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Genetic analysis of a wild-caught hybrid between non-sister *Heliconius* butterfly species

Kanchon K. Dasmahapatra*,
Armando Silva-Vásquez, Jae-Woo Chung
and James Mallet

Galton Laboratory, Department of Biology, University College London, London NW1 2HE, UK
**Author for correspondence* (*k.dasmahapatra@ucl.ac.uk*).

Interspecific hybridization occurs regularly in wild *Heliconius* butterflies, although hybrid individuals are usually very rare. However, hybridization generally occurs only between the most closely related species. We report a rare naturally occurring hybrid between non-sister species and carry out the first genetic analysis of such distant hybridization. Mitochondrial and nuclear genes indicate that the specimen is an F_1 hybrid between a female *Heliconius ethilla* and a male *Heliconius melpomene*, originating from a group of 13 species estimated to have diverged over 2.5 Myr ago. The presence of such distant natural hybrids, together with evidence for backcrossing, suggests that gene flow across species boundaries can take place long after speciation. Adaptive genes such as those involved in wing coloration could thus be widely shared among members of this highly mimetic genus.

Keywords: *Heliconius ethilla*; *Heliconius melpomene*; hybridization

1. INTRODUCTION

Evolutionary biologists generally accept the biological species concept, and this has led to hybridization between species being regarded as uncommon and unimportant (Mayr 1963). Although hybrids are, almost by definition, very rare on a per individual basis, recent surveys have shown that many species do in fact hybridize (Coyne & Orr 2004; Mallet 2005). On average, 10% of animal species and 25% of plant species are known to hybridize in the wild, and in some particularly charismatic groups, such as ducks, birds of paradise and North American *Papilio* butterflies, 40–75% of species are known to hybridize (Mallet 2005).

Many wild-caught interspecific hybrid specimens between *Heliconiina* butterflies are known. Hybrids occur in 35% of the 46 species in the genus *Heliconius* and 27% of the 73 species in the larger sub-tribe Heliconiinae (Mallet *et al.* 2007). These butterflies have been well studied owing to their bright wing coloration and extensive variation in wing pattern morphology both between species and among geographical races within species. The bright wing colours act as a warning of their unpalatability to potential predators. Many species share similar patterns with unrelated Heliconiinae and Ithomiinae, leading to impressive Müllerian mimicry rings (Bates 1862; Turner 1981; Beccaloni 1997; Joron & Mallet 1998). Although hybrids are rare within most *Heliconius* populations, the existence of naturally occurring hybrids is intriguing because it suggests that gene flow will be possible between species. Hence, this could lead to the transfer of adaptive genes between species (Gilbert 2003).

Hybridization in *Heliconius* has hitherto been examined genetically only in a few cases that involve closely related species: *Heliconius erato* and *Heliconius himera*, or *Heliconius melpomene* and *Heliconius cydno* (Jiggins *et al.* 1997; Bull *et al.* 2006; Kronforst *et al.* 2006). In all other cases, inferences of hybridization have been made on the basis of phenotypes of wild-caught specimens. In contrast, the extremely rare hybrids between *H. melpomene* and the more distant species in the 'silvaniform' *Heliconius* (Mallet *et al.* 2007) have never been examined genetically. In this paper, we describe a new rare hybrid formed between two distantly related *Heliconius* species in the *H. melpomene* group, recently caught in Peru, and use molecular markers to determine the parental types.

2. MATERIAL AND METHODS

We are engaged in an extensive study of heliconiine and ithomiine butterflies in the Departamento de San Martín, Peru (Mallet & Barton 1989; Joron *et al.* 2001; Whinnett *et al.* 2005). In addition to the pure species, subspecies and many interracial hybrids sampled, we recently caught a peculiar specimen, a putative interspecific hybrid from the *melpomene*–silvaniform group (specimen ID 06-921; table 1) near Moyobamba in Northern Peru. The hybrid specimen will be deposited in the collection at the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru. The remaining specimens analysed (table 1) are of common species, held at University College London and vouchered at UNMSM.

