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

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Heliconius charithonia is a widespread species which, unlike many *Heliconius*, is non-mimetic and shows little racial differentiation. Only one form, '*peruvianus*', which occurs in the dry forest habitats of western Ecuador and Peru, has a distinct and clearly mimetic colour pattern. Here it was shown that *H. peruvianus* was distinct from *H. charithonia bassleri* at allozyme loci (D = 0.25 over 22 loci). This differentiation was ten times greater than that between *H. charithonia* sampled from Ecuador and the Caribbean (D = 0.027) and was consistent with analysis of mitochondrial sequence data (3.4–4% sequence divergence between *H. peruvianus* and *H. charithonia*). One individual with a *H. peruvianus* colour pattern and allozyme genotype was collected in an area where *H. charithonia* was known to be common, demonstrating that contact between the taxa occurs in western Ecuador. Furthermore, the allozyme genotype of another individual was heterozygous for four of five diagnostic loci and was most likely an F1 hybrid between *H. charithonia* and *H. peruvianus*. These data imply that *H. charithonia* and *H. peruvianus* are distinct species which hybridize occasionally. 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.

ADDITIONAL KEY WORDS:—allopatry – butterfly – Ecuador – hybridization – hybrid zone – parapatry – Peru – speciation.

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INTRODUCTION

Neotropical dry forests are exceptionally rich in locally endemic species. However, the processes underlying this diversity 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. First, allopatric isolation in Pleistocene forest refuges (Haffer, 1969), and second, 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' predicts concordant genetic divergence between groups of recently evolved species and races, indicating vicariance. The 'parapatric adaptation hypothesis' on the other hand predicts 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 we present a comparative study of another species *Heliconius charithonia* (Linnaeus, 1767; Brower, 1994a). *H. charithonia* is not involved in Müllerian 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. charithonia bassleri* is replaced by the form *peruvianus* 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 (Brown & Comstock, 1952). The difference is adaptive, as *peruvianus* is a mimic of the ithomiine species, *Elzunia pavonii* (Butler) which is also a dry forest endemic (Poulton, 1931).

H. peruvianus was originally described as a separate species by Felder & Felder (1859), but subsequent authors have considered it a race of *H. charithonia* on the basis of genitalic characters (Eltringham, 1916; Comstock & Brown, 1950; Emsley, 1965). Parapatric sister taxa such as these provide an opportunity to investigate the early stages of speciation. Here patterns of distribution, host plant utilization, protein and mtDNA variation are examined to assess differentiation between *H. charithonia* and *H. peruvianus*. These results imply that *H. charithonia* and *H. peruvianus* are genetically isolated and should be considered distinct species.

METHODS

Collections were made in Ecuador at various times between 1993 and 1996 (CJ) and in the Caribbean during 1992 and 1993 (ND). For genetic analysis a total of

50 individuals were collected and frozen in liquid nitrogen 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 *H. peruvianus* correspond to those of Jiggins *et al.* (1996). Collections of *H. charithonia* were made in the Río Toachi valley, Prov. Pichincha. The Caribbean individuals (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 homogenized on ice in 80 µl 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, Mallet & Baltensweiler, 1995). Analysis of genetic distance was performed using BIOSYS (Swofford & 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 (Table 1). 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 *H. peruvianus* and *H. charithonia* were used for this analysis.

Passifloraceae are the sole host plants for *Heliconius* (Brown, 1981). To determine which hosts are utilized, 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 *H. peruvianus* and *H. charithonia* are associated with a concordant genetic break. Differentiation between *H. peruvianus* and *H. charithonia* over 22 loci (Nei's $D = 0.250$) was almost ten times more than that between samples of *H. charithonia* 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 1500 bp region spanning the COI and COII genes (Davies & Bermingham, in prep.). This also shows that *H. peruvianus* is distinct from *H. charithonia* (3.4–4% sequence divergence), whilst *H. charithonia* 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 *H. peruvianus* and *H. charithonia* is far greater than that expected through isolation by distance. The maximal genetic distance

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

	charithonia		*peruvianus*
	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

continued

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

	charithonia		*peruvianus*
	Ecuador	Caribbean	Ecuador
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

continued

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

	charithonia		*peruvianus*
	Ecuador	Caribbean	Ecuador
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

between populations of *H. charithonia* sampled from different Caribbean 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 400 km apart (Jiggins *et al.*, 1997).

