* are known from the area, which suggests that this individual may be a migrant from populations in the dry coastal forest 50km to the west.

There is also some evidence for hybridisation between *peruviana* and *charitonia* from the same valley. One individual with a *charitonia* wing pattern, collected at 1700m, has a genotype which is consistent with a *charitonia x peruviana* hybrid at four of five diagnostic loci (Table 2). This genotype is more likely to be an F1 than a *charitonia* (ΔlogL = 5.09, $p<0.05$) or *peruviana* (ΔlogL = 12.16, $p<0.05$).

It seems likely therefore that *peruviana* and *charitonia* are largely allopatric but do meet and hybridise occasionally. However even very occasional migrants between coastal and Andean forests would be sufficient to prevent the accumulation of the observed genetic distance through neutral divergence. Nei & Feldman (1972) showed that $D \approx \mu/m$, where D is genetic distance, μ is mutation rate and m is the rate of migration between populations. Therefore assuming an allozyme mutation rate of 10^{-5}, a genetic distance of D = 0.25 implies that $m \approx 4 \times 10^{-5}$. The proximity of known populations of *charitonia* and *peruviana* (less than 50km in western Ecuador), and the observed overlap (one *peruviana* individual collected in a sample of 22 *charitonia*), suggests that the migration rate is greater than that required to generate the genetic break under neutral divergence. This implies that there must be some pre- or post-zygotic isolation between *charitonia* and *peruviana*. *H. charitonia* and *H. peruviana* should be considered distinct species.

Larval host plants

There is no evidence for divergence in host plant use between *peruviana* and *charitonia*. *H. charitonia* and *H. peruviana* are the only *Heliconius* species known to feed on *Passiflora adenopoda*, which has defensive hooked trichomes (Gilbert, 1971, Table 3). *H. charitonia* also feeds on *P. lobata*, another species with hooked trichomes (Gilbert, 1991). However *peruviana* and *charitonia* are by no means

specialists and feed on a number of other species in the field (Young, 1976; Benson, Brown & Gilbert, 1975; Brown, 1981). Host plant records reported here show that *peruviana* feeds on all the previously recorded larval foodplants of *charitonia* encountered in Ecuador (Table 3).

Biogeographic implications

This study provides an interesting contrast with another species pair, *H. himera* and *H. erato*. *H. himera* is a sister taxon to the widespread *H. erato* which, like *peruviana*, is endemic to the dry forests of Ecuador and Peru (Jiggins et al., 1996). The level of genetic divergence is very similar between both species pairs at allozymes; D = 0.28 between *himera* and *erato* (Chapter 2) and D = 0.25 between *peruviana* and *charitonia*. In contrast mtDNA sequence divergence in the COI and COII genes is somewhat larger between *peruviana* and *charitonia* (3.4 - 4%) than between *himera* and *erato* (1.5 - 2%) (Brower, 1994; Davies & Bermingham, in prep.). The 1.5 - 4% sequence divergence indicates a time since divergence of 2 - 4 million years, coinciding with the late Tertiary and early Pleistocene (Brower, 1994 & 1996). Further evidence would be needed from other species pairs before this could be taken as good evidence for a shared vicariance event. There is however some evidence for the role of vicariance in another recent speciation event in the *charitonia* clade. *Heliconius hermathena,* which occurs in savanna regions of the amazon basin, is a contemporary example of divergence in an isolated dry forest fragment (Brown & Benson, 1977).

Both *himera* and *peruviana* are always associated with dry forest habitats, suggesting that ecology is a primary cause of the observed distribution patterns. However contact zones between *himera* and *erato*, and between *charitonia* and *peruviana* are not concordant. To the west of the Andes in Ecuador, *peruviana* occurs 450km further north than *himera* in dry coastal forest (Fig. 1), whilst to the east of the Andes, in the Marañon valley, contact between *charitonia* and *peruviana* occurs higher up the valley than that between *erato* and *himera* (Sheppard et. al., 1985; Mallet, 1993). These distinct local distribution patterns might be caused in part by known ecological differences between *erato* and *charitonia*. *H. charitonia* relies on colonising temporarily available habitat, such as seasonally dry cloud forests (Gilbert, 1991), whilst *erato* is common in more permanently suitable areas such as lowland wet forest. Despite the differences in the distribution patterns of *himera / erato* and

charitonia / peruviana, it remains true that in both cases speciation is associated with the switch from a humid to a dry forest habitat.

In both *himera* and *erato*, and *charitonia* and *peruviana,* the evolution of barriers to gene flow is associated with a shift in habitat and mimetic pattern. This suggests an adaptationist model of speciation would be most appropriate. However there is also limited evidence for a shared vicariance event in the two taxa. These species therefore seem to fulfil some of the predictions of both strict allopatric, and parapatric adaptationist modes of speciation. It seems likely that speciation in this case has resulted from the gradual accumulation of adaptive mimetic and ecological differences, which probably occurred at least partly in allopatry, suggesting that elements of both hypotheses may be true.

References

BENSON, W.W. 1982. Alternative models for infrageneric diversification in the humid tropics: tests with passion vine butterflies, In: Prance, G.T. (ed), *Biological Diversification in the Tropics*. pp. 608-640. Columbia Univ. Press, New York, NY.

BENSON, W.W., BROWN, K.S. AND GILBERT, L.E. 1975. Coevolution of plants and herbivores: passion flower butterflies. *Evolution*, 29, 659-680.

BEST, B.J. AND KESSLER, M. 1995. *Biodiversity and conservation in Tumbesian Ecuador and Peru*. Birdlife International, Cambridge, U.K.

BROWER, A.V.Z. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. *Proc. Natl. Acad. Sci., USA*, 91, 6491-6495.

BROWER, A.V.Z. 1996. Parallel race formation and the evolution of mimicry in *Heliconius* butterflies: a phylogenetic hypothesis from mitochondrial DNA sequences. *Evolution*, 50, 195-221.

BROWN, F.M. AND COMSTOCK, W.P. 1952. Some biometrics of *Heliconius charitonius* (Linnaeus) (Lepidoptera, Nymphalidae). *Am. Mus. Novitat.*, 1574, .

BROWN, K.S. 1979. *Ecologia Geográfica e Evoluçâo nas Florestas Neotropicais*. 2 vols. (Livre de Docencia) Universidade Estadual de Campinas, Campinas, Brazil.

BROWN, K.S. 1981. The biology of *Heliconius* and related genera. *Ann. Rev. Entomol.*, 26, 427-456.

BROWN, K.S. AND BENSON, W.W. 1977. Evolution in modern Amazonian non-forest islands: *Heliconius hermathena*. *Biotropica*, 9, 95-117.

COMSTOCK, W.P. AND BROWN, F.M. 1950. Geographical variation and subspeciation in *Heliconius charitonius* Linnaeus (Lepidoptera, Nymphalidae). *Am. Mus. Novitat.*, 1467, .

DAVIES, N. 1995. Origins of diversity: The evolutionary genetics of Caribbean butterflies. Ph.D. Diss, University of London.

DODSON, C.H. AND GENTRY, A.H. 1991. Biological extinction in Western Ecuador. *Ann. Missouri Bot. Gard.*, 78, 273-295.

ELTRINGHAM, H. 1916. On specific and mimetic relationships in the genus *Heliconius*. *Trans. Entomol. Soc. Lond.*, 1916, 101-148.

EMELIANOV, I., MALLET, J. AND BALTENSWEILER, W. 1995. Genetic differentiation in *Zeiraphera diniana* (Lepidoptera: Tortricidae, the larch budmoth): polymorphism, host races or sibling species? *Heredity*, 75, 416-424.

EMSLEY, M.G. 1965. Speciation in *Heliconius* (Lep., Nymphalidae): morphology and geographic distribution. *Zoologica, N.Y.*, 50, 191-254.

ENDLER, J.A. 1977. *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton, N.J.

FELDER, C. AND FELDER, R. 1859. Lepidopterologische Fragmente. *Weiner Ent Monatschr*, 3, 390-405.

GILBERT, L.E. 1971. Butterfly-plant coevolution: has *Passiflora adenopoda* won the selectional race with heliconiine butterflies? *Science*, 172, 585-586.

GILBERT, L.E. 1991. Biodiversity of a Central American *Heliconius* community: pattern, process, and problems, In: Price, P.W., Lewinsohn, T.M., Fernandes, T.W., and Benson, W.W. (eds), *Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions*. pp. 403-427. John Wiley & Sons, New York.

HAFFER, J. 1969. Speciation in amazonian forest birds. *Science*, 165, 131-137.

JIGGINS, C., MCMILLAN, W.O., NEUKIRCHEN, W. AND MALLET, J. 1996b. What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.*, 59, 221-242.

LAMAS, G.M. 1975. Supuesta extincion de una mariposa en Lima, Peru (Lepidoptera, Rhopalocera). *Rev. Peruana Entomol.*, 119-120.

MALLET, J., KORMAN, A., HECKEL, D.G. AND KING, P. 1993. Biochemical genetics of *Heliothis* and *Helicoverpa* (Lepidoptera: Noctuidae) and evidence for a founder event in *Helicoverpa zea*. *Ann. Entomol. Soc. Amer.*, 86, 189-197.

MALLET, J. 1993 Speciation, raciation, and color pattern evolution in *Heliconius* butterflies: evidence from hybrid zones. In *Hybrid Zones and the Evolutionary Process* . (ed. R.G. Harrison), pp. 226-260. New York: Oxford University Press.

NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, 89, 583-590.

NEI, M. AND FELDMAN, M. W. 1972. Identity of genes by descent within and between populations under mutation and migration pressures. *J. Theoret. Biol.*, 3, 460-465.

POULTON, E.B. 1931. An Ithomiine butterfly and its Heliconiine mimic taken flying together in N.W. Peru. *Proc. Roy. Entomol. Soc. Lond.*, 5, 91.

SHEPPARD, P.M., TURNER, J.R.G., BROWN, K.S., BENSON, W.W. AND SINGER, M.C. 1985. Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. *Phil. Trans. Roy. Soc. Lond. (B)*, 308, 433-613.

