Hybrid speciation in *Heliconius* butterflies? A review and critique of the evidence

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Abstract The evidence supporting the recent hypothesis of a homoploid hybrid origin for the butterfly species *Heliconius heurippa* is evaluated. Data from selective breeding experiments, mate-choice studies, and a wide variety of DNA markers are reviewed, and an alternative hypothesis for the origin of the species and its close relatives is proposed. A scenario of occasional red wing-pattern mutations in peripheral populations of *Heliconius cydno* with subsequent adaptive convergence towards sympatric mimicry rings involving *H. melpomene* and *H. erato* is offered as an alternative to the HHS hypothesis. Recent twists of this tale are addressed in a postscript.

Keywords Homoploid · Introgression · Admixture · Interspecific gene flow · Mimicry · Magic traits · Ecological speciation

The hybrid individuals cannot be distinguished from other individuals of *H. cydno*, indicating that multiple generations of backcrossing must have occurred. (Mavárez et al. 2006: Fig. S4)

If a similar scenario were involved in the origin of *H. heurippa*, then it would be very difficult to detect a significant signal of the hybrid founding event—the genome would primarily be derived from *H. cydno* despite the crucial role of introgression of the

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patterning genes in the formation of the novel lineage. (Jiggins et al. 2008)

One likely possibility is that *timareta* is another form that has stabilized after hybridization between Amazonian rayed *melpomene* and an unidentified, maybe extinct form of *cydno*. (Mallet 2009)

Some genuinely testable theories, when found to be false, are still upheld by their admirers—for example by introducing ad hoc some auxiliary assumption, or by reinterpreting the theory ad hoc in such a way that it escapes refutation. Such a procedure is always possible, but it rescues the theory from refutation only at the price of destroying, or at least lowering, its scientific status. (Popper 1968:37)

Heliconius butterflies (Lepidoptera: Nymphalidae) have long been celebrated for their bold aposematic coloration, Müllerian mimicry and striking intraspecific polymorphism (Bates 1862; Eltringham 1916; Turner 1976; Brown 1979). The diversity of wing color patterns exhibited by the co-mimetic pair *Heliconius erato* (L.) and *Heliconius melpomene* (L.) is unparalleled by any other insects, with at least ten highly distinct phenotypes shared between geographical races of the two species (Sheppard et al. 1985). The genetic architecture underlying this pattern diversity is likewise quite distinct from the norm. In "typical" butterfly wing patterns, the expression of pattern elements is more or less developmentally integrated over the entire wing surface (Monteiro et al. 1994; Beldade and Brakefield 2002) such that allelic variation in shapes, sizes and colors of the wing pattern may be expressed as single allelic "supergene" differences (Clarke and Sheppard 1960). By contrast, in *Heliconius*, different wing pattern elements are controlled by genes on separate chromosomes (Sheppard

et al. 1985; Jiggins et al. 2005; Kapan et al. 2006) and assort independently in experimental crosses, as well as in natural hybrid zones (Sheppard 1963; Turner 1971; Mallet 1993). This diversity of pattern elements under independent genetic control has been referred to as a "toolbox" for the generation of phenotypic diversity (Gilbert 2003).

Müllerian mimicry is an "honest" signal between unpalatable prey and potential predator, and is selectively beneficial to the taxa that employ it because a smaller number of individuals per species must be attacked before a predator learns the aposematic signal and ceases pursuit of insects exhibiting it (Müller 1879). Mimetic aposematism is maintained by positive frequency-dependent selection: the most abundant pattern in a given habitat confers the greatest protection to individuals that display it, and exhibiting a novel phenotype is strongly disadvantageous (Mallet and Barton 1989). The diversity of mimetic forms within and among *Heliconius* species in spite of selection to conform to a single aposematic pattern has thus been viewed as an evolutionary paradox (Ford 1953; Turner 1977; Joron and Mallet 1998), explained variously as a product of vicariance in Pleistocene refugia (Brown et al. 1974), selective divergence across ecotones (Endler 1982; Mallet 1993), or some combination thereof (Turner 1982, 1983).

Hybrid zones both challenge the axiomatic view of an orderly natural hierarchy imposed by taxonomy, and provide a natural laboratory for the study of speciation (Harrison 1990, 1993). For decades, there has been an interest in *Heliconius* hybridization, both to understand the diversity of phenotypes produced in hybrid zones, and to characterize the putative selective forces that constrain the width of hybrid zones between phenotypically divergent yet interfertile geographical races (Descimon and Mast de Maeght 1983; Sheppard et al. 1985; Mallet 1989; Mallet et al. 1990; Jiggins et al. 1997, 2001a; Linares 1997a; Blum 2008). More recently, the focus of hybrid zone research has expanded to explore putative interspecific hybridization events (Naisbit et al. 2001; Gilbert 2003; Salazar et al. 2005; Bull et al. 2006; Kronforst et al. 2006). Although to date there is only a single wild-caught specimen that has been conclusively determined to be of interspecific hybrid origin (Dasmahapatra et al. 2007), Mallet et al. (2007) have argued, based on the occurrence of phenotypically atypical specimens from natural history museums that they infer to be natural interspecific hybrids, that the species boundary in *Heliconius* is a porous continuum, ranging from relatively free hybridization between close relatives, and exhibiting a logarithmic decrease in hybridization events as genetic divergence increases.

Perhaps the most dramatic development in recent times regarding theories of diversification of *Heliconius*

butterflies was the widely-publicized paper by Mavárez et al. (2006; presaged by Salazar et al. 2005), proposing that the origin of the species *Heliconius heurippa* Hewitson stemmed from interspecific hybridization between geographical races of *Heliconius cydno* Doubleday and *Heliconius melpomene*. While self-perpetuating polyploid and/or parthenogenetic lineages may occasionally form as a result of hybridization between diploid sexual species (reviewed in Mallet 2007), these are typically instantly reproductively isolated from the parental lineage(s) by differences in chromosome number that prevent successful meiosis in backcrosses. The production of a reproductively-isolated, sexually reproducing, diploid species from two sexually reproducing parental species (known as homoploid hybrid speciation, HHS) is considered to be improbable, because chromosomal isolating mechanisms are not in place, and the genetic compatibility that allows hybridization between the parental species should not, in theory, promote the establishment of a reproductively isolated, genetically distinct yet sympatric hybrid offspring (Coyne and Orr 2004).

Most of the recently documented putative cases of HHS in animals (e.g., Nolte et al. 2005; Schwarz et al. 2005; Gompert et al. 2006; Kuusela et al. 2007) rely upon the hybrids' occupation of novel habitats where they have neither the opportunity to compete against nor to interbreed with their parental species. By contrast, in the *H. heurippa* case, the putative hybrid offspring species is sympatric with one of its parents, and is hypothesized (according to the Mavárez et al. 2006 model; see also Duenez-Guzman et al. 2009) to have competitively excluded the other. As discussed above, the origin of a novel, phenotypically distinctive hybrid species such as *H. heurippa* arising from parent species belonging to different Müllerian mimetic complexes ought to be further impeded by the hurdle of overcoming the strong positive frequency-dependent selection by predators that maintains homogeneity within and differences between the parental phenotypes (Mallet and Barton 1989). Although several reviews discussing the *H. heurippa* case have already been published (Mallet 2007, 2009; Jiggins 2008; Jiggins et al. 2008; Mavárez and Linares 2008), most advance hybrid speciation of *H. heurippa* as received wisdom or hedge the original hypothesis with modified concepts such as "hybrid trait speciation" or "ecological speciation," and none attempts to critically reevaluate the data purported to support the case. The object of this paper is to review the experimental and genetic evidence from the *H. cydno—H. melpomene* clade supporting the emerging *Heliconius* hybrid speciation paradigm, and to explore alternative hypotheses for the origin of *H. heurippa*.

Systematic background

Historically, members of the geographically variable species *H. cydno* and *H. melpomene* have been viewed as intrinsically partitioned by ecological differences into separate habitats, larval host plants and mimicry complexes (e.g., Smiley 1978; Mallet and Gilbert 1995; Srygley and Ellington 1999). The two species are broadly sympatric in their ranges from southern Mexico to Venezuela and western Ecuador, and each is involved in Müllerian mimicry with one or more members of the *H. erato-H. sara* "pupal mating" clade (Brown 1981; see Fig. 1). *H. cydno* is a comimic of black and white or black and yellow forms of *H. sapho* (Drury) and *H. eleuchia* Hewitson, while *H. melpomene* participates in the famous geographically diverse red, yellow and black association with *H. erato* (Eltringham 1916). There is one notable exception to this pattern: in the upper Cauca Valley, Colombia, *H. melpomene*, *H. sapho* and *H. eleuchia* are absent, and *H. erato chestertonii* Hewitson lacks a red forewing band and mimics *H. cydno weymeri* Staudinger form "*gustavi*,"[1] both species exhibiting a satiny bluish black ground coloration with a yellow transverse hindwing stripe.