DNA was extracted from a single leg of the hybrid specimen using a QIAamp DNA Micro Kit (QIAGEN), and from one-third of the thorax of all other specimens using the DNeasy Blood and Tissue Kit (QIAGEN). Approximately 2200 bp of mtDNA comprising *CoI* and 5′ end of *CoII* genes were amplified by PCR in three sections for the hybrid individual, representatives of *Heliconius melpomene amaryllis*, *Heliconius elevatus* and each of the four sympatric silvaniform species: *Heliconius numata*; *Heliconius ethilla*; *Heliconius pardalinus*; and *Heliconius hecale*. In addition, five nuclear loci (*Tektin*, *Rpl5*, *Tpi*, *Mpi* and *inv*) were amplified in the hybrid, representatives of *H. melpomene* and *H. ethilla*. PCR products were cleaned and cycle sequenced with the PCR primers using the BIG DYE TERMINATOR v. 3.1 Cycle Sequencing Kit (Applied Biosystems). Table S1 of the electronic supplementary material contains details of the primers used, PCR conditions and sequence accession numbers.

Apart from mtDNA and *Tektin*, all other loci spanned one or more intron regions. Indels in the intronic regions often resulted in the amplification of alleles with different sizes from a single individual. Unless sequence quality was low, the indel could be readily identified and the two alleles deconvoluted using information from the double-peak signals following the indel (Flot *et al.* 2006). See the electronic supplementary material for details of this procedure.

3. RESULTS

The putative hybrid (figure 1) is a male, and its wing pattern is unlike that of other local *melpomene*–silvaniform group species. Instead, the markings appear to combine those of *H. m. amaryllis* (the local 'postman'-patterned *melpomene* race) and of a local

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Table 1. Details of samples and collection localities.

specimen ID	species	collection locality	latitude	longitude
06-921	*Heliconius* hybrid	Rumiyacu, near Moyobamba	06°05′23″ S	076°58′09″ W
04-286	*Heliconius melpomene*	Bosque von Humboldt	08°49′48″ S	075°03′28″ W
04-288	*Heliconius melpomene*	Bosque von Humboldt	08°49′48″ S	075°03′28″ W
02-366	*Heliconius melpomene*	Davidcillo	06°14′47″ S	076°15′58″ W
02-944	*Heliconius melpomene*	Puente Serranayacu	05°40′48″ S	077°40′50″ W
02-1839	*Heliconius melpomene*	Boca Toma Río Shilcayo	06°27′20″ S	076°20’40″ W
02-1850	*Heliconius melpomene*	Shapaja	06°34’29″ S	079°16’48″ W
02-1882	*Heliconius melpomene*	Chumia	06°36’57″ S	076°11’07″ W
02-1894	*Heliconius melpomene*	km 30, Tarapoto-Yurimaguas	06°24’33″ S	076°18’24″ W
02-2060	*Heliconius melpomene*	km 26, Yurimaguas-Tarapoto	05°58’30″ S	076°14’15″ W
02-3	*Heliconius ethilla*	Boca Toma Río Shilcayo	06°27’20″ S	076°20’40″ W
02-975	*Heliconius ethilla*	km 10, Tarapoto-Yurimaguas	06°27’18″ S	076°17’46″ W
02-1483	*Heliconius ethilla*	La Antena, km 16, Tarapoto-Yurimaguas	06°27’19″ S	076°17’54″ W
02-2037	*Heliconius numata*	km 26, Yurimaguas-Tarapoto	05°58’30″ S	076°14’15″ W
02-364	*Heliconius elevatus*	Davidcillo	06°14’47″ S	076°15’58″ W
05-1196	*Heliconius pardalinus*	Urahuasha	06°27’43″ S	076°19’36″ W
02-1330	*Heliconius hecale*	km 7.2, Pongo-Barranquita	06°17’41″ S	076°13’53″ W