SWOFFORD, D.L. AND SELANDER, R.B. 1989. *BIOSYS-1. A Computer Program for the Analysis of Allelic Variation in Population Genetics and Biochemical Systematics.* Release 1.7 ed. Illinois Natural History Survey, Champaign, Illinois.

YOUNG, A.M. 1976. Studies on the biology of *Heliconius charitonius* L. in Costa Rica. *Pan-Pacific Entomol.*, 52, 291-303.

Table 1; Allele frequencies. Sample sizes and allelic mobilities relative to the commonest allele are also shown. The suffix s = slow and f = fast.

	charitonia		*peruviana*
	Ecuador	Caribbean	Ecuador
GPI	**Glucose-6-phosphate isomerase**		
(N)	21	7	29
250	-	0.071	-
200	0.024	0.071	-
180	0.048	0.286	0.052
100	0.833	0.429	0.690
80	-	-	0.138
60	0.095	0.143	0.121
GOT-f	**Glutamate oxaloacetate transaminase**		
(N)	20	7	29
100	0.075	-	1.000
80	0.925	0.929	-
60	-	0.071	-
GOT-s	**Glutamate oxaloacetate transaminase**		
(N)	21	7	29
-20	-	-	0.069
-35	-	-	0.069
-100	1.000	1.000	0.862
PGM	**Phosphoglucomutase**		
(N)	21	7	28
120	0.214	0.214	-
100	0.667	0.357	-
90	0.071	0.357	-
80	-	-	0.107
75	0.048	0.071	0.786
70	-	-	0.071
65	-	-	0.036
MPI	**Mannose-6-phosphate isomerase**		
(N)	20	7	27
110	0.075	-	0.407
100	0.35	0.429	0.537
90	0.575	0.571	0.056
MDH-s	**Malate dehydrogenase**		
(N)	21	7	29
190	-	-	0.034
100	1.000	1.000	0.948
20	-	-	0.017
MDH-f	**Malate dehydrogenase**		
(N)	21	5	28

110	0.262	-	0.125
100	0.714	1.000	0.875
90	0.024	-	-
G-3	**3-phosphoglycerate dehydrogenase**		
(N)	8	4	19
180	0.063	-	-
160	0.313	-	-
100	0.625	1.000	1.000
6-PGD	**6-phosphogluconate dehydrogenase**		
(N)	21	7	28
100	1.000	1.000	1.000
PK	**Pyruvate kinase**		
(N)	21	7	29
125	-	0.071	-
100	1.000	0.929	0.983
85	-	-	0.017
AK	**Adenylate kinase**		
(N)	21	7	29
100	1.000	1.000	0.983
85	-	-	0.017
ENO	**Enolase**		
(N)	21	7	29
100	1.000	0.929	0.966
45	-	0.071	0.034
ACON-f	**Aconitase**		
(N)	21	7	29
100	0.857	1.000	1.000
90	0.143	-	-
ACON-s	**Aconitase**		
(N)	21	7	29
-55	-	-	0.034
-100	1.000	1.000	0.966
HBDH	**β-Hydroxy-butyrate dehydrogenase**		
(N)	13	3	13
100	1.000	1.000	1.000
IDH	**Isocitrate dehydrogenase (NADP)**		
(N)	17	7	28
140	-	-	0.036
130	0.294	0.143	0.232
100	0.706	0.857	0.732
ME	**Malic enzyme**		
(N)	21	7	27
140	0.310	-	-

130	0.190	-	-
110	0.500	0.357	0.019
100	-	0.429	0.963
90	-	0.214	0.019
α-GPD	**α-Glycerophosphate dehydrogenase**		
(N)	21	7	28
120	-	-	0.018
100	1.000	1.000	0.982
GPT	**Glutamate pyruvate transaminase**		
(N)	21	7	28
150	-	-	0.018
100	0.119	-	0.982
75	0.881	1.000	-
FUM	**Fumarase**		
(N)	21	7	28
-100	1.000	1.000	0.946
-50	-	-	0.054
LA-f	**Leu-Ala peptidase**		
(N)	21	7	28
100	0.976	1.000	-
90	0.024	-	1.000
LA-s	**Leu-Ala peptidase**		
(N)	21	7	28
110	0.024	-	-
105	0.071	-	0.036
100	0.905	1.000	0.911
90	-	-	0.054

		Population allele frequencies		Single individual genotypes		
		Ecuador (allopatric)	Ecuador (allopatric)	Hybrid	Ecuador (sympatric)	
Phenotype		*charitonia*	*peruviana*	*charitonia*	*charitonia*	*peruviana*
Ref. #				3984	3919	3918
Sex				male	male	female
Locus	Mobility					
GOT-f	80	0.925	-	80 / 100	80 / 80	100 / 100
	100	0.075	1.0			
PGM	100	0.667	-	75 / 100	100 / 100	75 / 80
	75	0.048	0.786			
	80	-	0.107			
ME	110	0.5	-	110 / 110	110 / 130	100 / 100
	100	-	0.963			
	130	0.19	-			
GPT	75	0.881	-	75 / 100	75 / 100	100 / 100
	100	0.119	0.982			
LA-f	100	0.976	-	100 / 90	100 / 100	90 / 90
	90	0.024	1.0			

Table 2; Sample genotypes. The loci shown are those with > 0.5 frequency difference between the most common allele in *peruviana* and *charitonia*. Note that not all alleles are shown for each locus, for further details see Table 1. Individual no. 3984 has a genotype which is more likely to be an F1 than a *charitonia* (ΔlogL = 5.09, $p<0.05$) or *peruviana* (ΔlogL = 12.16, $p<0.05$). The *peruviana* individual caught in sympatry with *charitonia* is most likely derived from the *peruviana* as opposed to the local *charitonia* population (ΔlogL = 28.56, $p<0.05$).

	charitonia	*peruviana*
P. adenopoda	M,P,Ea	Ep
P. lobata	P	-
P. rubra	J	Ev
P. hahnii	V, P	-
P. suberosa	J, V	L, Ep
P. perfoliata	J	-
P. manicata		Eb
P. tacsonioides	J	-

Table 3; Host plant records. Data from Benson, Brown & Gilbert (1975), Mallet, pers. comm. and collections in Ecuador. M = Mexico; P = Panama; V = Venezuela; J = Jamaica; L = Lima, Peru; Ea = Ecuador, Alluriquin (Prov. Pichincha); Ep = Ecuador, Piñas (Prov. El Oro); Ev = Ecuador, Vilcabamba (Prov. Loja); Eb = Ecuador, Alamor (Prov. Loja). *P. lobata* was previously included in the genus *Tetrastylis*. - these *Passiflora* species were not found in western Ecuador.

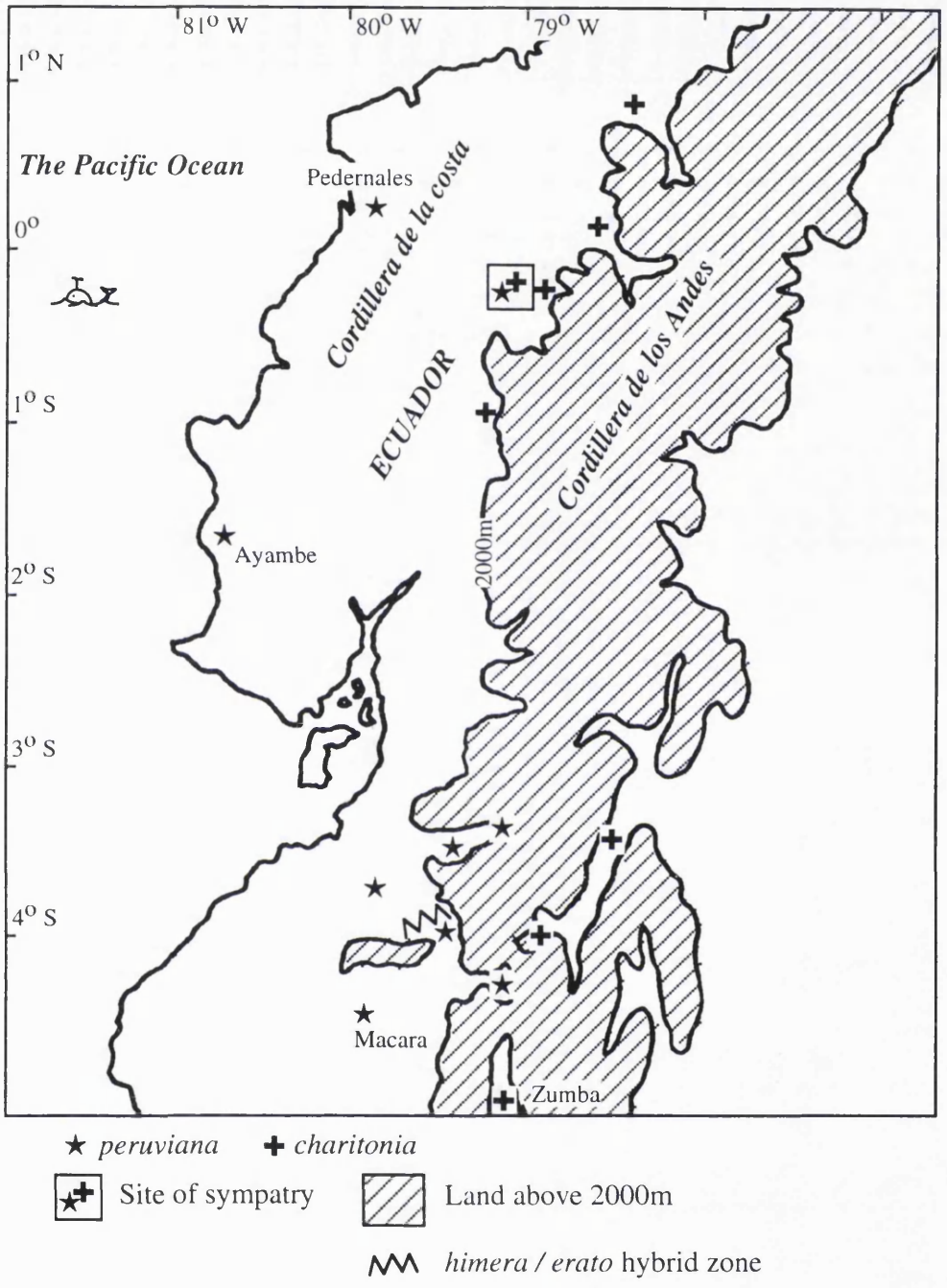


Figure 1. Collections of *charitonia* and *peruviana* in Ecuador.
Data collected by the author, K. Willmott and D. Kapan

CHAPTER 7

Conclusions: What causes speciation?