Although *H. cydno* and *H. melpomene* have long been considered to be closely-related (e.g., Stichel and Riffarth 1905), Brower (1996a) presented the first empirically-supported cladogram examining relationships among geographical races of *H. cydno*, *H. melpomene*, and closely-related "species" *H. heurippa*, *H. pachinus* Salvin and *H. timareta* Hewitson (none of which, based upon the mtDNA sequences, appeared to be any more differentiated from *H. cydno* than the intraspecific variation among races within *H. cydno*, *H. melpomene* or *H. erato*). Brower (1996b) described *H. tristero*, a fourth *H. cydno* satellite, from the upper Putumayo valley in southeastern Colombia, geographically between the ranges of *H. heurippa* and *H. timareta*. The phylogenetic relationships implied by mtDNA COI-COII sequences in these papers suggest that *H. melpomene* is paraphyletic with respect to *H. cydno* and its satellites, with *H. cydno* and *H. melpomene* from Central America and Amazonia more closely related to one another than to *H. melpomene* from French Guiana. The paraphyletic *H. melpomene* pattern has generally been supported in subsequent work (Bull et al. 2006; Beltrán et al. 2007, Quek et al. 2010), although the sister-relationship between the *H. cydno* clade and a particular *H. melpomene* clade is not stable.

Heliconius heurippa was described in 1854 by W. C. Hewitson, based on material from eastern Colombia.

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[1] The genus *Heliconius* has been anointed with over 2,000 names for subspecies, races, forms and aberrations of its 47 species. Names such as form "*gustavi*" indicated in quotation marks are infrasubspecific quadrinomia that have no status in zoological nomenclature.
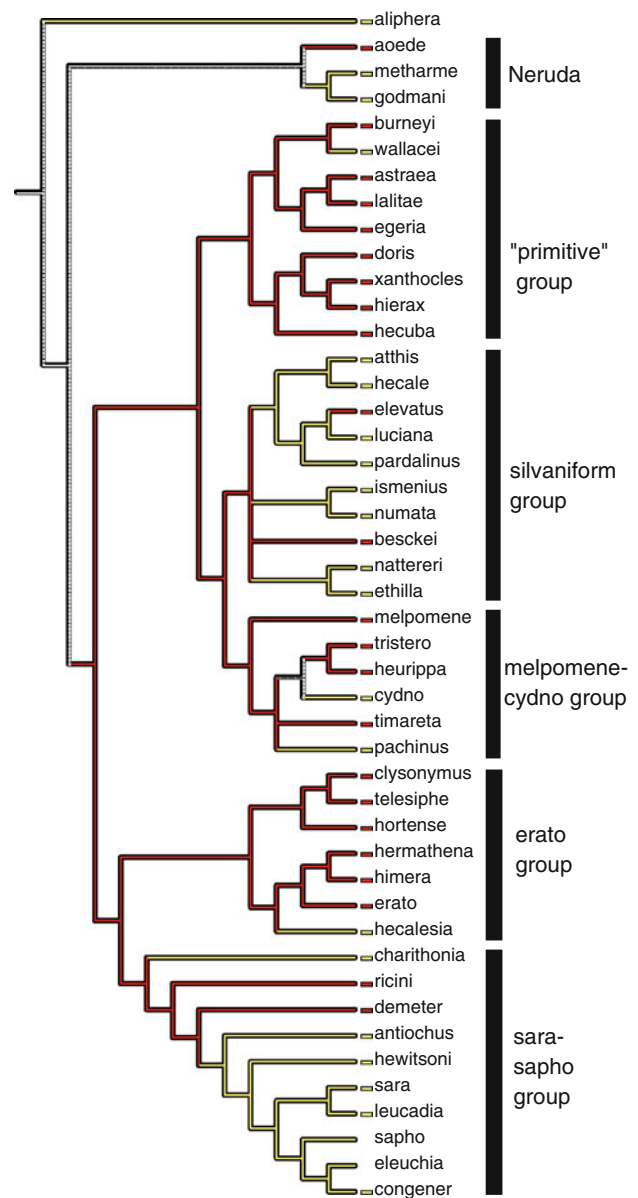
Fig. 1 Phylogenetic relationships among species of the genus *Heliconius*, as inferred by Beltrán et al. (2007), with presence or absence of red wing pattern elements optimized (assuming accelerated transformation) on internal nodes (red = red; yellow = no red; striped = equivocal). Basal red dots on the ventral surface of the hindwing are not considered "pattern elements." If the gain and loss of red pattern elements has occurred parsimoniously, it was present in the ancestor of the genus, lost at least six times, but unequivocally regained only once (in *H. elevatus* Nöldner). Note that this cladogram represents *H. melpomene* as a single terminal and thus does not reflect its inferred paraphyly discussed in the text

The species, along with the eastern Ecuadorian *H. timareta*, was viewed by subsequent authors as a member of the "*melpomene*-group" (Riffarth 1901; Stichel 1906; Neustetter 1929; Emsley 1965; but see Brown 1979), based upon the presence of a red forewing band, although as early as 1901, Riffarth considered it to "stand between"

H. melpomene and *H. cydno*. *H. heurippa* is sympatric with *H. melpomene melpomene*[2] in the foothills above Villavicencio in Meta Prov., Colombia (I have collected both species at the same locality). As far as is known, *H. heurippa* is allopatric with *H. cydno*, the races in closest proximity being *H. cydno wanningeri* Neukirchen across the Cordillera Oriental of the Andes to the west (which is too high for easy dispersal), and *H. cydno cordula* Neustetter on the eastern slope of the Cordillera Oriental to the north in northern Colombia and western Venezuela (Salazar et al. 2005 suggest that this race may abut with *H. heurippa* near Yopal). Based on historical collection records, there is no evidence of natural hybridization in the wild between *H. heurippa* and *H. cydno* or *H. melpomene* (Mallet et al. 2007).

The toolbox

As noted above, Gilbert (2003) described a qualitative model for the evolution of wing pattern diversity in *Heliconius* as the "toolbox," the "tools" being "those pattern genes that behave essentially the same" in *H. melpomene* and *H. cydno*. Gilbert's scheme hypothesizes that after some initial diversification via mutation, that "hybridization and introgression would have begun to accelerate in importance and quickly would have replaced mutation as the proximate generator of novel pattern genotypes in *Heliconius*." There is thus an unexplained reduction of the rate of wing-pattern altering mutations after the initial "ur-phenotypes" are established (the ur-*cydno* pattern is bluish black with white or yellow forewing and hindwing bands, and the ur-*melpomene* pattern is brownish black with at least some red pattern elements). White pattern elements observed in *H. melpomene* races, such as the hindwing marginal band of *H. melpomene cythera* Hewitson are hypothesized to have originated by introgression from sympatric *cydno* races (e.g., *H. cydno alithea* Hewitson), while red bands or rays in *H. cydno*-cognate taxa such as *H. heurippa* and *H. timareta* are thought to have originated via introgression from various *H. melpomene* forms.

While intuitively pleasing, Gilbert's tool-swapping scheme is supported neither by phylogenetic evidence nor by comparative genomic data. Mapping the distribution of red wing pattern elements shows that presence of red pigment is plesiomorphic in *Heliconius* (Fig. 1) (the presence of red basal spots on the ventral surface of the

hindwing may be a plesiomorphic feature of the entire subtribe). Thus, *Heliconius* species that do not exhibit red bands and/or rays are likely to have lost them. This is particularly relevant in the *H. cydno*-*H. melpomene* group, since it implies that the red wing pattern elements (or at least the genes that encode them) of putative "hybrid" taxa such as *H. timareta* and *H. heurippa* could be ancestral. Note also that while hybridization could explain novel patterns in those species, it cannot explain them in the equally phenotypically diverse *H. erato* or the *sapho* clade, members of which have no sympatric relatives with which they can exchange alleles (Gilbert 2003). In these taxa, all novel phenotypes, such as the white hindwing band in *H. erato cyrbia* Godart (the mimic of *H. melpomene cythera*) must have arisen by mutations.

From a genomic perspective, several recent papers have begun to reveal that the underlying genetic architecture of wing patterns in *Heliconius* is conserved between *H. erato* and *H. melpomene* (Joron et al. 2006; Papa et al. 2008; Baxter et al. 2010; Counterman et al. 2010), indicating that homologous genes have independently produced convergent phenotypes in different clades (an instance of homoiology, sensu Plate 1928; cf. Hennig 1966). If the alleles encoding, for example, red forewing patches (or absence thereof) have evolved independently in *H. erato* and *H. melpomene*, then there is no reason to assume that wing-pattern elements should not also arise in an unparsimonious manner within species, a hypothesis advanced by Brower (1996a). Thus, although hybridization may be a source of novel genetic variation, it is not unreasonable to invoke the occasional parallel switching on or off of shared genes responsible for similar-looking wing patterns as another potential source of novel variation within *Heliconius* species.

Evidence for hybrid speciation

Three types of evidence have been used in support of the hypothesis of hybrid origin of *H. heurippa*: the "recreation" of the *heurippa* phenotype by selective interbreeding of *H. melpomene* and *H. cydno* in the lab, studies of the species' capacity to interbreed with its putative parent species, and patterns of genetic variation in sequences of mtDNA, several nuclear genes, and batteries of microsatellite loci. These will each be examined in turn.

"Re-creating" the *H. heurippa* phenotype in the laboratory

Mavárez et al. (2006) selected a true-breeding hybrid laboratory strain with a yellow and red banded forewing phenotype similar to that of *H. heurippa*, by crossing captive *H. cydno cordula* with *H. melpomene melpomene*,

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[2] *Heliconius melpomene melpomene* (L.) is a geographically widespread, "polyphyletic" entity (cf. Brower 1996a) probably deserving of multiple subspecific names. Honey and Scoble (2001) consider the lectotype of *H. melpomene* (L.) to have originated from the Guianas. The similar-looking but genetically distinct forms from eastern Panama and northern Colombia appear to be unnamed.

backcrossing F1 males to *H. cydno*, and selecting *heurippa*-like backcross individuals for subsequent matings. After an unstated number of generations, homozygous *heurippa*-like phenotypes were produced, which the authors claim breed true when crossed to *H. heurippa* (only one representative of this cross is illustrated).