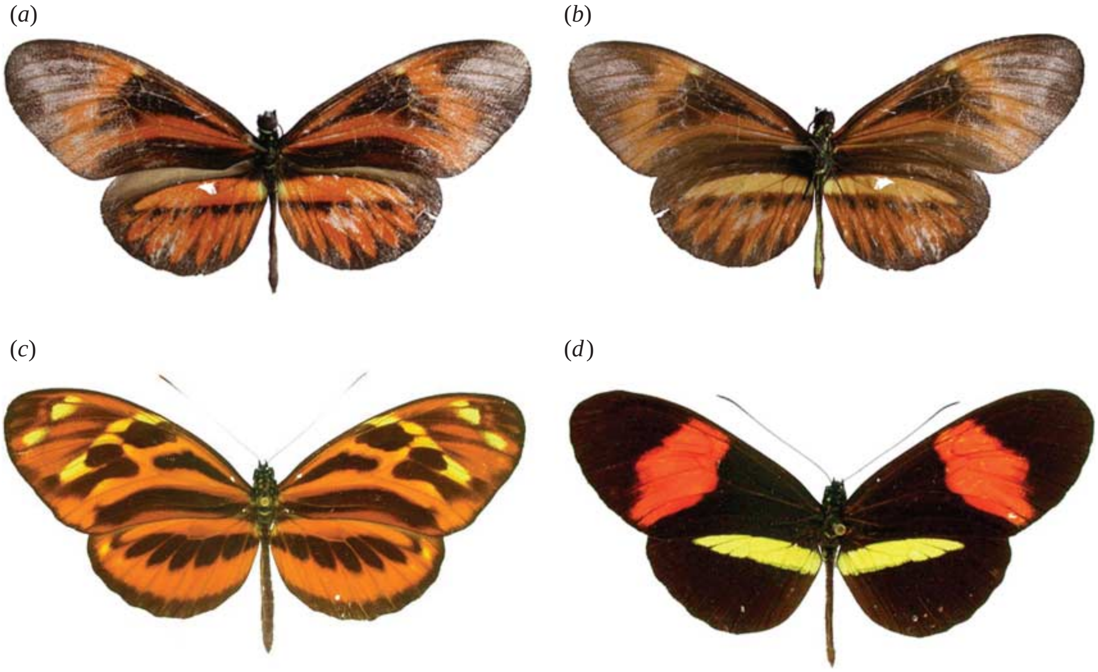
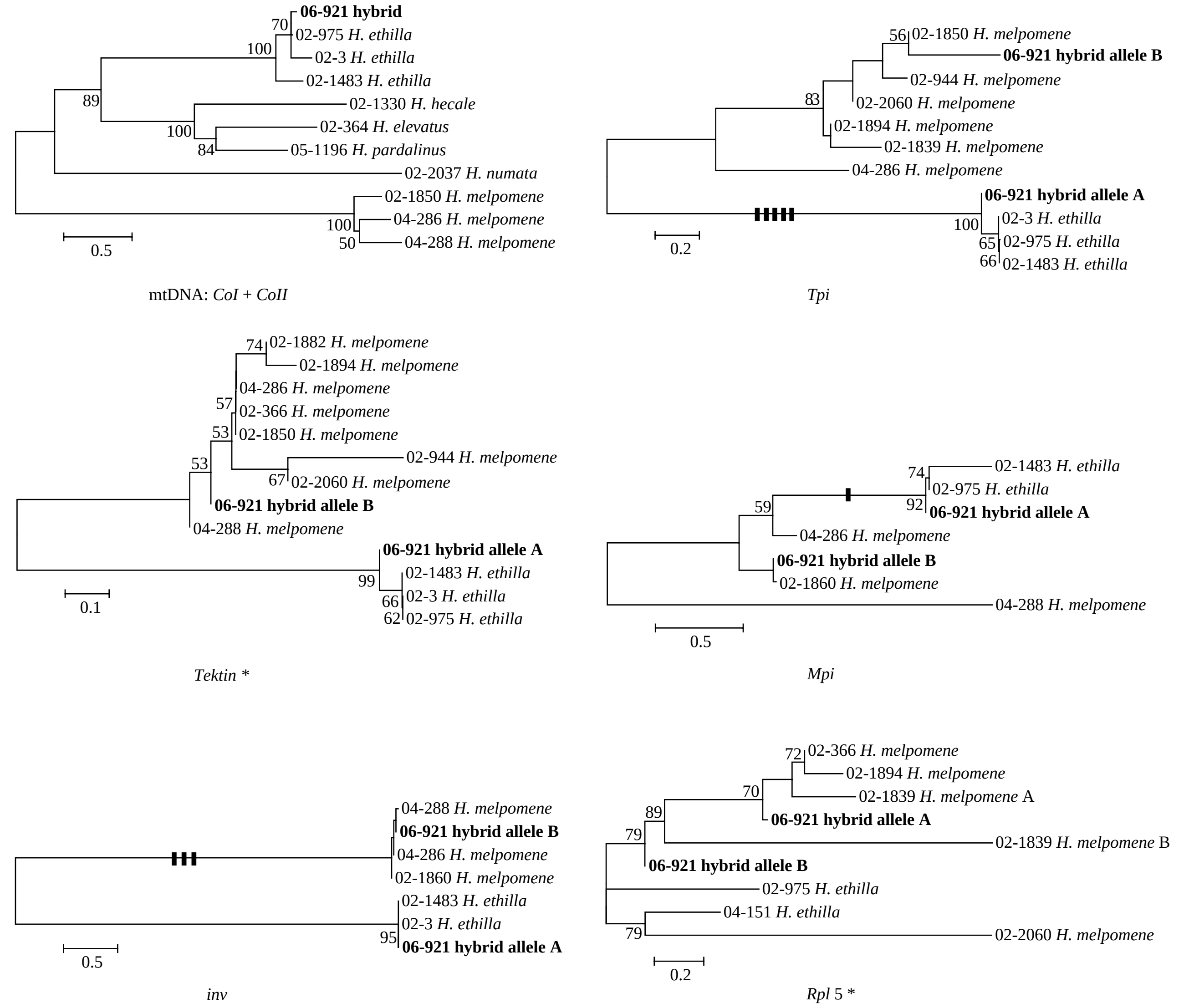