Natural hybridisation between species offers an opportunity to study the processes of speciation. *H. himera* and *H. erato* show marked differences in colour pattern, ecology, allozyme loci and mitochondrial DNA haplotype. The hybrid zone described here is unusual in that these genetic differences are maintained within overlapping populations, where hybrid genotypes are found at low frequency. This pattern contrasts with many other hybrid zones which have been studied, where genetic differences break down within sympatric populations. The *himera x erato* hybrid zone therefore provides a good system in which to analyse the barriers to gene flow that allow species to coexist.

Laboratory experiments suggest that speciation is most likely to occur as a pleiotropic effect of divergent ecological selection (Rice & Hostert, 1993). This prediction would seem to be borne out by a number of recent field studies showing the evolution of reproductive isolation as a consequence of niche shifts. For example, hybrids between several species of Darwins finches are strongly selected against as a result of disruptive ecological selection (Grant & Grant, 1996). Host races of *Rhagoletis, Chrysoperla* and *Eurosta,* and feeding morphs of sticklebacks have all evolved strong premating barriers to gene flow as a direct result of ecological divergence (Tauber & Tauber, 1989; Craig et al., 1993; Feder et al., 1994; Schluter 1994 & 1995).

These studies suggest that the ecological divergence plays a much greater role in speciation than hybrid sterility or inviability, which are commonly considered to be of primary importance (Coyne & Orr, 1989). Recently evolved species of sticklebacks, Darwin's finches and monkeyflowers show no evidence for any intrinsic hybrid sterility or inviability (Schluter, 1993; Bradshaw et. al., 1995; Grant & Grant, 1996). Indeed, when disruptive ecological selection is relaxed, hybrids between *Geospiza fortis, G. scandens* and *G. fuliginosa* may have increased fitness over parental genotypes (Grant & Grant, 1996). The results of the present study support this, as *H. himera* and *H. erato* show no evidence for any hybrid sterility or inviability. Three

generations of hybrid crosses showed no reduction in hatch rate, larval survival or fertility relative to parental control crosses (McMillan et. al., 1997). Genomic incompatibilities may be a likely result, but not necessarily a primary cause of speciation.

The evidence from this study also supports an emphasis on the role of niche shifts in speciation. Host plant adaptation has been touted as the most likely mode of ecological speciation in phytophagous insects (Bush, 1993), however there was no evidence for shifts in host plant use between either *himera* and *erato*, or *peruviana* and *charitonia*. Instead, in both species pairs, the evolution of reproductive isolation is associated with a shift in mimetic pattern and biotope. The *himera x erato* contact zone is closely correlated with a habitat transition from wet to dry forest, which suggests that parapatry is maintained in part by ecological adapation. Similarly *peruviana* is associated with dry forest habitats, whilst its sister species, *charitonia*, is generally found in humid or moist montane forest.

Both species pairs also show differences in their mimetic colour patterns. Crosses between *himera* and *erato* show that major gene control of pattern elements is similar to that found in previous studies of *H. erato* races, and the loci are probably homologous. This suggests that similar genetic processes are involved in the morphological divergence of species and races.

Furthermore changes in habitat and warning colour probably also generate post-mating barriers to gene flow between *himera* and *erato*. The virtually complete linkage disequilibrium between markers in hybrid zone populations is hard to explain in view of 5-10% hybridisation and the lack of evidence for hybrid inviability or sterility. A possible explanation is that the hybrid zone results from extremely recent contact, such that extensive introgression has not yet occurred. However we now have evidence that the zone has been stable for 14 years, so this seems unlikely (Table 1). The barrier to gene flow is most easily explained by selection against hybrids due to divergence in habitat and warning colour.

It is possible that the primary source of postmating isolation results from frequency dependent selection on warning colour (Mallet et al., 1997). In the centre of inter-racial hybrid zones, where mating is random, hybrids form most of the population

(Fig. 1A) and therefore levels of predation are similar on all phenotypes. In contrast, where there is strong premating isolation between warning colour phenotypes, hybrids are the rarest forms throughout the hybrid zone, and therefore selection against hybrids will be strong in all areas (Fig. 1B). Thus, when assortative mating is associated with differences in warning colour this may generate strong disruptive selection.

Whilst there is convincing evidence that changes in habitat and warning colour play a role in generating current barriers to gene flow, it remains unclear whether they have played a directly causal role in speciation. *H. erato* is found in dry savanna habitats in the llanos of Venezuela (Benson, 1982) and has undergone widespread adaptive radiation of colour pattern, which shows that neither habitat nor warning colour divergence necessarily leads to speciation. Instead racial divergence of colour pattern and ecology might be seen as an essential precursor to species formation. In much the same way, incompatibilities such as Haldane's rule can evolve between races without leading to speciation (e.g. *Chorthippus paralellus*, Hewitt et. al., 1987). It may be that speciation only results when boundaries between divergently selected taxa somehow become associated with assortative mating.

Therefore the evolution of premating isolation can be considered the catalyst of speciation. A similar conclusion was reached by Paterson (1985) who emphasised the primary importance of 'species recognition systems' as the vital attribute of a species. In the case of *Heliconius himera* and *H. erato* it is possible that divergence in mating preferences evolved as a direct result of ecological adaptation to the different habitats. This would support the prediction of Rice and Hostert (1993) that speciation occurs as a pleiotropic effect of divergent ecological selection. Alternatively, changes in mating preferences may have been driven by sexual selection, and have subsequently become associated with the ecological discontinuity. The evolutionary origins of assortative mating, and the means by which it becomes associated with ecological and warning colour boundaries must remain the greatest outstanding question in our understanding of the speciation of *Heliconius himera* and *Heliconius erato*.

References

Benson, W.W. 1982 Alternative models for infrageneric diversification in the humid tropics: tests with passion vine butterflies. In *Biological Diversification in the Tropics* . (ed. G.T. Prance), pp. 608-640. New York, NY: Columbia Univ. Press.

Bradshaw, H.D., Wilbert, S.M., Otto, K.G. & Schemske, D.W. 1995 Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*). *Nature* **376**, 762-785.

Bush, G.L. 1993 A reaffirmation of Santa Rosalia, or why are there so many kinds of *small* animals. In *Evolutionary Patterns and Processes* . (ed. D.R. Lees & D. Edwards), pp. 229-249. London: Linnean Society of London.

Coyne, J.A. & Orr, H.A. 1989 Two rules of speciation. In *Speciation and its Consequences* . (ed. D. Otte & J.A. Endler), pp. 180-207. Sunderland, Mass.: Sinauer Associates.

Craig, T.P., Itami, J.K., Abrahamson, W.G. & Horner, J.D. 1993 Behavioral evidence for host-race formation in *Eurosta solidaginis*. *Evolution* **47**, 1696-1710.

Descimon, H. & Mast De Maeght, J. 1984 Semispecies relationships between *Heliconius erato cyrbia* Godt. and *H. himera* Hew. in southwestern Ecuador. *J. Res. Lepid.* **22**, 229-239.

Feder, J.L., Opp, S.B., Wlazlo, B., Reynolds, K., Go, W. & Spisak, S. 1994 Host fidelity is an effective premating barrier between sympatric races of the apple maggot fly. *Proc. Natl. Acad. Sci., USA* **91**, 7990-7994.

Grant, P.R. & Grant, R.B. 1996 High survival of Darwin's finch hybrids: Effects of beak morphology and diets. *Ecology* **77**, 500-509.

Hewitt, G.M., Butlin, R.K. & East, T.M. 1987 Testicular dysfunction in hybrids between parapatric subspecies of the grasshopper *Chorthippus parallelus*. *Biol. J. Linn. Soc.* **31**, 25-34.

Mallet, J. 1996 The genetics of diversity at and below the species level. In *Biodiversity: Biology of Numbers and Difference*. (ed. K.J. Gaston), p. 13-47. Oxford: Blackwell.

Mallet, J., McMillan, W.O. and Jiggins, C.D. 1997. Mimicry and warning colour at the boundary between microevolution and macroevolution. In. *Endless Forms: Species and Speciation*. (eds. S. Berlocher & D. Howard). pp. 00-00. Oxford University Press, Oxford.

McMillan, W.O., Jiggins, C.D. and Mallet, J. 1997. What initiates speciation in passion vine butterflies? *Proc. Natl. Acad. Sci., USA*, submitted.[see Appendix]

Paterson, H.E.H. 1985 The recognition concept of species. In *Species and speciation.* (ed. E.S. Vrba). Transvaal Museum Monograph No. 4: Pretoria.

Rice, W.R. & Hostert, E.E. 1993 Laboratory experiments on speciation: What have we learned in 40 years? *Evolution* **47**, 1637-1653.

Schluter, D. 1993 Adaptive radiation in sticklebacks: size, shape, and habitat use efficiency. *Ecology* **74**, 699-709.

Schluter, D. 1994 Experimental evidence that competition promotes divergence in adaptive radiation. *Science* **266**, 798-801.