Mavárez et al. indicate that there are three homologous genes shared between *H. cydno* and *H. melpomene* with allelic differences responsible for the major color pattern differences between *H. cydno cordula* and *H. melpomene melpomene*: B/b, presence/absence of a red forewing band; N^N/N^B, presence/absence of a yellow forewing band (heterozygotes intermediate); and Br/br, expression of the brown "forceps" on the ventral hindwing. The wild type genotype of *H. cydno cordula* is bbN^{N}N^{N}BrBr, while the wild type genotype of *H. melpomene melpomene* is BBN^{B}N^{B}brbr, and the genotype of the *heurippa*-like hybrids is BBN^{N}N^{N}brbr. Mavárez et al. apparently assume that the alleles making up this genotype are identical by descent with the alleles that produce the same phenotype in *H. heurippa*, but there are subtle differences between the phenotypes, such as the shape of the yellow band and the shape and the color of the red band, that cast some doubt upon that assumption. As has been corroborated by genome mapping efforts (e.g., Baxter et al. 2008), the genes responsible for various pattern elements are the same among divergent, mimetic *Heliconius* clades, albeit with different homoiologous alleles. There is thus no reason to assume that alleles resulting in a mimetic or otherwise convergent wing pattern element are IBD among all taxa that exhibit that phenotype, nor, more specifically, any reason why alleles for the expression of a red forewing patch and the masking of the brown hindwing "forceps" could not have arisen independently in *H. heurippa*. Since the loci responsible for the patterns are the same, selecting a hybrid strain of *H. melpomene* x *H. cydno* that looks like *H. heurippa* is no more evidence of the latter's hybrid origin from those species than would a selected strain of *H. erato* that looks like *H.heurippa* be evidence that *H. heurippa* is descended from *H. erato*.[3]

Intrinsic barriers to gene exchange

There is substantial evidence that *Heliconius melpomene* and *H. cydno* are capable of mating and producing offspring in the laboratory (Brown 1981; Mallet et al. 1998; Gilbert 2003), and there is likewise mounting circumstantial evidence that the two species occasionally hybridize in the wild (Salazar 1993; Mallet et al. 1998, 2007). However,

Gilbert (2003) noted that "it is possible to keep *cydno* and *melpomene* in the same 13 ft × 21 ft greenhouse for years without the occurrence of interspecific courtship or mating." Controlled mating experiments produce the same result: Naisbit et al. (2001) and Jiggins et al. (2001b) showed, respectively, in no-choice and tetrad mating experiments in the lab that *H. melpomene rosina* Boisduval and *H. cydno chioneus* Bates from Panama are completely behaviorally isolated from one another: males of one species will not court females of the other. Mavárez et al. (2006; their Table 2) reported similar results for attempted crosses between *H. melpomene melpomene* and *H. cydno cordula* from allopatric populations on the eastern slope of the Colombian Andes: in tetrad mate choice experiments, *H. cydno* and *H. melpomene* never mated with one another.

Mavárez et al. also performed reciprocal tetrad experiments to test *H. heurippa*'s capacity to mate with its two hypothetical parental species. They found that neither *H. cydno* nor *H. melpomene* males will mate with *H. heurippa* females, that *H. heurippa* males will not mate with *H. melpomene* females, but that male *H. heurippa* will mate with female *H. cydno*. In summary, *H. cydno* and *H. melpomene* are prezygotically isolated from one another, as are *H. melpomene* and *H. heurippa*. *H. cydno* and *H. heurippa* are not prezygotically isolated. Note that all of these experiments assess the mating predilections of males, while the females employed are usually sacrificial virgins with neither the opportunity, nor apparently the will, to discriminate. Salazar et al. (2005) indicate that "sexually mature females show a low mating probability with males that are not from their own species" and "*H. heurippa* females show strong assortative mating when tested against both *H. melpomene* and *H. cydno*." Thus, in addition to male courtship, it is likely that in the wild, mate choice behavior of females of these species also contributes to premating isolation.

If these prezygotically isolated species are forced to mate or happen to mate out of desperation in the insectary, what is the fate of the resultant offspring? Offspring of the *H. cydno* x *H. melpomene* cross are viable and partially infertile, following Haldane's rule: heterogametic females are sterile (Linares 1989; Nijhout et al. 1990). Salazar et al. (2005) performed crosses between both of these species and *H. heurippa*, finding complete hybrid viability and fertility in the *H. cydno* x *H. heurippa* crosses, and an asymmetrical pattern of offspring fertility in the *H. melpomene* x *H. heurippa* crosses. Male *H. melpomene* x female *H. heurippa* F1 females are sterile, while male *H. heurippa* x female *H. melpomene* F1 females are fertile (males from both crosses are viable and fertile). The patterns of pre- and postzygotic mating success described here are summarized in Fig. 2.

Mavárez et al. (2006) stated, "… the phenotype of *H. heurippa* reproductively isolates it from both parental species." This is simply not the case (see Mavárez et al.

[3] Oberthür (1902) illustrates a number of natural hybrids between *H. erato hydara* Hewitson and *H. erato erato* from French Guiana reminiscent of the *H. heurippa* pattern (red proximal-yellow distal forewing band).
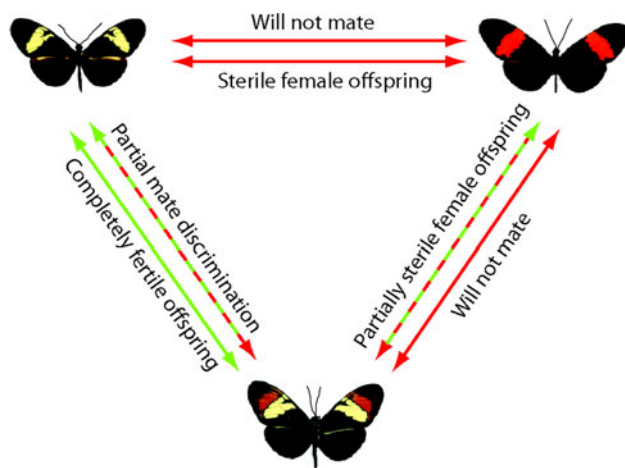
Fig. 2 Schematic summary of pre-and postzygotic isolation among *H. cydno cordula* (*top left*), *H. melpomene melpomene* (*top right*), and *H. heurippa* (*bottom*). Red arrows indicate isolation, green arrows indicate compatibility. Barred arrows indicate asymmetrical patterns

2006, Table 2). While it is true that neither *H. melpomene* nor *H. cydno* males will mate with *H. heurippa* females, *H. heurippa* males are perfectly willing and able to mate with *H. cydno* females (although, as noted, "mature" *H. cydno* females might reject attempted matings by *H. heurippa* males). Therefore, it is likely that *H. heurippa* and *H. cydno* remain distinct from one another in nature due solely to their geographical disjunction from one another. These authors also claimed that their study provided, "the first example of a hybrid trait causing pre-mating isolation through assortative mating." In fact, the trait that causes (partial) isolation between *H. cydno* and *H. heurippa* is the symplesiomorphic aversive response to red wing pattern elements by *H. cydno* males, a behavior that also deters them from mating with *H. melpomene* females (Jiggins et al. 2001b). *H. heurippa* males share with *H. cydno* the positive courtship stimulus of white/yellow wing pattern elements (also a symplesiomorphy), which explains why they will pursue *H. cydno* females but not *H. melpomene* females.

Patterns of genetic differentiation

Multiple "known" loci, as well as several batteries of microsatellite and AFLP markers, have been examined to assess the genetic structure of the *melpomene-cydno* group and the *H. heurippa* hybrid speciation hypothesis. The strategy employed in this paper was to obtain and reanalyze all relevant sequence data for each gene for which a *H. heurippa* has been sequenced, using the parsimony algorithm as implemented in TNT (Goloboff et al. 2003) (1,000 random addition replicates; gaps encoded as a fifth character; equal weights). Sequence data were transcribed from GenBank. Terminals are labeled with individual voucher codes as reported in the GenBank annotations or

corresponding publications. Note that some sequences were published on more than one occasion and are represented in GenBank with more than one accession code. Aligned data matrices for each of the genes are available as Nexus files at http://www.mtsu.edu/~abrower/datasets. Alignments were performed by eye.

Data which were unavailable for reanalysis were evaluated based upon the authors' descriptions of them in the original publications.

Mitochondrial DNA

As noted earlier, phylogenetic analysis of mtDNA COI-COII sequences has usually placed *H. heurippa* in a clade with *H. cydno,* nested in turn within a paraphyletic *H. melpomene* (Brower 1996a, b; Beltrán et al. 2007; Quek et al. 2010). There has evidently been some confusion about this pattern. Bull et al. (2006), in a rather contorted argument that contradicted their own results, claimed that "mutual monophyly (of *H. cydno* and *H. melpomene*) cannot be rejected," and Quek et al. (2010), citing Brower (1996a), stated that "early mtDNA studies indicated a sister species relationship," despite the cited paper's clearly stating, "… intraspecific variation in *H. melpomene* is complicated by the apparent paraphyly of the species with respect to *H. cydno* and its close relatives …."

To clarify the current understanding of relationships implied by this gene region, all published COI–COII sequences for the *melpomene-cydno* complex (371ingroup exemplars, from papers cited above, as well as Beltrán et al. 2002; Kronforst et al. 2006; Giraldo et al. 2008; Chamberlain et al. 2009) were extracted from Genbank, aligned (no length variation) and analyzed via parsimony (MP). A strict consensus tree is presented in Fig. 3.