Figure 1. (*a*) Dorsal and (*b*) ventral wing colour patterns of the hybrid specimen 06-921. Dorsal wing colour patterns of the putative parent species (*c*) *H. ethilla aerotome* and (*d*) *H. melpomene amaryllis*.

silvaniform species: *H. numata*; *ethilla*; *hecale*; or *pardalinus*. Compared with *H. m. amaryllis*, the forewing crimson colours have become burnt orange and these orange markings extend over both fore- and hindwings, rather than being restricted to a forewing bar. The yellow hindwing bar of the latter species has also become orange on the upperside, although the yellow is almost fully expressed on the underside. Compared with *Heliconius ethilla aerotome* (identified as the other parent, see below), the orange markings are much reduced, and on the hindwings narrowed into 'rays' reminiscent of those found in races of *H. melpomene* such as *Heliconius melpomene aglaope*, which occurs over the mountain range in the Amazonian lowlands to the northeast of the capture site. The spotty melanic markings of *H. ethilla* are also broadened in the putative hybrid to form black smears, particularly in the central part of the forewing. Apart from the underside-expressed yellow hindwing bar, the underside and upperside patterns are similar. This hybrid is similar to a specimen in the Natural History Museum, London, originally named as a separate species '*Heliconius hippola*' Hewitson (Mallet *et al.* 2007).

A BLAST search of the mtDNA sequence revealed 99.5% similarity to *H. ethilla*. Subsequent comparison with mtDNA sequences obtained from locally caught specimens of the four potential silvaniform species showed unambiguously that the hybrid possessed a *H. ethilla* mitochondrial sequence (figure 2; table S2, electronic supplementary material). Diagnostic sequence differences between *H. melpomene* and *H. ethilla* were not



mtDNA: *CoI* + *CoII* *Tpi*

Tektin * *Mpi*

inv *Rpl* 5 *

Figure 2. Neighbour-joining trees for six loci showing the relationship between alleles found in the hybrid and other melpomene–cydno–silvaniform group species. Nodes with 50% or greater bootstrap support are labelled. Bold vertical lines indicate diagnostic indels between *H. melpomene* and *H. ethilla*. Scale bars represent percentage sequence divergence. Asterisks, additional information in the electronic supplementary material.

found at *Rpl5*, but were present at the other four nuclear loci. The hybrid individual was found to possess both a *melpomene*- and an *ethilla*-type allele at each of these four diagnostic nuclear loci (figure 2).