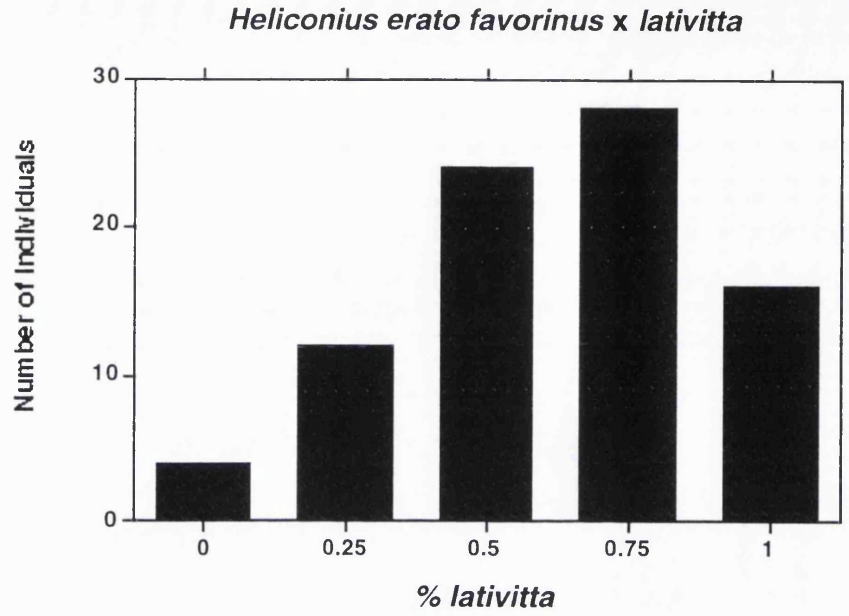
Schluter, D. & Nagel, L.M. 1995 Parallel speciation by natural selection. *Amer. Nat.* **146**, 292-301.

Tauber, C.A. & Tauber, M.J. 1989 Sympatric speciation in insects: perception and perspective. In *Speciation and its consequences* . (ed. D. Otte & J.A. Endler), pp. 307-344. Sunderland, Massachusetts: Sinauer.

	Phenotype		
Date	*cyrbia*	*himera*	Hybrids
Jun 82	31	11	5
Nov 92	36	3	3
Dec 93	13	2	0
12 Jan 94	10	3	0
18 Jan 94	6	0	0
May 94	47	6	4
Oct 94	11	0	0
Dec 94	9	1	1
Jan 95	9	0	2
Jul 96	83	11	1

Table 1. Temporal stability of contact between *himera* and *erato*. Numbers of individuals collected at Site 4 from 1982 to 1996 are shown. A test for heterogeneity on this table is significant (G_{18}=30.02, $p<0.05$). This might indicate movement of the cline over time, but is more likely explained by uneven collecting effort by Descimon and Mast de Maeght in 1982, leading to a deficit of the common *cyrbia* phenotype (Descimon & Mast de Maeght, 1984). When the 1982 data is excluded there is no evidence for heterogeneity (G_{16}=20.50, NS). Contact between *himera* and *erato* is therefore a stable phenomenon with little evidence for cline movement.

A)



B)

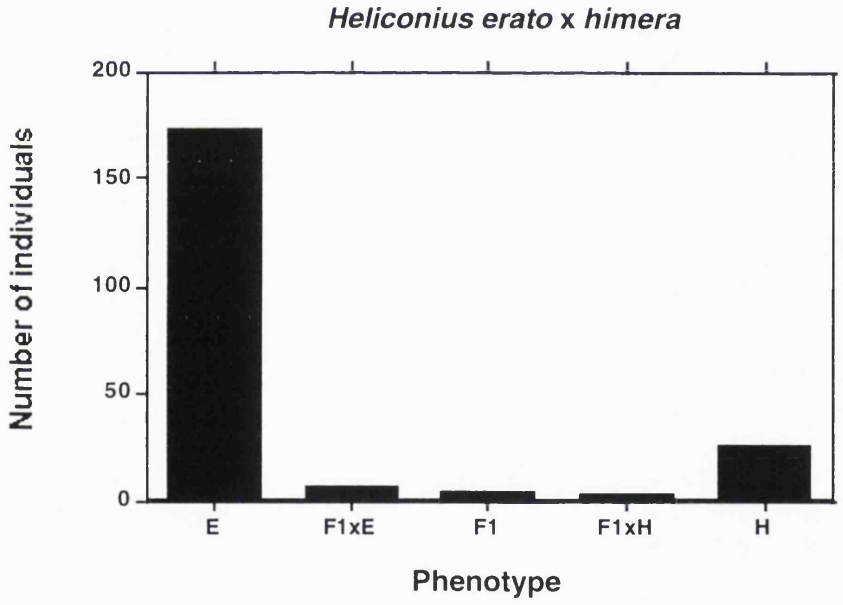


Figure 1. Frequency of phenotypes in A) inter-racial and B) inter-species hybrid zones. The rarity of hybrid phenotypes throughout the *himera* x *erato* zone will result in much stronger disruptive selection against hybrids than in the inter-racial case. Figures adapted from Mallet (1996).

APPENDIX

What initiates speciation in passion-vine butterflies?

A paper submitted to *Proceedings of the National Academy of Science (USA)*
by W. Owen McMillan, Chris D. Jiggins, James Mallet

Abstract

Studies of the continuum between geographic races and species provide the clearest insights into the causes of speciation. Here we report on mate choice and hybrid viability experiments in a pair of warningly-colored butterflies, Heliconius erato and H. himera, that maintain their genetic integrity in the face of hybridization. Hybrid sterility and inviability have been unimportant in the early stages of speciation of these two Heliconius. We find no evidence of reduced fecundity, egg hatch, or larval survival in three generations of hybrid crosses. In addition, hybrid offspring develop at a rate intermediate to the two pure types and there is no strong evidence for asymmetric mortality of males and females (i.e. Haldane's rule) in F1, F2, or backcross broods. Instead, speciation in this pair appears to have been catalyzed by the association of strong mating preferences with divergence in warning coloration and ecology. In mate choice experiments, matings between the two species are a tenth as likely as matings within species. F1 hybrids of both sexes mate frequently with both pure forms. However, male F1 progeny from crosses between H. himera mothers and H. erato fathers have somewhat reduced mating success. The strong barrier to gene flow provided by divergence in mate preference is probably enhanced by frequency-dependent predation against hybrids similar to the type known to occur across inter-racial hybrid zones of H. erato. In addition, the transition between this pair falls at the boundary between wet and dry forest and rare hybrids may also be selected against because they are poorly adapted to either biotope. These results add to a growing body of evidence that challenge the importance of genomic incompatibilities in the earliest stages of speciation.

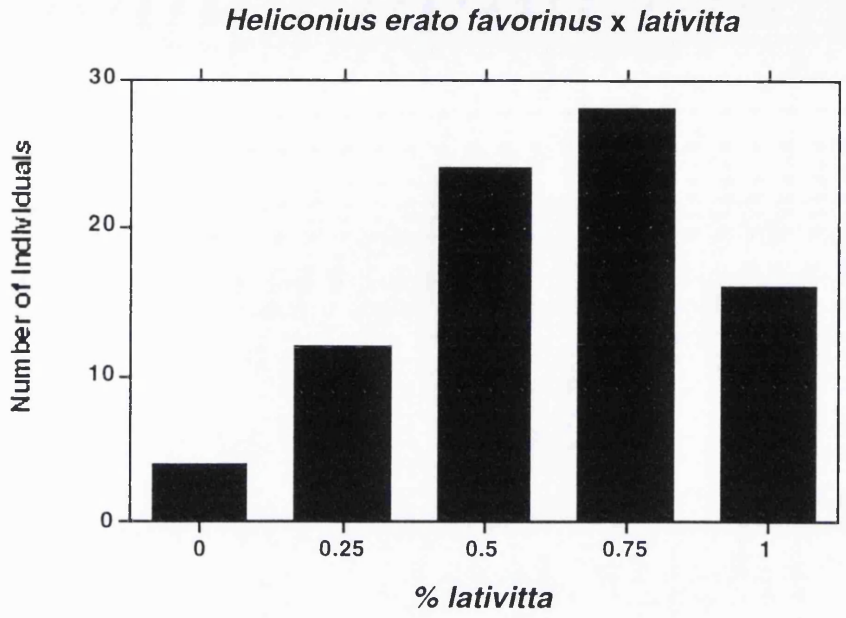
Introduction

Empirical research on speciation has mostly involved either laboratory hybridization experiments on Drosophila or studies of natural hybrid zones in taxa as diverse as crickets, butterflies, frogs, birds, and mice. This work has yielded valuable insights into the nature of species and racial differences, but the proximal causes of speciation remain unclear. Many Drosophila studies, for example, have examined species pairs between which there are multiple behavioral and genetic incompatibilities, making it hard to distinguish causes of speciation from differences that have evolved subsequently (**1**). In addition, with the possible exception of Hawaiian and cactophilic Drosophila (**2, 3**), the behavior and ecology of wild Drosophila and its importance in speciation is still poorly understood (**1**). Many hybrid zones, on the other hand, have been studied intensively both in the field and via laboratory experiments (**4-6**). However, in most of these studies, genetic differences between pure forms break down in narrow zones composed mainly of hybrids and a good argument can be made that speciation has not occurred. Indeed, as many of these zones are thought to represent secondary contact, these pairs may better highlight a failure to speciate rather than giving insight into speciation. Fewer studies have examined very closely-related taxa which can maintain their genetic integrity despite persistent hybridization. Taxa in this intermediate stage have only recently acquired to ability to remain distinct and are of extreme interest because they are more likely to reveal the traits that actually initiated speciation.

Here we examine the incipient stages of speciation of a pair of Heliconius butterflies. Heliconius erato is a common, brightly colored butterfly of secondary and gallery forests throughout the New World tropics. Like other members of the genus, H. erato is unpalatable to predators and shows strong Müllerian mimicry to other unpalatable species (**7-10**). Racial diversification within this species has reached extraordinary proportions. Nearly 30 parapatric races are described with ranges spanning thousands to a few hundred square kilometers. These races correspond almost exactly to those of its usual Müllerian co-mimic, H. melpomene (**7-10**). Races differ, often strikingly, in warning color patterns, although they mate randomly in narrow contact zones (**11-13**). Other than loci coding for wing pattern elements there is little genetic distinction between races: speciation has clearly not happened (**13-15**).