The salient features of this tree are the same as those revealed by Brower (1996a): a basal *melpomene* clade from the Guianas, and then a polytomy comprising *melpomene* clades from southeastern Brazil, Amazonia, and the trans-Andean region and a clade of *cydno* cognates. Thus *H. melpomene* in the broad sense is still "paraphyletic" with respect to *H. cydno* and its satellites. Note that under the Phylogenetic Species Concept of Nixon and Wheeler (1990), two such entities are indeed considered to be "sister taxa"[4] (see Brower 1999). As Brower (1996a) found, there is little resolution within each of these clades, and no obvious correspondence between haplotypes and wing-patterns or finer scale geography. *H. heurippa,*

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[4] I stated above that *H. melpomene* and *H. cydno* are not "sister species" and here am saying that they are "sister taxa." This is not a contradiction, since *H. heurippa*, *H. tristero* and *H. timareta* are viewed as "species" and form a clade with *H. cydno.* The paraphyletic *H. melpomene* is sister to that clade. While paraphyletic taxa are undesirable, current nomenclature and ranks are maintained pending a taxonomic revision.
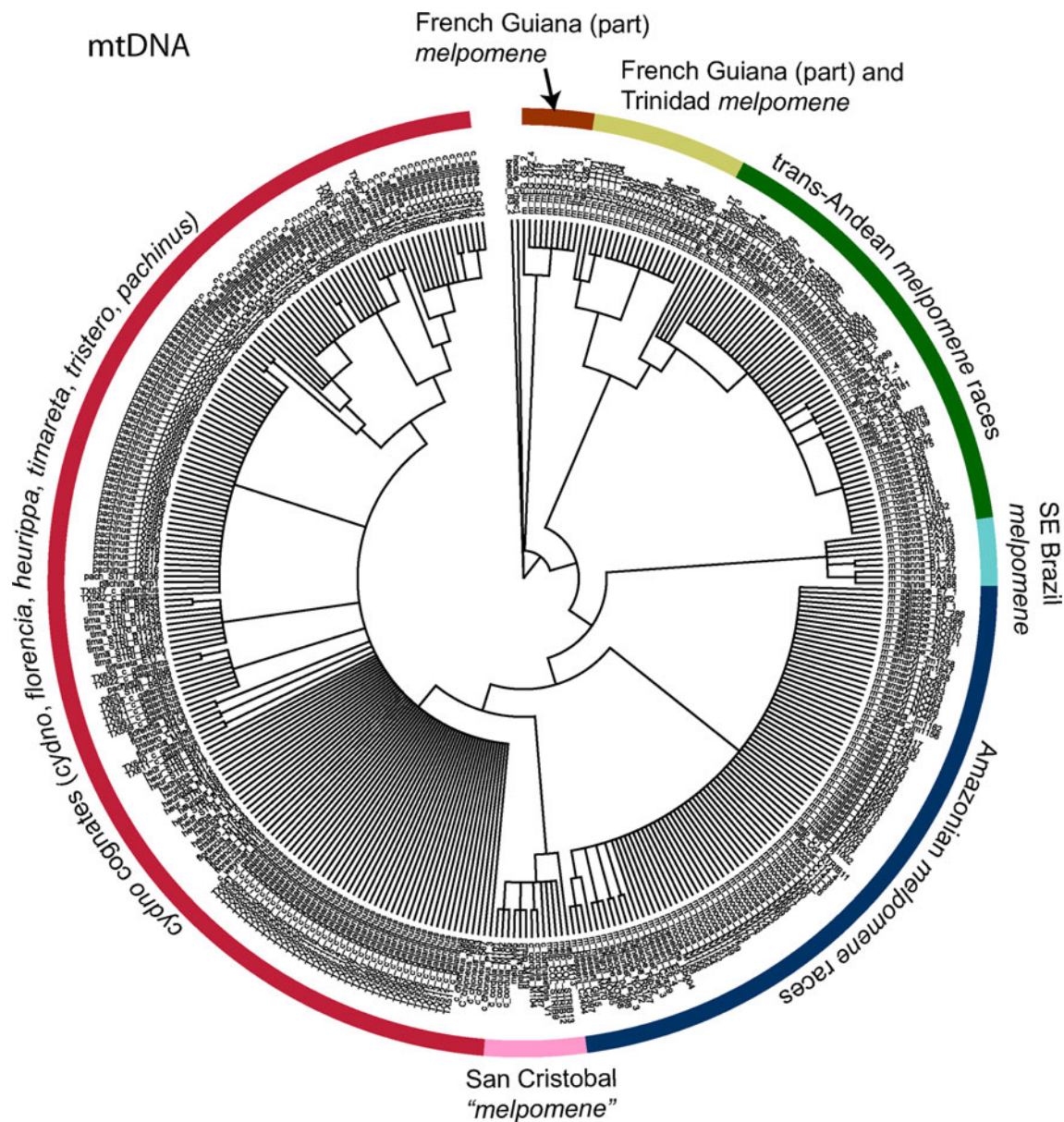
Fig. 3 Phylogenetic relationships of mtDNA COI–COII haplotypes. Strict consensus of 98 trees; length = 559 steps; CIx = 0.4846; RI = 0.9715. *H. cydno* and relatives form a clade embedded among various clades representing geographically separated groups of *H. melpomene* races. There is no implied introgression

H. timareta, and *H. tristero* individuals are interspersed within the *H. cydno* clade. Two "new" clades appear: a second clade of *H. melpomene* individuals from French Guiana and Trinidad (which will not be discussed here), and a cluster of "*H. melpomene*" haplotypes from Chirajara, Cundinamarca, Colombia and Santa Ana, Mérida, Venezuela, which is sister to the *H. cydno* clade. More of these anon.

Triose phosphate isomerase

Tpi is a polymorphic, sex-linked nuclear gene, a ∼530 bp region of which was developed for examining *Heliconius*

relationships by Beltrán et al. (2002). Additional members of the *H. melpomene-H. cydno* complex have been sequenced by Flanagan et al. (2004), Bull et al. (2006), Kronforst et al. (2006), Dasmahapatra et al. (2007), Giraldo et al. (2008), Kronforst (2008) and Salazar et al. (2008). 186 sequences were extracted from Genbank and analyzed via MP. Unlike the mtDNA, the *Tpi* gene region contains a length-variable segment spanning an intron. Alignment is not trivial, and approximately 2/3 of the phylogenetically informative sites occur in the noncoding region.

Analyses of these data, either including the gaps as a fifth character state or treating them as "missing," reveal a

weakly supported clade of alleles from various *H. melpomene* races, embedded within a paraphyletic cluster of alleles from *H. cydno* races, *H. timareta* and *H. heurippa* (Fig. 4). There is no apparent correlation of the gene tree topology with finer-scale phenotypic or geographical patterns. Single alleles are shared by multiple geographical races of *H. melpomene* and *H. cydno*, and among *H. heurippa* and *H. timareta*. Alternate alleles from some individuals are highly divergent from one another (although no alleles are shared between *H. cydno* and *H. melpomene*). There is no evidence for interspecific *H. melpomene*—*H. cydno* hybridization at this locus.

Mannose-6-phosphate isomerase

Beltrán et al. (2002) also developed a region of the autosomal *Mpi* as a marker for examining relationships in

Heliconius. The amplified region is composed of short exon sequences (9 and 13 codons) flanking a highly length-variable intron ranging from <100 to >400 bp in the current alignment. Additional sequences generated by Flanagan et al. (2004), Bull et al. (2006), Kronforst et al. (2006) and Kronforst (2008) were compiled into a matrix of 118 ingroup taxa plus five outgroups. The data exhibit long, highly similar insertions and deletions that are shared among a variety of taxa, including a 250 bp deletion shared among *H. cydno chioneus* and *H. melpomene rosina*, both from Panama, and *H. melpomene melpomene* from French Guiana. There is also a 65 bp insertion which occurs in several *H. pachinus*, *H. cydno chioneus* and *H. cydno galanthus* Bates, as well as one *H. melpomene rosina*.