An F_1 hybrid should be heterozygous at all nuclear genes, bearing alleles of each parental type. In contrast, a backcross hybrid should only be heterozygous at half its nuclear genes and homozygous for one parental type over its remaining nuclear genes. As the hybrid is heterozygous at four diagnostic nuclear loci, this indicates that the individual is unlikely to be a backcross or F_2 hybrid (binomial $p=0.5^4=0.0625$); an F_1 hybrid between *H. ethilla* and *H. m. amaryllis* is 16 times more likely than any second-generation hybrid.

4. DISCUSSION

Although interspecific hybridization is a common phenomenon among heliconiine butterflies, at the individual level, hybridization is rare, usually comprising less than 1 in 1000 wild individuals (Mallet *et al.* 2007). Most wild hybrids are either intraspecific (between different wing pattern races) or between closely related, usually sister taxa (Mallet *et al.* 2007). Only 10 putative hybrid specimens have been documented between *melpomene* and silvaniform species, and only four are putatively between *H. melpomene* and *H. ethilla* (Mallet *et al.* 2007). However, no molecular verification of these rare and distant hybrids has hitherto been carried out, so the identity of the parents and whether they are in fact hybrids or aberrations is in doubt (Mallet *et al.* 2007). Here, genetic evidence for natural hybridization between such distant non-sister *Heliconius* species (*H. melpomene* and *H. ethilla*) has been obtained for the first time.

Heliconius ethilla and *H. melpomene* are approximately 5% different at the mtDNA studied. Assuming that this gene evolves in a clock-like manner at 2% Myr^{-1} (Brower 1994), this suggests hybridization events occurring *ca* 2.5 Myr after speciation has occurred. The occurrence of a wild adult F_1 hybrid between these species indicates that such hybrids can develop normally and survive in the wild. Some putative wild hybrids between *melpomene* and silvaniform group butterflies are considered to be backcrosses

(Mallet *et al.* 2007), providing evidence that some such hybrids may be fertile and capable of reproduction. Two of the four putative hybrids between *H. melpomene* and *H. ethilla* known from Colombia are clearly not F_1 hybrids, and are presumably backcrosses to *H. ethilla* (Mallet *et al.* 2007). Although those putative hybrids have not been analysed genetically, the more *ethilla*-like patterns, the strong expression of yellow coloration in the forewing (which is recessive in hybrids) and strongly *ethilla*-like hindwing pattern all provide clear evidence of backcrossing to *ethilla* in nature. The other two hybrids are *H. hippola*-like and are presumably F_1 hybrids (Mallet *et al.* 2007). Backcrosses via male F_1 hybrids in a complex cross involving *H. melpomene* and the silvaniforms *H. hecale* and *H. atthis* (the latter close to *H. ethilla*) have been obtained in captivity, although female hybrids were reported to be sterile (Mallet *et al.* 2007), indicating that such backcrossing is possible. This potential for backcrossing may result in transfer of genes between species.

As hybridization is regular, species boundaries in *Heliconius* have the potential to be porous. Within the *melpomene–cydno* group, hybridization and backcrossing has led to interspecific introgression at some, but not all, genomic regions (Bull *et al.* 2006; Kronforst *et al.* 2006), and has apparently produced at least one hybrid species (Mavárez *et al.* 2006). If extensive hybridization among these two closely related species can cause adaptive genes to introgress as a result of hybridization, rarer hybridization between more distant species, such as between the silvaniform and the *melpomene–cydno* group species, as here, may also play a role. Closely related *Heliconius* species are often members of different colour pattern mimicry rings, and very similar and apparently homoplasious mimetic patterns are often found between related non-sister species; for instance, *H. elevatus* (a silvaniform) shares almost identical ray patterns with some races of *H. melpomene* and *Heliconius timareta*. *Heliconius besckei* (also a silvaniform), in contrast, shares a *melpomene amaryllis*-like postman pattern with races of *H. melpomene* and *H. timareta*. Our data therefore contribute evidence for the intriguing possibility that mimetic wing patterns may be shared via introgression among distant as well as closely related species (Gilbert 2003; Mallet *et al.* 2007).

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