Heliconius himera represents an intermediate step in the transition from a race to a species in this group. It has variously been considered a race of H. erato, which it replaces in the dry thorn-scrub habitats of Andean valleys in Southern Ecuador and

A)



B)

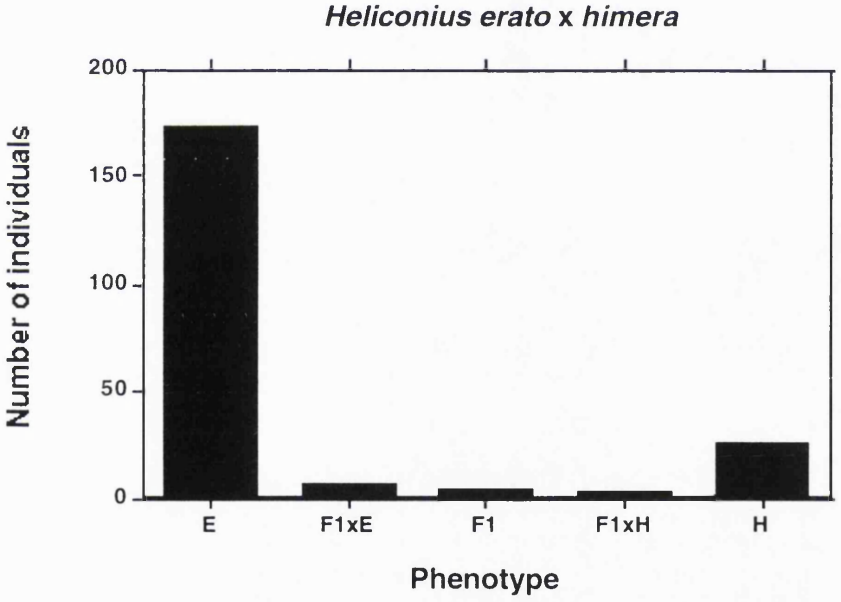


Figure 1. Frequency of phenotypes in A) inter-racial and B) inter-species hybrid zones. The rarity of hybrid phenotypes throughout the *himera* x *erato* zone will result in much stronger disruptive selection against hybrids than in the inter-racial case. Figures adapted from Mallet (1996).

APPENDIX

What initiates speciation in passion-vine butterflies?

A paper submitted to *Proceedings of the National Academy of Science (USA)*
by W. Owen McMillan, Chris D. Jiggins, James Mallet

Abstract

Studies of the continuum between geographic races and species provide the clearest insights into the causes of speciation. Here we report on mate choice and hybrid viability experiments in a pair of warningly-colored butterflies, Heliconius erato and H. himera, that maintain their genetic integrity in the face of hybridization. Hybrid sterility and inviability have been unimportant in the early stages of speciation of these two Heliconius. We find no evidence of reduced fecundity, egg hatch, or larval survival in three generations of hybrid crosses. In addition, hybrid offspring develop at a rate intermediate to the two pure types and there is no strong evidence for asymmetric mortality of males and females (i.e. Haldane's rule) in F1, F2, or backcross broods. Instead, speciation in this pair appears to have been catalyzed by the association of strong mating preferences with divergence in warning coloration and ecology. In mate choice experiments, matings between the two species are a tenth as likely as matings within species. F1 hybrids of both sexes mate frequently with both pure forms. However, male F1 progeny from crosses between H. himera mothers and H. erato fathers have somewhat reduced mating success. The strong barrier to gene flow provided by divergence in mate preference is probably enhanced by frequency-dependent predation against hybrids similar to the type known to occur across inter-racial hybrid zones of H. erato. In addition, the transition between this pair falls at the boundary between wet and dry forest and rare hybrids may also be selected against because they are poorly adapted to either biotope. These results add to a growing body of evidence that challenge the importance of genomic incompatibilities in the earliest stages of speciation.

Introduction

Empirical research on speciation has mostly involved either laboratory hybridization experiments on Drosophila or studies of natural hybrid zones in taxa as diverse as crickets, butterflies, frogs, birds, and mice. This work has yielded valuable insights into the nature of species and racial differences, but the proximal causes of speciation remain unclear. Many Drosophila studies, for example, have examined species pairs between which there are multiple behavioral and genetic incompatibilities, making it hard to distinguish causes of speciation from differences that have evolved subsequently (**1**). In addition, with the possible exception of Hawaiian and cactophilic Drosophila (**2, 3**), the behavior and ecology of wild Drosophila and its importance in speciation is still poorly understood (**1**). Many hybrid zones, on the other hand, have been studied intensively both in the field and via laboratory experiments (**4-6**). However, in most of these studies, genetic differences between pure forms break down in narrow zones composed mainly of hybrids and a good argument can be made that speciation has not occurred. Indeed, as many of these zones are thought to represent secondary contact, these pairs may better highlight a failure to speciate rather than giving insight into speciation. Fewer studies have examined very closely-related taxa which can maintain their genetic integrity despite persistent hybridization. Taxa in this intermediate stage have only recently acquired to ability to remain distinct and are of extreme interest because they are more likely to reveal the traits that actually initiated speciation.

Here we examine the incipient stages of speciation of a pair of Heliconius butterflies. Heliconius erato is a common, brightly colored butterfly of secondary and gallery forests throughout the New World tropics. Like other members of the genus, H. erato is unpalatable to predators and shows strong Müllerian mimicry to other unpalatable species (**7-10**). Racial diversification within this species has reached extraordinary proportions. Nearly 30 parapatric races are described with ranges spanning thousands to a few hundred square kilometers. These races correspond almost exactly to those of its usual Müllerian co-mimic, H. melpomene (**7-10**). Races differ, often strikingly, in warning color patterns, although they mate randomly in narrow contact zones (**11-13**). Other than loci coding for wing pattern elements there is little genetic distinction between races: speciation has clearly not happened (**13-15**).

Heliconius himera represents an intermediate step in the transition from a race to a species in this group. It has variously been considered a race of H. erato, which it replaces in the dry thorn-scrub habitats of Andean valleys in Southern Ecuador and

Northern Peru, or a separate species (**16**). Both genetic and morphological analyses confirm the close relationship between H. himera and H. erato (**15, 17**). MtDNA differences between H. himera and H. erato fall at the edge of the range of inter-racial differences and suggest a divergence time of approximately 1 million years (**15**). In contrast to inter-racial contact zones within H. erato, parental types predominate in the hybrid zones between H. erato and H. himera. In the contact zone between H. himera and H. erato cyrbia in Ecuador, hybrids (F1 and backcrosses) make up only 9% of the population (**16**). Similarly, hybrids are known to be rare in contact zones of H. himera/H. e. favorinus and H. himera/H. e. lativitta in Peru (**13**). In the Ecuador hybrid zone, morphologically "pure" erato and himera are also "pure" at allozyme and mtDNA loci (Jiggins, C., McMillan, W. O. & Mallet J., in prep.). This pair has clearly reached the crucial stage in speciation where emerging forms can maintain multi-locus genotypic differences when in contact. Genetic differences can continue to accumulate, opening the possibility for continued divergence that may eventually permit widespread coexistence of the two species.

Rapid racial evolution has occurred repeatedly throughout Heliconius and especially in H. erato. This propensity could be an important factor contributing to the approximately 10-fold greater species diversity within Heliconius relative to ancestral heliconiine groups. Nonetheless, the link between racial evolution and speciation is poorly characterized in Heliconius. We report on mate choice and breeding experiments that, in combination with field data, examine the relative roles that mating behavior, hybrid inviability, sterility and ecology play in early stages of the transition from race to a species.

Materials and methods

Mating and breeding experiments were performed in outdoor insectaries in Vilcabamba, Ecuador (altitude 1600m), a dry forest region within the range of H. himera, between August 1994 and March 1995.

Mating behavior. Two types of mate-choice experiments, "single-male" and "multiple-male", were designed to study competition between females and males, respectively. The four types of males and females used in these experiments were designated as follows: E= erato, H = himera, EH = F1 progeny of erato female x himera male, and HE = the reciprocal F1 progeny. In each single-male experiment, a male was caged with two newly eclosed virgin females in 1x1x2 m^3 outdoor

insectaries. In multiple-male experiments a single newly eclosed virgin female was placed in a much larger (3x2x2 m^3) cage containing 3 H, 3 E, and 3 F1 males. Mating experiments were monitored hourly. Most matings occurred within the first hour, but occasionally took until the following day. In single-male experiments, individuals were used only once. In multiple-male experiments, the mated pair and a randomly chosen male from each of the two classes that did not mate were removed and replaced with three new males (1 H, 1 E and 1 F1). Wild-caught and reared males (≥ 7 days old) were used in single-male experiments but only reared males (≥ 7 days old) were used in multiple-male experiments. Most of the 415 individual H and E used in these experiments were collected, or raised from individuals collected at least 25 km from the hybrid zone. However, 43 pure individuals (19 male and 12 female E, and 4 male and 8 female H) were collected from the hybrid zone or were the offspring of one parent collected from hybrid zone populations. Because mate preference was very strong, even in individuals collected away from the hybrid zone, we used both sets of individuals to estimate mating probabilities (see below).