Most of the authors working with *Mpi* have viewed the sharing of alleles among these species as evidence for
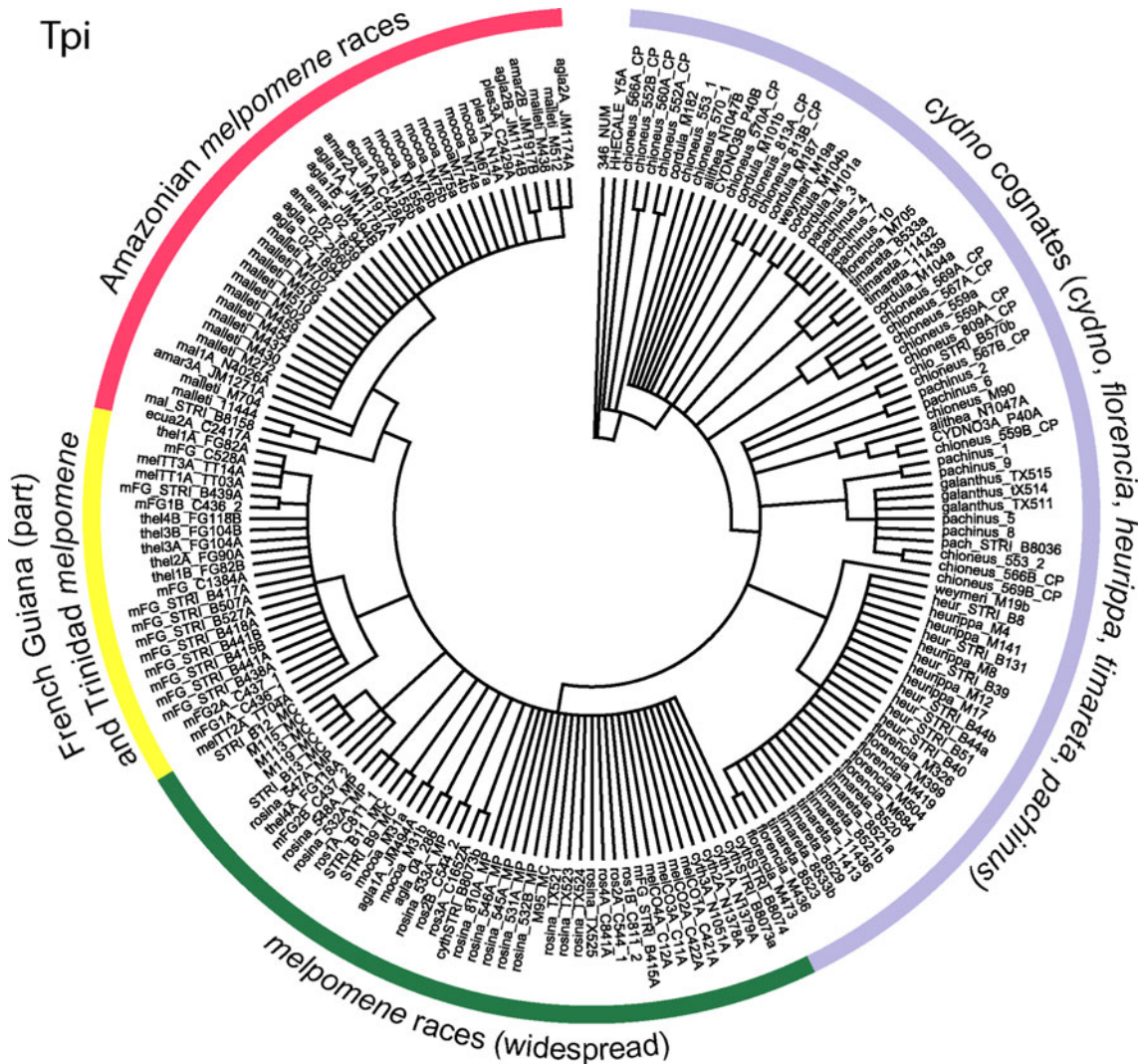
Fig. 4 Phylogenetic relationships of *Tpi* alleles. Strict consensus of 174 trees; length = 612 steps; CIx = 0.6927; RI = 0.9685. *H. melpomene* alleles form a clade with *H. cydno* and relatives forming a paraphyletic basal grade. There is no implied introgression
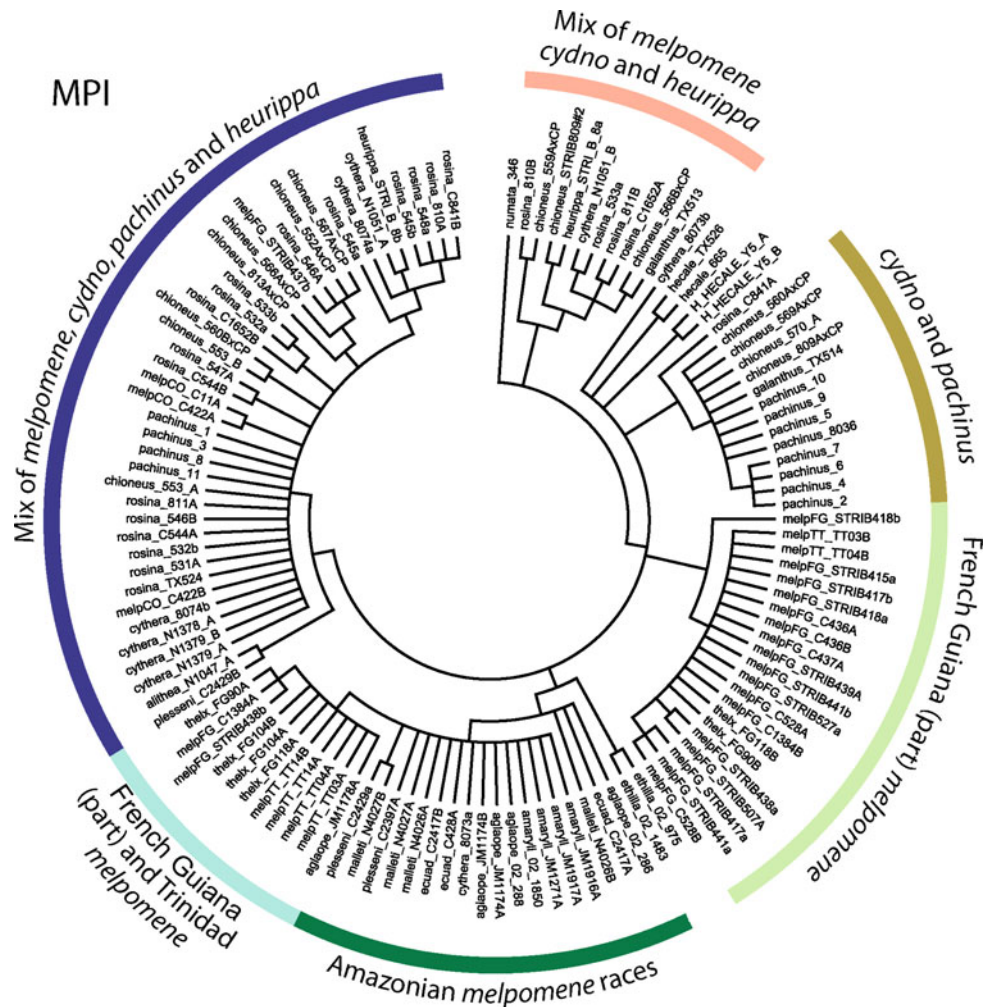
current interspecific gene flow. How hybridization explains the distribution of the 250 bp deletion in specimens from Central America and French Guiana (more than 2,000 km apart) is not clear. Further, when one takes into account the phylogenetic distribution of alleles present in heterozygous individuals, the pattern suggests that the diversity of alleles is nearly panmictic, not only among members of the *H. cydno*-*H. melpomene* group, but also among outgroup *H. hecale* (Fabricius) and *H. ethilla* (Godart). In regards to the "hybrid speciation" question, the alleles from the single *H. heurippa* sampled for this gene fall into two distinct clades (Fig. 5), each of which contains alleles from both *H. cydno* and *H. melpomene*. Given that the patterns of change in the *Mpi* marker are incoherent with respect to species boundaries and biogeography and because the variation is evidently not driven by single nucleotide substitutions, it seems premature to ascribe much evidentiary value to phylogenetic interpretations of this gene region.

Distal-less

Kronforst et al. (2006) and Mavárez et al. (2006) have examined two sets of sequences from the autosomal developmental gene *Dll*, each study interpreting the distribution of alleles to support the hypothesis of interspecific gene flow. Kronforst's specimens were all Costa Rican, and Mavárez's were all from the eastern slopes of the Andes in Colombia and Venezuela. Again, the bulk of informative variation in this gene region comes from a length-variable intron. Data from both these studies comprising 99 ingroup alleles were combined and analyzed via MP with gap characters from the intron included as a fifth character state. Mavárez et al. (2006) reported "no allele sharing between *H. cydno* and *H. melpomene*, whereas the *H. heurippa* genome appears as an admixture, sharing allelic variation with both putative parental species." The tree based on the combined dataset (Fig. 6; see also