When the level of differentiation is compared with a survey of allozyme genetic distances for pairwise comparisons of Lepidoptera species and populations, the distance between *H. charithonia* and *H. peruvianus* is well within the range of differentiation found between species and greater than virtually all the conspecific comparisons (Emelianov *et al.*, 1995). Furthermore, Turner, Johnson & Eanes (1979) studied allozyme differentiation among a number of *Heliconius* species and showed the probability of genetic identity varied from 0.49 to 0.87. The evidence suggests that the genetic break between *H. charithonia* and *H. peruvianus* (Nei's $D = 0.250$) is greater than would be expected solely as a result of isolation by distance.

The distribution of H. peruvianus *and* H. charithonia

Sheppard *et al.* (1985) provide anecdotal evidence that *peruvianus* and *charithonia* are sympatric in the Marañon valley (north east Peru). Here the first evidence for contact between *H. peruvianus* and *H. charithonia* west of the Andes is reported. A single specimen with a *H. peruvianus* wing pattern and genotype was collected at Alluriquín, in the Río Toachi valley (780 m), flying alongside an individual of *H. charithonia* (Table 2; Fig. 1). Unfortunately no more individuals were collected at this site. In the same valley *H. charithonia* is found at 1700 m (Hacienda Hesperia) and 750 m (Tinalandia; Willmott, pers. comm.). No other individuals of *H. peruvianus* are known from the area, which suggests that this individual may be a migrant from populations in the dry coastal forest 50 km to the west.

There is some evidence for hybridization between *H. peruvianus* and *H. charithonia* from the same valley. One individual with a *H. charithonia* wing pattern, collected at 1700 m, has a genotype which is consistent with a *H. charithonia x H. peruvianus* hybrid at four of five diagnostic loci (Table 2). This genotype is more likely to be

TABLE 2. Sample genotypes. The loci shown are those with >0.5 frequency difference between the most common allele in *peruvianus* and *charithonia*. 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 *charithonia* ($\Delta\log L = 5.09$, $P<0.05$) or *peruvianus* ($\Delta\log L = 12.16$, $P<0.05$). The *peruvianus* individual caught in sympatry with *charithonia* is most likely derived from the *peruvianus* as opposed to the local *charithonia* population ($\Delta\log L = 28.56$, $P<0.05$)

		Population allele frequencies		Single individual genotypes		
		Ecuador (allopatric)	Ecuador (allopatric)	Hybrid	Ecuador (sympatric)	
Phenotype		*charithonia*	*peruvianus*	*charithonia*	*charithonia*	*peruvianus*
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			

an F1 than a *H. charithonia* ($\Delta\log L = 5.09$, $P<0.05$) or *H. peruvianus* ($\Delta\log L = 12.16$, $P<0.05$).

It seems likely therefore that *H. peruvianus* and *H. charithonia* are largely allopatric but do meet and hybridize 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 *H. charithonia* and *H. peruvianus* (less than 50 km in western Ecuador), and the observed overlap (one *H. peruvianus* individual collected in a sample of 22 *H. charithonia*), suggests that the migration rate very likely 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 *H. charithonia* and *H. peruvianus*. *H. charithonia* and *H. peruvianus* should be considered distinct species.