Estimation of mating probabilities. We used a novel likelihood approach to estimate the relative probability of mating between pure species and F1 progeny. Likelihood gives a powerful means of estimating parameters and support intervals across multiple experiments and our analysis could also be useful in many other studies of mating behavior between different strains or species. We estimated the probability, P_{IxJ}, of a mating between *I*-type females and *J*-type males relative to the probability of matings within erato, P_{ExE}, setting P_{ExE} to 1.00 (similarly, P_{HxH} was set to 1.00 in multiple-male experiments involving himera females). These relative mating probabilities were then combined to give actual mating probabilities, which, in any experiment, must sum to one. For example, the actual probabilities of E males mating with E and EH females in the single-male experiments (see Table 1a), were then: $\Pi_{ExE} = 1/(1 + P_{EHxE})$ and $\Pi_{EHxE} = P_{EHxE}/(1 + P_{EHxE})$, respectively. Similarly, the actual probabilities that E females mate with E, EH, HE, and H males in multiple-male experiments (Table 1b) were given by: $\Phi_{ExE} = 1/(1 + P_{ExEH} + P_{ExHE} + P_{ExH})$, $\Phi_{ExEH} = P_{ExEH}/(1 + P_{ExEH} + P_{ExHE} + P_{ExH})$, $\Phi_{ExHE} = P_{ExHE}/(1 + P_{ExEH} + P_{ExHE} + P_{ExH})$ and $\Phi_{ExH} = P_{ExH}/(1 + P_{ExEH} + P_{ExHE} + P_{ExH})$, respectively. These probabilities were estimated using likelihood (**18**). For example, the likelihood of results in the two experiments described above were $\Pi_{ExE}^{13}\ \Pi_{EHxE}^{5}$, and $\Phi_{ExE}^{11}\ \Phi_{ExEH}^{2}\ \Phi_{ExHE}^{6}\ \Phi_{ExH}^{0}$, respectively (data are from Tables 1a, b). The likelihood over all experiments was maximized, and used to estimate all P_{IxJ} parameters and compare different models of

mating. Twice the difference in maximum log-likelihood ($G= 2\Delta \log_e L$) between two models asymptotically follows a χ^2 distribution, permitting comparison of models differing in numbers of parameters (**18**). The reliability of our estimates of mating behavior were given "support limits", i.e. the parameter values at which the $\log_e$ likelihood dropped two units below the maximum. Support limits are asymptotically equivalent to 95% confidence limits.

Hybrid sterility and inviability. Our breeding experiments were conducted under ambient conditions. Each mated female was kept in an individual 1x1x2 m^3 insectary that contained ample artificial nectar, pollen resources, and larval foodplants. Artificial nectar consisted of a 10% sugar solution displayed in small red and yellow plastic cups. Sugar solution was checked daily and replenished as necessary. In addition, fresh nectar and pollen resources were provided by cut Lantana sp. flowers. Lantana flowers were replaced every 2-3 days. Potted Passiflora rubra and P. punctata, each utilized by wild populations of both erato and himera (**16**), was provided for oviposition. Eggs were collected daily and the larvae were raised individually in small plastic pots to prevent cannibalism. Developing larvae were fed ample new growth of P. rubra. Larval pots were cleaned daily and new food was added as needed. A small twig taped to the top of each pot provided a suitable pupation site. Pupae were housed together by brood in larger plastic cages until emergence. We avoided inbreeding by crossing only unrelated individuals.

To control for environmental fluctuations in our breeding experiments, hybrid crosses were reared simultaneously with pure crosses as controls in complexes of six cages. Groups of hybrids and controls reared together in this way were designated as a replicate. The number of eggs laid per day, hatching success, larval survival and developmental times of hybrid or hybrid-mated crosses could then be examined relative to pure crosses raised in the same cage complex and during the same period. We used hierarchical G-tests to examine differences in egg hatch and larval survival i) between different F1 crosses (HE vs. EH), ii) between different pure crosses (HH vs. EE), and iii) between hybrids and controls. The F2 crosses were not raised under this experimental design, but were compared directly to pure broods of himera and erato females collected from the wild and reared simultaneously.

Results

Mating behavior. Although the mating behavior of H. himera in the wild is still poorly researched, mating in H. erato is known to occur shortly after female eclosion (**19**). Male H. erato actively search for larvae and pupae on host plants and have been observed in greenhouses to "pupal-mate", i.e. sit on pupae, and then mate with females as they eclose (**20**). Pupal-mating never occurred with himera and erato in our insectary, even when females were allowed to develop on host plants growing in all-male cages. Nonetheless, in our mating experiments with newly eclosed females, mating was often rapid and lacked obvious courtship elements. In both single- and multiple-male experiments, males flew aggressively towards females, sometimes hovering briefly, before landing and attempting to mate. Females could and often did reject males by spreading their wings and raising their abdomens out of reach of male claspers.

There were no differences in the relative probabilities of mating between single-male and multiple-male experiments ($G= 4.68$, $p>0.5$, $df= 6$). Under both experimental designs inter-specific matings were rare, occurring in only 8 of 84 mating experiments (Table 1). Because the type of mating experiment did not influence the relative probabilities of different mating combinations, we could reduce the number of P_{IxJ} mating probabilities to be estimated from 21 in the full model to 15. This 15-parameter model was reduced still further since all but 4 of the P_{IxJ}'s were not informatively different from P_{ExE} (see Table 2). Under this final model, inter-specific matings were approximately a tenth as likely as within-species mating (Table 2).

Matings between hybrids and pure forms occurred more frequently in our experiments. As an example, F1 males mated pure females in 19 of 42 multiple-male experiments (Table 1). However, most of these matings involved HE males (progeny of himera females and cyrbia males). Mating models where the two reciprocal F1 types were considered the same were significantly worse than models where the two F1 types were considered separately ($G= 19.69$, $P<0.01$, $df= 7$), indicating heterogeneity between the F1 hybrid classes. This heterogeneity was the result of reduced mating probabilities in only some crosses with EH males (Table 2). In particular, ExEH matings were less than half as probable as conspecific matings and the probability of EHxEH matings was reduced still further (Table 2). The reason for the mating asymmetry between F1 males was unclear. Because males are the homogametic sex in butterflies, the two F1 classes differ only at cytoplasmically

inherited genes. Whatever the reason, the mating probabilities involving F1 males were, on average, much greater than those between erato and himera (Table 2).

Hybrid sterility and inviability. Our experiment examined fitness parameters in over three generations of hybrid crosses. Nearly 4500 eggs were laid over our 8-month breeding experiment generating 2475 adults. Egg-to-adult developmental time was approximately 29 days and not significantly different between males and females. Mortality was generally highest in the early larval stages.

All hybrid crosses proved both as viable and fertile as pure crosses (Table 3). Hybrid crosses laid fertile eggs and, with the possible exception of F2 crosses, fecundity, as estimated by number of eggs laid per day, did not differ significantly among conspecific, interspecific, and hybrid matings (Table 3). Moreover, hybrid egg hatch was high, averaging 90%, and similar to controls (Table 3). There was strong brood-dependent variation in hatching success within F1, BC1, and both types of pure crosses [within F1 broods: $G= 85.33$, $p< 0.001$, $df= 19$; within BC1 broods: $G= 70.07$, $p< 0.001$, $df= 16$; within HH broods: $G=32.70$, $p< 0.01$, $df= 13$; within EE broods: $G= 33.38$, $p< 0.001$, $df= 11$]; however, in standardized comparisons the only significant differences in egg hatch between pure and hybrid crosses was between F3+ and their controls (Figure 1a). In this case, egg hatch was significantly higher among the hybrid crosses ($G= 13.48$, $p< 0.0001$, $df= 1$).

Hybrid larval survival was also similar to that of controls. Only HxE crosses showed a slight reduction of larval survival relative to HxH crosses when averaged over the entire experimental period (Table 3). This apparent difference, as well as an apparent increase in survival of F2 and F3+ crosses, however, was not obvious in comparisons between hybrid and pure crosses raised together. As with egg-hatch, there was significant variation in survival among broods in some hybrid and control crosses [within F1 broods: $G= 65.64$, $p< 0.001$, $df= 19$; within BC2 broods: $G= 139.35$, $p< 0.001$, $df= 15$; within HH broods: $G= 42.32$, $p< 0.001$, $df= 13$; within EE broods: $G= 40.06$, $p< 0.001$, $df= 11$]. This variation was particularly marked because of a disease outbreak. The epidemic was localized to individual hybrid and control broods (Figure 1b). Largely because of this disease, there were strong differences in larval survival between controls and hybrids in F1 and BC2 replicates ($G=15.69$, $p< 0.001$, $df= 5$; $G=54.85$, $p< 0.0001$, $df= 4$, respectively). However, differences between F1 hybrids and controls were most strongly influenced by higher hybrid larval survival in replicate I ($G= 5.86$, $p< 0.05$, $df= 1$) and a higher control survival in replicate IV ($G=8.57$, $p< 0.001$, $df= 1$) (Figure 1b). Lower larval survival

of BC2 hybrids compared with their controls were chiefly due to diseased hybrid broods in replicates I and II (Figure 1b) ($G= 32.4$, $p< 0.0001$, $df= 1$ and $G=18.73$, $p< 0.0001$, $df= 1$, respectively). BC2 replicates that were largely unaffected by disease did not differ significantly in larval survival. With the exception of a barely significant reduction in the number of females among BC2 hybrids overall ($G= 4.9$, $p< 0.05$, $df= 1$), all crosses produced approximately equal numbers of males and females (Table 3).

Egg-to-adult developmental times, potentially sensitive to incompatibilities within hybrid genomes, were also similar between hybrids and controls (Table 3). In standardized comparisons, there were significant differences in developmental time between himera and erato, and hybrid larvae developed at a roughly intermediate rate. The observed difference in egg-to-adult developmental time between himera and erato probably reflects an underlying adaptive difference. Heliconius himera and H. erato live in markedly different biotopes: H. himera is found in drier forests at altitudes up to 400m higher that those of H. erato (**16**). All larvae were reared under ambient conditions within the himera biotope in Vilcabamba. In this environment, ExE larvae took 1.3 ± 0.4 days longer than HxH larvae to reach adulthood ($n= 11$, brood comparisons). In general, the more erato genes in a hybrid cross the longer the egg-to-adult developmental time and there was a significant regression of the extra development time (in days, relative to HxH controls) against the proportion of erato genes (slope = 1.10, $r = 0.56$, $p= 0.001$, $df= 66$) (Fig 2). Heliconius himera was also more active in the early morning and late evening suggesting that physiological adaptation to different environmental conditions extends to adults (Davison, A., McMillan, W. O., Griffin, A. S., Jiggins, C. D. & Mallet, J., in prep.).