Fig. 5 Phylogenetic relationships of *Mpi* alleles. Strict consensus of 236 trees; length = 777 steps; CIx = 0.7341; RI = 0.9442. While there are several clades containing only *H. melpomene* alleles, all clades containing *H. cydno* alleles also contain multiple *H. melpomene* alleles
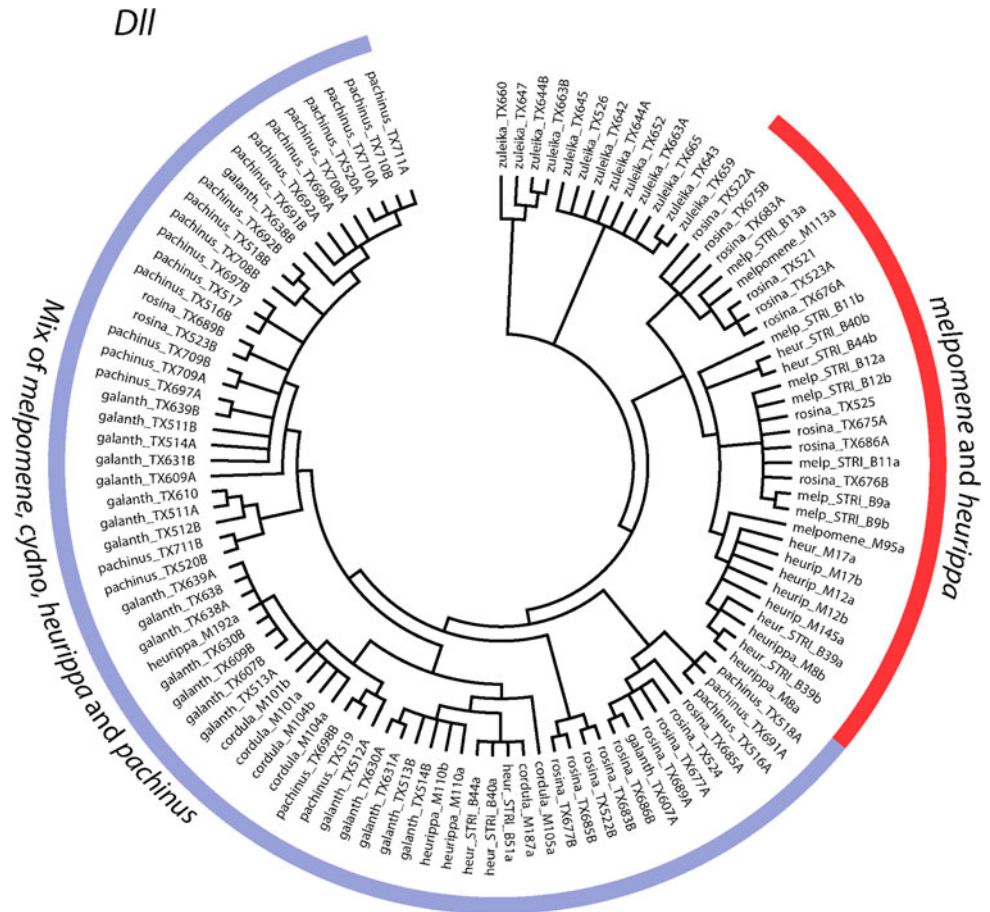
Kronforst et al. 2007) complicates the interspecific hybridization scenario by revealing nearly identical alleles shared not only among sympatric *H. melpomene* and *H. cydno*, but also between Costa Rican and eastern Colombian *H. melpomene,* and between Costa Rican *H. cydno galanthus* and *H. heurippa*. Such a pattern suggests that ancestral polymorphism could also explain the maintenance of allelic diversity across the ranges of these taxa. It seems that further sampling of this locus from the geographical diversity of *H. melpomene* and *H. cydno* forms will be necessary before the extent and mode of gene flow throughout the clade may be clearly discerned.

Invected

The data for this gene have the same provenances as the *Dll* data, and were also invoked by their respective authors as evidence of the origin of *H. heurippa* via interspecific hybridization. Once again, most informative variation in the *inv* fragment comes from a length-variable intron. The two data sets were combined into a matrix of 67 ingroup alleles × 479 bp and analyzed via MP with gap characters from the intron included as a fifth character state. The combined tree (Fig. 7; see also Kronforst et al. 2007) exhibits near

reciprocal monophyly of *H. cydno*-group alleles and *H. melpomene* alleles, but several *H. heurippa* alleles and one *H. pachinus* allele cluster among the *H. melpomene* alleles. This pattern represents the most convincing evidence of hybridization from any of the genes discussed so far. However, it is again evident that very closely-related alleles are distributed between Venezuela and Costa Rica, suggesting not only sympatric interspecific hybridization, but also extreme long-distance dispersal or some other mechanism that results in homologous (or convergent) intron variation among remote areas. Further, no *H. heurippa* allele is identical to any *H. melpomene* allele, and one of the *H. heurippa* alleles is sister taxon to all *H. melpomene* alleles save one, suggesting that if these alleles were acquired via hybridization, the hybridization events happened long ago and on several separate occasions. As in *Dll*, the entire picture of allelic diversity at this locus may not be represented by the limited geographical samples examined to date.

White

Sequences of the ommochrome biosynthesis gene *w* for 37 ingroup alleles were obtained from Kronforst et al. (2006) and Salazar et al. (2008), representing the same taxa as *Dll*

Fig. 7 Phylogenetic relationships of *Invected* alleles. Strict consensus of 267 trees; length = 387 steps; CIx = 0.6655; RI = 0.9322. *H. melpomene* and *H. cydno* are not monophyletic with respect to the outgroup. *H. heurippa* alleles are distributed throughout the tree
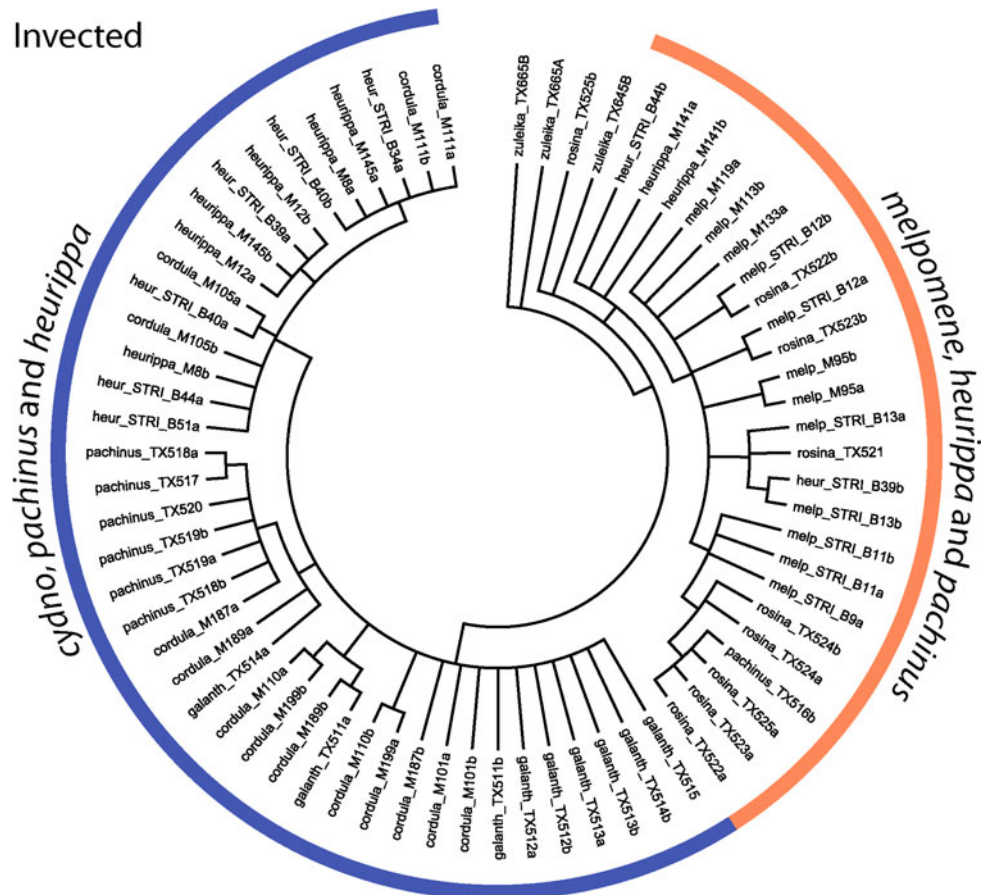
and *inv*. Again, the aligned region is short (434 bp) and contains a length-variable intron. Parsimony analysis resulted in a consensus tree (Fig. 8) with all species' alleles polyphyletic with respect to one another, and no obvious geographical structure. If the distribution of alleles among species at this locus is due to hybridization, then *H. cydno-H. melpomene* group members are exchanging alleles not only among themselves, but also with the outgroup *H. hecale*.

Scalloped

Sequences of a region of this putative developmental gene representing 46 ingroup alleles were obtained from Kronforst et al. (2006) and Salazar et al. (2008), representing the same taxa as the preceding three genes. The aligned region is 555 bp and contains a length-variable intron. Parsimony analysis resulted in a consensus tree (Fig. 9) with all species' alleles polyphyletic with respect to one another, and no obvious geographical structure.

Wingless and elongation factor 1-alpha

Sequence data for these genes have been useful for a wide variety of higher-level studies of butterfly relationships

(e.g., Brower 2000a; Wahlberg et al. 2009), but have proven to exhibit little variability within or among closely-related *Heliconius* species (Brower and Egan 1997; Beltrán et al. 2007). 33 *wg* sequences and 31 *Ef-1 alpha* sequences representing the *H. cydno-H. melpomene* group were obtained from Genbank, aligned and analyzed, but resulted in largely unresolved polytomies (data not shown).

Microsatellite and AFLP data

Microsatellites and AFLP data are the allozymes of the twenty-first century, and their use as a source of evidence to infer population structure has resurrected many of the dubious phenetic approaches that were superceded by cladistics in the late 1970s. Unlike sequences of known genes, these markers are "anonymous," their putative homology is not testable, and they cannot be compared across studies in the manner performed for the loci discussed above. Their inscrutability is further exacerbated by the unfortunate tendency by researchers who employ them to not make available the raw data for critical reexamination. Thus, the would-be intersubjective corroborator is relegated to deciphering recondite graphical summaries and discursive interpretations as a means of evaluating these "data." Several of the following studies did not

Fig. 8 Phylogenetic
relationships of *white* alleles.
Strict consensus of 8 trees;
length = 351 steps;
CIx = 0.7299; RI = 0.9343.
Little resolution is evident
among included species

examine *H. heurippa*, but the patterns found among other *Heliconius* species bear upon the interpretation of the *H. heurippa* story.