Larval host plants

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

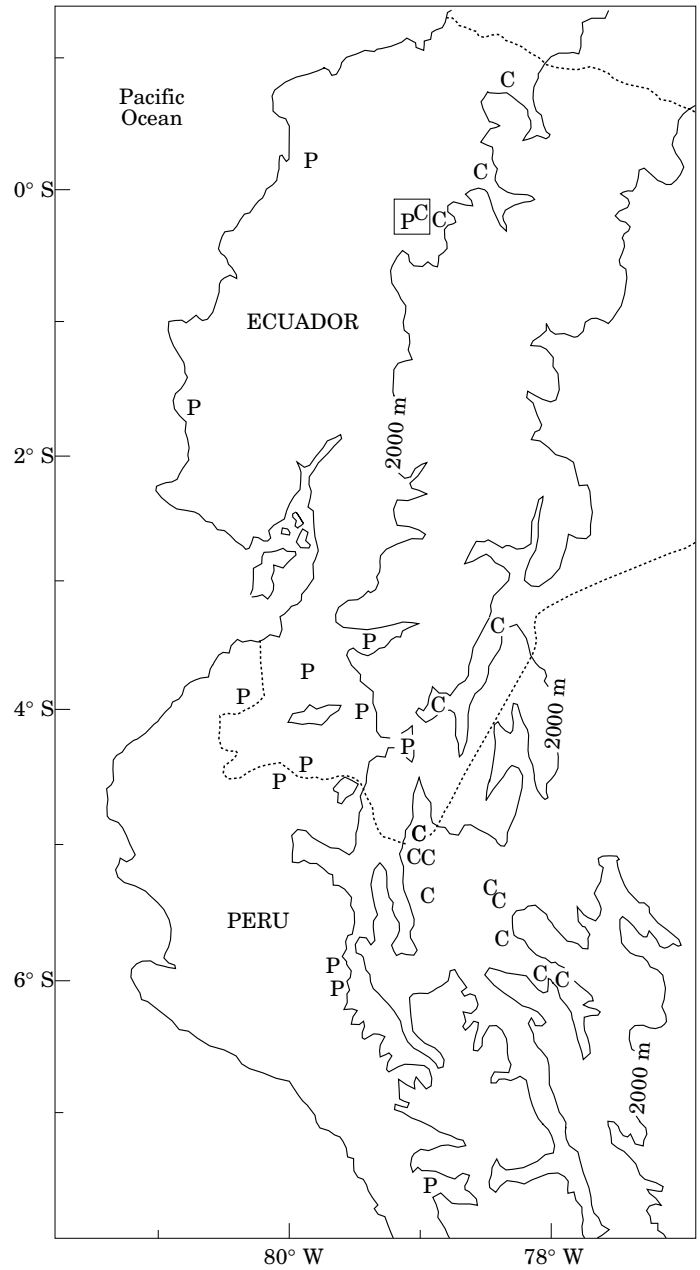

Figure 1 Distribution of *H. charithonia* (c) and *H. peruvianus* (p) in Peru and Ecuador. The box shows Alluriquín where both species were found in sympatry. *H. peruvianus* previously extended as far south as Lima (12°S) but is now considered extinct there (Lamas, 1975).

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 *H. peruvianus* feeds on all the previously recorded larval foodplants of *H. charithonia* encountered in Ecuador (Table 3).

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, Alluriquín (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

	charithonia	*peruvianus*
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	—

Biogeography and speciation

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 *H. peruvianus*, 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 *H. himera* and *H. erato* (Jiggins *et al.*, 1997) and $D = 0.25$ between *H. peruvianus* and *H. charithonia*. In contrast mtDNA sequence divergence in the COI and COII genes is somewhat larger between *H. peruvianus* and *H. charithonia* (3.4–4%) than between *H. himera* and *H. erato* (1.5–2%) (Brower, 1994b; 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, 1994b, 1996). Further evidence would be needed from other species pairs before this could be taken as good evidence for a shared vicariance event. However, vicariance is suggested by another recent speciation event in the *H. charithonia* 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 *peruvianus* are always associated with dry forest habitats, suggesting that ecology is a primary cause of the observed distribution patterns. However contact zones between *himera/erato*, and *charithonia/peruvianus* are not concordant. To the west of the Andes in Ecuador, *H. peruvianus* occurs 450 km further north than *H. himera* in dry coastal forest (Fig. 1), whilst to the east of the Andes, in the Marañon valley, contact between *H. charithonia* and *H. peruvianus* occurs higher up the valley than that between *H. erato* and *H. himera* (Sheppard *et al.*, 1985; Mallet, 1993). These distinct local distribution patterns might be caused in part by known ecological differences between *H. erato* and *H. charithonia*. *H. charithonia* relies on colonising temporarily available habitat, such as seasonally dry cloud forests (Gilbert, 1991), whilst *H. erato* is common in more permanently suitable areas such as lowland wet forest. Despite the differences in the distribution patterns of *himera/erato* and *charithonia/peruvianus*, it remains true that in both cases the species boundary is associated with a change from humid to dry forest habitat.

In both the *himera/erato*, and *charithonia/peruvianus* species pairs, the evolution of

barriers to gene flow is associated with a shift in habitat and mimetic pattern. This suggests an adaptationist model of speciation. However there is also limited evidence for a shared vicariance event. 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.

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