Discussion

Today, H. W. Bates is remembered as the discoverer of mimicry. However, his original treatise (**7**), in which he describes mimetic patterns in Amazonian butterflies, is primarily concerned with speciation. He believed that species and geographic races form a continuum and that heliconiine and ithomiine butterflies best demonstrated the transition from geographic variation to speciation. Here we show that for himera and erato, the step most critical for this transition is the evolution of strong mate preference and that classical hybrid inviability or sterility plays, at best, a minor role. Our breeding experiments show virtually no reductions of fecundity, egg

hatch, larval survival, or developmental time of hybrids. Mating preferences, in contrast, provide an approximately 90% effective barrier to the production of hybrids. The only possible evidence of classical hybrid incompatibility was the lower mating propensity of EH hybrid males in some crosses. The very strong barrier to the production of hybrids tally with the observation that hybrids make up only 9% of individuals in the zones of overlap between the two species (**16**). Further indication of strong mating preferences comes from females collected (n= 35) in the contact zones. Of the four 'pure-species" females that were not mated by conspecifics, only one produced an F1 progeny. The remaining three hybrid matings involved F1 males.

It is tempting to attribute the divergence in mating preferences we observe to changes in warning coloration. Vision is well developed in butterflies and colors can attract Heliconius from distances of 20 meters or more (**21**); however, differences in wing patterns, as great as those that exist between himera and erato, have not resulted in assortative mating among the many geographic races of erato (**11-13**). In addition, as Bates discovered over a hundred years ago, within any given area of the neotropics an extraordinary number of different species share nearly identical color patterns. This convergence would seem to make color pattern a poor signal for sexual communication in mimetic butterflies like Heliconius. Changes in chemically mediated signals, such as pheromones or cuticular hydrocarbons, are thought to be important in mate selection in other butterflies (**22**). Divergence in these molecules may similarly underlie the strong differences in mate preference we observe in erato and himera and this pair will provide a tractable model for future study of the cues responsible for the evolution of mate choice.

Even if change in warning-coloration does not directly cause divergence in mate choice, it is probably a very important component of speciation in this pair. This is because mating preferences alone cannot fully explain the continued maintenance of genetic differences between erato and himera in areas of overlap. Ten percent hybridization per generation would quickly erode the distinctness of these two species in areas of contact unless hybrids were eliminated from the population. Strong frequency-dependent selection by predators ($s \approx 0.5$) removes rare color pattern phenotypes in inter-racial hybrid zones of H. erato (**13**), and is probably important in the erato/himera hybrid zone as well. Where assortative mating limits hybrid numbers, as in this case, strong frequency-dependent selection becomes an even more potent source of "post-mating isolation". Additionally, the transition from erato to himera occurs in the boundary between wet and dry forests and hybrids may be

poorly adapted to either biotope. The very narrow transition (≈ 5 km) between species, is further evidence for extremely strong selection coefficients (s ≈ 1.0) (**16**).

Thus, the transition from a race to a species in these Heliconius appears to have been catalyzed by the association of strong mating preferences with divergence in warning color and ecology. Divergence in mate preference in this pair probably evolved as a by-product of ecological or sexual selection rather than through direct selection against hybridization (i.e. reinforcement, sensu Dobzhansky, **23**). There are at least three reasons to doubt reinforcement in this case. First, many strongly selected hybrid zones between races of H. erato are stable over thousands of generations but have not resulted in assortative mating (**11**). Second, the two species hybridize over only a very narrow region relative to the total geographic range of each species, a situation that makes reinforcement theoretically difficult (**24,** but see also **25**); and lastly, in most of our experiments strong mate preference occurred between erato collected > 25 km, and himera collected > 60 km from the hybrid zone.

Taxa which have evolved the ability to remain distinct in the face of hybridization offer ideal systems in which to identify proximal causes of speciation. Interspecific hybridization of this kind is common in taxa as diverse as butterflies, birds, and coral reef fishes (**26-28**). Whether divergence in mate choice and ecology precedes the evolution of sterility and inviability, as in this case, needs to be tested in many more pairs of incipient species. However, a growing number of studies demonstrate that genomic incompatibilities leading to hybrid sterility or inviability have been relatively unimportant in the early stages of speciation (**29-34**). Rapid divergence of mate preference through sexual selection (**35**) may account for the extraordinary diversity of Hawaiian Drosophila (**29**) and may have driven the divergence in pollinators in monkeyflowers (**30**). In other groups, ecological divergence also seems to be necessary for the maintenance of genetic distinctiveness in sympatry (**31, 32**). Indeed, long term study of Darwin's finches underscores the importance of adaptation in speciation. Despite strong mating preferences, when disruptive ecological selection was relaxed because of climatic changes following the 1983/84 El Niño, hybrids of three species of finches (Geospiza fortis, G. scandens, and G. fuliginosa) increased in abundance leading to an erosion of morphological distinctions (**36**). Even in Drosophila, where hybrid inviability and sterility seem to evolve rapidly, strong mating discrimination has preceded any genomic incompatibility (I=0) in at least 7 out of 17 closely-related sympatric pairs (Nei's D < 0.3) (**1**).

The idea that the evolution of hybrid sterility and inviability is important in speciation dates to the very beginning of the modern synthesis (**37, 38**) and has become entrenched in theories of speciation largely because of the wide spread application of the biological species concept (**38**). Our findings, together with other recent studies, however, challenge the generalization that genomic incompatibilities are important causes of speciation. Inviability and sterility may be the eventual result of cladogenesis (**39**), but their relevance as initiators of speciation may have been overemphasized.

References

1. Coyne, J. A. & Orr, H. A. (1989) Evolution **43**, 362-381.
2. Kaneshiro, K. Y. & Boake, C. R. B. (1987) Trends Ecol. Evol. **2**, 207-212.
3. Etges, W. J. (1992) Evolution **46**, 1945-1950.
4. Barton, N. H. & Hewitt, G. M. (1985) Ann. Rev. Ecol. Syst. **16**, 113-148.
5. Barton, N. H. & Hewitt, G. M. (1989) Nature **341**, 497-503.
6. Harrison, R. G. (1990) in Oxford Survey of Evolutionary Biology, Vol. 7, eds. Futuyma, D. & Antonovics, J. (Oxford University Press, Oxford), pp. 69-128.
7. Bates, H. W. (1862) Trans. Linn. Soc. Lond. **23**, 495-566.
8. Brown, K. S., Sheppard, P. M., & Turner, J. R. G. (1974) Proc. Roy. Soc. Lond. (B) **187**, 369-378.
9. Turner, J. R. G. (1981) Ann. Rev. Ecol. Syst. **12**, 99-121.
10. P. M. Sheppard, Turner, J. R. G., Brown, K. S., Benson, W. W. & Singer, M. C. (1985) Phil. Trans. Roy. Soc., Lon (B) **308**, 433-613.
11. Turner, J. R. G. (1971) Evolution **25**, 471-482.
12. Mallet, J. & Barton, N .H. (1989) Evolution **43**, 421-431.
13. Mallet, J. (1993) in Hybrid Zones and the Evolutionary Process, ed. Harrison, R. G. (Oxford University Press, New York), pp. 226-260.
14. Turner, J. R. G., Johnson, M. S. & Eanes, W. F. (1979) Proc. Natl. Acad. Sci., USA **76**, 1924-1928.
15. Brower, A. V. Z. (1994) Proc. Natl. Acad. Sci., USA **91**, 6491-6495.
16. Jiggins, C., McMillan, W. O., Neukirchen, W. & Mallet, J. (1996) Biol. J. Linn. Soc., 59:221-242.
17. Emsley, M. G. (1965) Zoologica, N.Y. **50**, 191-254.
18. Edwards, A. W. F. (1972) Likelihood (Cambridge University Press, Cambridge).
19. Mallet, J. (1986) Oecologia **68**, 210-217.
20. Gilbert, L. E. (1972) Proc. Natl. Acad. Sci., USA **69**, 1403-1407.
21. Mallet, J., Barton, N., Lamas, G., Santisteban, J., Muedas, M. & Eeley, H. (1990) Genetics **124**, 921-936.
22. Vane-Wright, R. I. & Boppré, M. (1993) Phil. Trans. Roy. Soc., Lon (B) **340**, 197-205.
23. Dobzhansky, T. (1940) Am. Nat. **74**, 312-321.
24. Butlin, R. (1989) in Speciation and Its Consequences, eds. Otte, D. & J. A. Endler (Sinauer Associates, Sunderland, MA), pp. 158-179.

25. Kelly, J. K. & Noor, M. A. F. (1996) Genetics **143**, 1485-1497.
26. Grant, P. R. & B. R. Grant (1992) Science **256**, 193-197.
27. Pyle, R. L. & Randel, J. E. (1994) Environ. Biol. Fishes **41**, 127-145.
28. Guillaumin, M. & Descimon, H. (1976) in Les Problèmes de l'Espèce dans le Regne Animal, Vol. 1, eds. Bocquet, C., Génermont, J. & Lamotte, M. (Société Zoologique de France, Paris), pp. 129-201.
29. Hoy, R. R., Hoikkala, A. & Kaneshiro, K. (1988) Science **240**, 217-219.
30. Bradshaw, H. D., Wilbert, S. M., Otto, S. G., & Schemske, D. W. (1995) Nature **376**, 762-765.
31. Schluter, D. (1994) Science **266**, 798-801.
32. Grant, B. R. & Grant, P. R. (1987) Biol. J. Linn. Soc. **32**, pp.247-270.
33. Feder, J. L., Opp, S. B., Wlazlo, B., Reynolds, K., Go, W. & Spisak, S. (1994) Proc. Natl. Acad. Sci., USA **91**, 7990-7994.
34. Bush, G. L. (1994) Trends Ecol. Evol. **9**, 285-288.
35. Andersson, M. (1994) Sexual Selection (Princeton University Press, Princeton).
36. Grant, B. R. & Grant, P. R. (1996) Ecology **77**, 500-509.
37. Dobzhansky, T. (1937) Genetics and the Origin of Species (Columbia University Press, New York).
38. Mayr, E. (1942) Systematics and the Origin of Species (Columbia University Press, New York).
39. Turelli, M. & Orr, H. A. (1995) Genetics **140**, 389-402.

Acknowledgments

We thank the government of Ecuador, INEFAN, Museo Ecuatoriano de Ciencias Naturales, Peter Wilson, and Fundación Arcoiris. Help in raising butterflies was provided by Angus Davison, Ashleigh Griffin, José Carpio Ayala, Carlos Roberto Carpio Ayala, María del Carmen Cabrera, María Soledad Arias Díaz. We thank Jeff Feder, Kevin Fowler, and Andrew Pomiankowski for comments on earlier drafts. Funded by BBSRC, UK.