Mavárez et al. (2006) examined 12 microsatellite loci from five populations of *H. cydno* and *H. melpomene* and one population of *H. heurippa* using Structure 2.1 (Pritchard et al. 2000), a Bayesian model that simultaneously infers how many clusters of similar multilocus genotypes exist in the data and assigns individuals to those clusters. They reported that the "best model" specified three clusters, allowed for admixture and independent estimations of allele frequencies. They did not report whether or not the "best model" was significantly more likely than any other.[5] Nor do they mention the interesting

phenomenon that the degree of inferred admixture among *H. heurippa*, *H. melpomene* and *H. cydno* appears to be highest between the populations of the latter two that are the most geographically remote (Pipeline Road, Panama) from the range of *H. heurippa*. In sum, the methods used to analyze these data were so inadequately described that the results are indeterminate. Their diagram (their Fig. 1) shows individuals of the three species organized into blocks of three different colors, suggesting that *H. cydno*, *H. melpomene* and *H. heurippa* are different from one another. But how different? Are these differences nested?

Mavárez et al. (2006, their supplementary Fig. 4) conducted a separate, also inadequately–described, microsatellite analysis of 36 *H. melpomene melpomene*, 44 *H. cydno cordula* and 9 putative *cydno* x *melpomene* hybrids from San Cristóbal, Venezuela. The microsatellite profiles of these "hybrid" specimens in Structure are apparently identical to those of *H. cydno cordula* (see epigram #1), suggesting under the hybrid speciation hypothesis that the only alleles from *H. melpomene* they

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[5] Mavárez et al. (2006) did not indicate the provenance of their microsatellites and whether or not they meet the assumptions of the Structure algorithm (Hardy–Weinberg equilibrium and no linkage), so there is no way to evaluate the meaning or validity of their interpretations of the data. The microsatellite markers may have been selected from among those reported in Mavárez and González (2006), most of which have a significant heterozygote deficiency and thus would not satisfy the Structure assumptions. Structure also assumes that the populations are of equal size, while Mavárez et al. sampled 168 *H. melpomene*, 165 *H. cydno* but only 46 *H. heurippa*. Pritchard et al. (2000) also caution that inference of K (the number of

Footnote 5 continued

populations) is difficult to infer from a limited number of markers when there is admixture, as in this case.

Fig. 9 Phylogenetic relationships of *scalloped* alleles. Strict consensus of 147 trees; length = 295 steps; CIx = 0.7677; RI = 0.9521. Little resolution is evident among included species
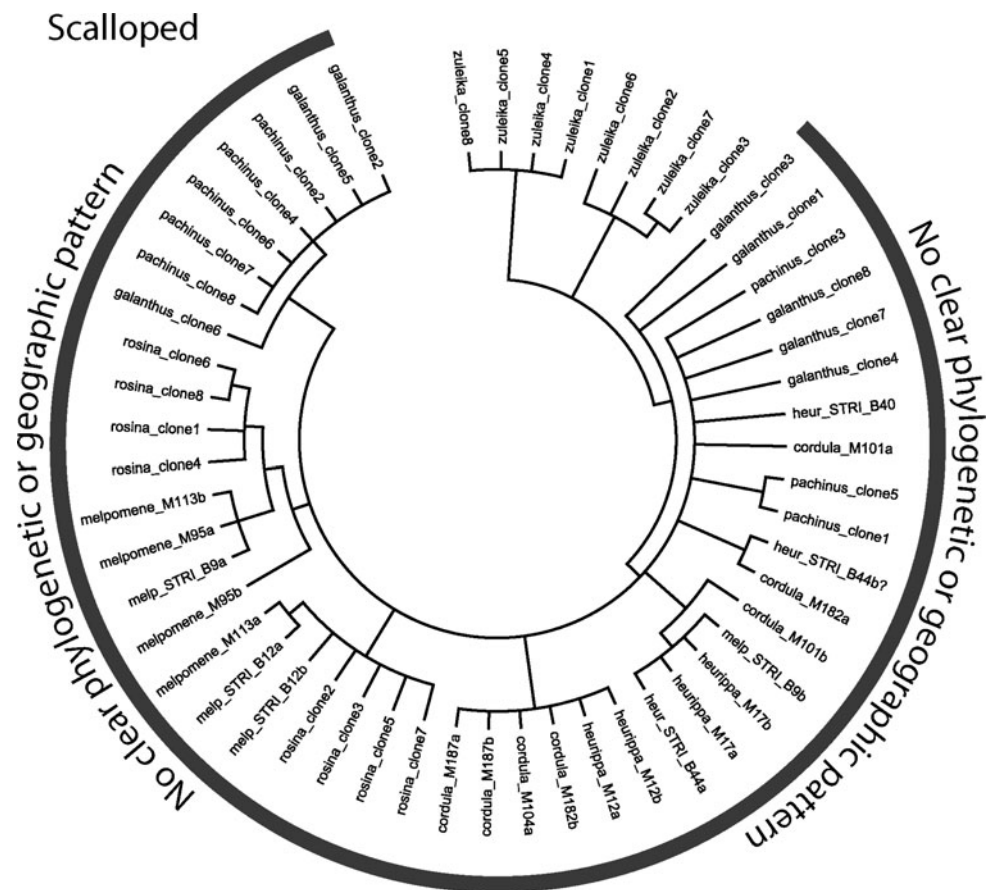
have retained from their interbreeding are specifically those responsible for their "hybrid" wing patterns.

Jesús Mavárez (pers. comm., Nov. 2005) reported results of a to-date unpublished Factorial Analysis of Correspondence based on microsatellite variation at 12 loci for the two specimens of *H. tristero* known at that time,[6] compared to *H. melpomene* and *H. cydno* from Panama, Colombia and Venezuela. *H. tristero* clustered within *H. cydno* and not within *H. melpomene*, and no *H. melpomene* individual ever fell within the *H. cydno* cluster. He also indicated that his results show that *H. pachinus* and *H. timareta* are also closely related to *H. cydno*.

Kronforst et al. (2006, 2007) examined a large data set of AFLP loci to assess interspecific gene flow among Costa Rican *H. pachinus*, *H. cydno galanthus* and *H. melpomene rosina*. They found (2006) "multiple instances of mixed ancestry in all three species," but concluded (2007) based on the same data that all their analyses "supported a close genetic relationship between *H. pachinus* and *H. cydno* and none suggested a genetic contribution from *H. melpomene* in the origin of *H. pachinus*." They drew an important distinction between hybridization as an incidental process

and hybridization as a source of evolutionarily significant genetic variation, ruling out the latter in *H. pachinus*, but invoked the *inv* and *Dll* data discussed above to suggest that *H. heurippa* may have a hybrid origin.

Giraldo et al. (2008) used microsatellite data in Structure to sort an unspecified number of individuals of *H. melpomene malleti* Lamas and *H. florencia* Giraldo et al.[7] into populations, and found five individuals with microsatellite profiles containing markers assigned to multiple species that they interpreted to be hybrids. As in Mavárez et al. (2006), there is no way to assess the robustness of this interpretation from the data as presented.

Chamberlain et al. (2009) examined microsatellites from samples of *H. cydno alithea*, *H. cydno galanthus* and *H. pachinus* to look for possible genetic differences between yellow and white polymorphic forms of the

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[6] Aliquots of genomic DNA from the holotype and paratype specimens of *H. tristero* were sent to Mavárez by the author.

[7] The taxon described as *Heliconius timareta florencia* Giraldo, Salazar, Jiggins, Bermingham and Linares, 2008 (Giraldo et al. 2008) exhibits a typical Amazonian "dennis-ray" wing pattern like that of the "*strandi*" form of the polymorphic *H. timareta timareta*, but it occupies a disjunct distribution (in Caquetá, Colombia) and does not form a clade with other "*H. timareta*" individuals in the mtDNA, *Tpi* or microsatellite analyses of Giraldo et al. (2008; see Figs. 3 and 4). Thus, it (and the other Amazonian *H. cydno* cognates) should either be subsumed as a race of *H. cydno*, or ranked as a species, as I have done here (see Brower 1996b, 2000b).

former. They didn't find any, but interestingly, when they ran Structure on their data allowing admixture, as in the studies reviewed above, all of their *H. cydno galanthus* specimens appear as "hybrids" (sharing alleles from allopatric *H. cydno alithea* and *H. pachinus*!) with an equal or higher degree of admixture than most of the "hybrid" specimens discussed above (their Fig. 3b).

Most recently, Quek et al. (2010) have used 3,186 AFLP loci to examine geographical structure among populations of *H. melpomene* throughout its range. Their NJ phenogram for these data is largely congruent with trees inferred from mtDNA (e.g., Fig. 3), although bootstrap values indicate rather high instability of relationships among most races and geographical areas. Specimens from "Colombia" represent three different geographical areas—the Pacific slope, the Magdalena Valley and the eastern slope of the Andes, and it would have been a surprise if they did form a "clade." Quek et al. also performed a Structure analysis of the same data, which as in the studies discussed above revealed "admixture" between various sampled individuals, including *H. cydno* from Panama and *H. melpomene* from southeastern Brazil.

So, to summarize these various Structure analyses, it seems that when one wants to find admixture to support one's hypothesis, then one can do so. Or when one wants to ignore admixture to support one's hypothesis, then one can do so. It is indeed an excellent type of evidence that is so compliant to the desired interpretations of the researcher.

In general, the available genetic evidence support a limited degree of shared polymorphism between *H. melpomene* and *H. cydno* and its various offshoots. Kronforst et al. (2006) summed up the pattern nicely: "Our results indicate that the three *Heliconius* species [Costa Rican *H. cydno*, *H. pachinus* and *H. melpomene*] studied here hybridize frequently enough to leave recognizable evidence of admixture and introgression but not enough to erode species boundaries." However, studies limited to populations of different species from a single geographical region may reveal shared alleles, but these patterns cannot be interpreted as the necessary result of recent hybridization when the same or closely-related alleles are also shared among remotely allopatric populations, implying additional historical processes besides exchange of alleles among sympatric relatives. The patterns are also made difficult to interpret by the fact that most of the informative variation in the genes studied occurs in length-variable intron regions. It is clear that these length variants do not evolve one nucleotide at a time, but the "gap characters" are treated as though they did (as in the above reanalyses) or ignored altogether as missing data. It is extremely doubtful that either of these approaches adequately captures the character state transformations as they have occurred, but until more is known about the tempo and

mode of intron evolution, there does not seem to be a more defensible alternative. Again, when the assumptions of the analytical approach are not met by the patterns of variation in the data, one is treading on thin epistemological ice to interpret the results as an accurate reflection of history.