Table 1: Observed and expected number of matings between pure and F1 individuals in single-male (A) and multiple-male experiments (B). Expected numbers of matings (in parentheses) are based on the relative mating probabilities given in Table 2. H represents a H. himera individual and E represents a H. erato cyrbia individual. The female parent of F1 individuals is given first.

A: Single-male experiments

	males used			
Female choices	E	EH	HE	H
E	20 (19.2)	3 (3.0)	4 (4.0)	4 (2.4)
H	1 (1.8)	7 (7.0)	4 (4.0)	17 (18.6)
E	13 (9.0)	1 (2.4)	1 (1.5)	----
EH	5 (9.0)	3 (1.6)	2 (1.5)	----
E	2 (2.5)	3 (0.9)	2 (1.5)	----
HE	3 (2.5)	0 (2.1)	1 (1.5)	----
H	----	13 (11.6)	0 (1.5)	10 (8.0)
EH	----	2 (3.4)	3 (1.5)	6 (8.0)
H	----	3 (2.5)	2 (1.5)	2 (1.0)
HE	----	2 (2.5)	1 (1.5)	0 (1.0)

B: Multiple male experiments

	males used			
Females	E	EH	HE	H
E	11 (10.6)	2 (2.7)	6 (4.3)	0 (1.4)
H	1 (1.0)	4 (6.9)	7 (4.1)	11 (11.0)

Table 2: Estimates (with support limits) of the relative mating probabilities (P_{IxJ}) between erato, himera and F1 individuals. By convention, all probabilities were estimated relative to the probability of ExE matings, which was set to 1. To evaluate which P_{IxJ}'s were informative, they were first ranked in order of increasing information (i.e. $\Delta \log_e L$). Each P_{IxJ} was then set to 1 in turn in order of increasing rank, after which the likelihood was re-maximized around the remaining parameters. P_{IxJ} parameters that, when set to 1, decreased the maximum log likelihood less than two units were judged non-informative, and were set permanently to 1. The final reduced model with 4 mating parameters was justified because it resulted in only a small likelihood change from the complete 15-parameter model (G= 18.66, df= 11, p> 0.05).

	males			
Females	E	EH	HE	H
E	[1]	0.43 (0.17, 0.95)	1	0.13 (0.04, 0.33)
EH	1	0.28 (0.09, 0.80)	1	1
HE	1	1	1	1
H	0.09 (0.02, 0.25)	1	1	1

Table 3: Summary of measures of viability of pure and hybrid crosses. The female parent of a cross is given first. Averages and 95% confidence limits of number of eggs/day, egg hatch, larval survival, developmental time, and sex ratio are taken over all hybrid and control crosses in each category. The two different F1 crosses (HE and EH) were examine separately. Similarly, backcross broods (BC1) were grouped into crosses between F1's and eratos [BC1(E)] or between F1's and himeras [BC1(H)]. All BC2 (crosses between BC1 and pure individuals) and F3+ (crosses between F2's and pure types) broods were evaluated together. Egg hatch and larval survival for all individual crosses is presented in Figure 1.

Cross type (n)	Eggs/ brood (S.D)	Eggs/day*	% Eggs hatching	% larval survival	Development time (days)†	Sex ratio (female/total)
HH (14)	61 (26.0)	2.6 ± 0.8	92.7 ± 3.3	66.7 ± 8.1	28.5 ± 0.9	0.49 ± 0.05
EE (12)	38 (22.1)	1.9 ± 0.4	90.3 ± 5.5	57.5 ± 12.3	29.8 ± 1.0	0.48 ± 0.06
EH (12)	46 (23.6)	1.8 ± 0.6	83.7 ± 9.8	64.1 ± 9.8	29.3 ± 0.8	0.48 ± 0.06
HE (8)	55 (35.0)	2.4 ± 1.2	89.2 ± 4.7	53.8 ± 12.7	29.2 ± 1.1	0.45 ± 0.06
BC1(E) (7)	70 (19.7)	2.8 ± 1.2	87.3 ± 9.0	67.8 ± 7.8	30.0 ± 1.0	0.51 ± 0.06
BC1(H) (10)	41 (23.1)	2.2 ± 1.2	90.0 ± 7.1	62.3 ± 4.7	28.9 ± 1.2	0.53 ± 0.07
BC2 (16)	44 (24.2)	2.0 ± 0.7	85.7 ± 12.3	53.8 ± 14.9	28.1 ± 0.6	0.44 ± 0.06
F2 (7)	40 (16.3)	1.5 ± 0.7	92.0 ± 9.6	84.5 ± 6.7	30.2 ± 2.0	0.51 ± 0.08
F3+ (6)	46 (15.4)	2.5 ± 1.2	99.3 ± 1.8	83.4 ± 10.0	28.1 ± 0.7	0.50 ± 0.08

* In comparisons of hybrids and control broods reared together, only F2 crosses laid significantly fewer eggs/day relative to erato and himera ($t = 2.547$, $p = 0.04$, $df = 6$).

† Egg-adult developmental time for each cross was estimated using the mean developmental time of five male and five female butterflies.

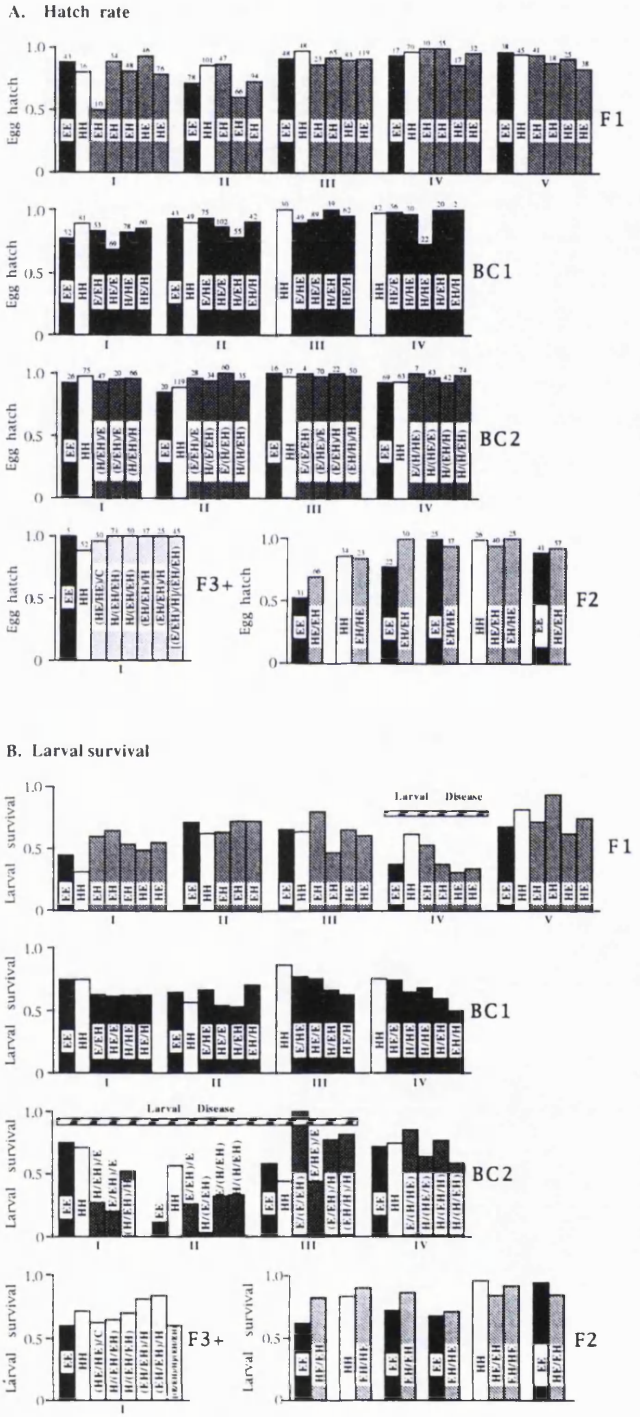


Figure 1: Proportion of egg-hatch (A) and larval survival (B) for hybrid and pure matings. Cross type is given within each bar. Hybrid individuals are designated by a combination of letters, where the female parent of each cross is listed first. The number of eggs laid per female is listed above the proportion hatching bar in A. Individuals raised together are designated by roman numerals.

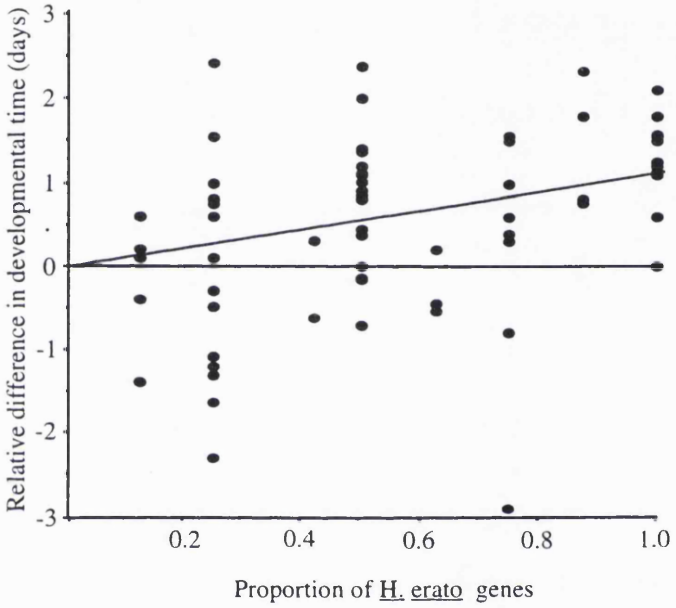


Figure 2: Excess larval developmental time (in days) as a function of the proportion of erato genes. Each point represents the mean egg-to-adult developmental time of 5 females and 5 males from a single cross. Differences in mean developmental time were calculated relative to the developmental time of HxH crosses reared at the same time. The regression line was calculated forcing the y-intercept through the origin.