Thirty years ago, Turner et al. (1979) found a high degree of shared polymorphism among *Heliconius* species using allozymes, and argued that the genes related to wing patterns must be decoupled or behave independently from the remainder of the genome. This pattern has been corroborated by recent genomic studies (Baxter et al. 2010), who found limited signatures of selection around specific loci associated with wing pattern elements. It therefore may not be possible to obtain a clear understanding of the evolution of mimetic phenotypes in these butterflies until we are able to examine gene genealogies for the genes that are responsible for the wing pattern elements themselves. I predict that the allele producing a red band on the forewing of *H. heurippa* will not be homologous (IBD) to that of sympatric *H. melpomene melpomene*, a pattern that would lay to rest the *H. heurippa* HHS hypothesis.

Hopeless monster: the origin and fate of an interspecific hybrid

But let us ignore all of the above and suppose, for the moment, that *H. heurippa* is the homoploid hybrid offspring of *H. melpomene melpomene* and *H. cydno cordula*. What events would need to take place for the origination and establishment of a genetically homogeneous *H. heurippa* population, sympatric with one of its parental species?

First, we would need an interspecific mating between the parental species—something that occurs rarely, and only when one of the species is rare, thereby limiting its mating options (Gilbert 2003). Since *H. heurippa* is currently sympatric with *H. melpomene* and not with *H. cydno*, it is parsimonious to suppose that *H. cydno* was the rare parental species. F1 individuals of this cross have a red forewing band with a thin dusting of yellow scales on its proximal margin (a pattern much more *H. melpomene*-like than *H. cydno*-like). One would suppose, therefore, that selection would favor the fine-tuning of the color pattern to be more *H. melpomene*-like in subsequent generations, since that is the common parent and a participant in the local Müllerian mimicry scheme with the even more common *H. erato hydara* Hewitson. However, the *H. heurippa* pattern is a worse mimic of *H. melpomene* than the F1 (so much so that it is often referred to as "nonmimetic"), so the normally strong positive frequency-dependent selection on the wing pattern would apparently either have been relaxed or have been overwhelmed by some alternative selective force counter to the positive frequency-dependent pull of Müllerian mimicry.

Next our F1 generation would need mates. Since female offspring of both reciprocal crosses of the parental species are sterile, F1 individuals cannot mate with one another to produce F2 offspring. Instead, the F1 hybrid males need to backcross to rare *H. cydno* females in order for their offspring to gain the *cydno*-like mtDNA that is observed in *H. heurippa*. The phenotypes of these F1-backcrosses exhibit 25% red forewing bands (Bb/$N^N N^B$) 25% red-and-yellow forewing bands (Bb/$N^N N^N$), and 50% yellow bands only (bb/N^N-). Once again, selection must favor individuals with the "non-mimetic" *H. heurippa* pattern over those with patterns like the parentals. In order to become homozygous for the *H. heurippa* phenotype (BB/$N^N N^N$/brbr), two "*H. melpomene*" color pattern alleles need to become fixed, despite indications that backcrossing, at least initially, must be to *H. cydno*. A further complication to this scenario is the observation by Naisbit et al. (2001) that F1 hybrid males prefer to mate with F1 hybrid females over females of either of their parental strains. That would seem like a clear path to prezygotic isolation and speciation (cf. Melo et al. 2009) except that *H. cydno* x *H. melpomene* F1 females are, of course, sterile and such matings are completely fruitless.[8]

Duenez-Guzman et al. (2009) found that when their model considered "color patterns learned as a whole," "hybrids never achieve high frequencies … and both parental species coexist sympatrically". Predators do indeed perceive the entire butterfly, as attested by the enormous selection coefficient against novel phenotypes found in a Peruvian *H. melpomene* hybrid zone (Mallet and Barton 1989), despite the fact that the butterflies on either side are both black with red and yellow markings. Jacamars (Galbulidae), which are likely to be the main predators responsible for maintaining Müllerian mimicry in *Heliconius*, have acute perception of small pattern differences (Langham 2004, 2006).

So, in addition to overcoming the problem of selection against novel phenotypes by predators, incipient "*H. heurippa*" hybrids would also need to mate with females of an allopatric geographical race of *H. cydno* with whom they would prefer not to, and who would prefer to not mate with them. This does not seem like a recipe for success.

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[8] It is relevant to observe that most of the putative *H. melpomene* × *H. cydno* hybrid specimens compiled by Mallet et al. (2007) or illustrated elsewhere (e.g., Ackery and Smiles 1976; Brown and Fernandez-Yepez 1985; Posla-Fuentes 1993; Salazar 1993) do not appear to be F1 hybrids. Given that interspecific hybridization is a rare event, and the various selective disadvantages of being a hybrid discussed here, one would suppose that most of the "interspecific hybrid" specimens encountered by collectors would be F1 individuals like the one analyzed by Dasmahapatra et al. (2007). Intriguingly, this does not seem to be the case.

H. heurippa, hybrid species? An alternative hypothesis

As reviewed above, all of the genetic and breeding evidence indicate that *H. heurippa* is more closely-related to *H. cydno* than it is to *H. melpomene*. If *H. heurippa* were the only *H. cydno*-cognate to exhibit red wing pattern elements, then the homoploid hybridization hypothesis with introgression of some *H. melpomene* wing pattern alleles into a mainly *H. cydno* background might provide a reasonable, if unlikely, explanation for its existence. But what are the chances of the same improbable phenomenon occurring independently multiple times in other forms with different wing patterns? Could there be another explanation for this general pattern?

It is becoming increasingly evident that an allopatric or parapatric string of taxa occurs between 800 and 1,200 m. along the Amazonian slope of the northern Andes with red wing pattern elements and "*H. cydno* genes" (see Mallet 2009 for a distribution map). These are briefly described from south northwards: *H. timareta*, with differentiated races in northeastern Peru and eastern Ecuador is often characterized, like *H. heurippa*, as "non-mimetic," but at least some of its forms are very good mimics of nearby *H. melpomene* and *H. erato* races. Mallet (2009) mentions another unnamed *H. cydno* cognate from the Mayo valley in San Martín, Peru which, based upon its mimetic wing pattern, has long been conflated with the local *M. melpomene amaryllis* Felder & Felder. In the Putumayo valley of southeastern Colombia, *H. tristero* is a dead-ringer for *H. melpomene mocoa* Brower and *H. erato dignus* Stichel. Not far to the northeast in the Río Hacha valley occurs the recently-described *H. florencia*, a very good mimic of *H. melpomene malleti* and *H. erato lativitta* Butler. Giraldo et al. (2008) illustrate an unnamed *H. cydno* cognate with a yellow forewing band like that of *H. cydno cordula* (and no red pattern elements) from the Río Pata valley near the Cordillera de Los Pichados. *H. heurippa* itself occurs in the Meta valley above Villavicencio. Then there is a gap, where no *H. cydno* cognate is known, until the range of *H. cydno cordula* in the Cordillera de Mérida in northern Colombia and western Venezuela. But, remember the "*H. melpomenes*" with red forewing patches but *H. cydno* mtDNA from Chirajara (sympatric with *H. heurippa*) and Santa Ana, Venezuela (Salazar et al. 2008) and the "hybrids" from San Cristóbal, Venezuela (latter two sites about 15 km apart)? What if these specimens represent another *H. cydno* cognate that mimics *H. melpomene melpomene* and *H. erato hydara* and occurs along the eastern slope of the northern Colombian Andes? That would explain both the "hybrid" wing patterns and the genetic similarity of the San Cristóbal "hybrids" to *H. cydno* much more simply than the "multiple generations of backcrossing" invoked by Mavárez et al. (2006). In

general, given that the red wing pattern elements of these various taxa occur on different parts of the wing and are controlled by different genes (Sheppard et al. 1985), it seems all the more unlikely that they can all be explained as the result of HHS, since each of those hybridization-speciation events would need to occur independently.

Here is an alternative scenario for the origin of all these cis-Amazonian *H. cydno* cognates. Many authors have argued that *H. cydno* (and *H. melpomene*) have been subordinate participants in their mimicry rings, evolving to converge on the wing patterns of more abundant members of the "pupal mating" group—*H. sapho/H. eleuchia* and *H. erato*, respectively. Since *H. melpomene* is paraphyletic with respect to *H. cydno*, and its basal lineages occur in southeastern Brazil and the Guianas, it is reasonable to hypothesize that *H. melpomene* had a broad distribution before the origin of *H. cydno*. *H. cydno* and *H. melpomene* diverged from one another west of the Andes (perhaps even in southern Mexico, where *H. melpomene* does not occur), and *H. cydno's* wing pattern converged on that of *H. sapho*. *H. cydno* began to expand its range southward and eastward, evolving to mimic a variety of locally common aposematic *Heliconius* (or even ithomiines such as *Elzunia humboldt* (Latreille); cf. Linares 1997b), but its distribution was limited by the distribution of its black-and-white comimics, which are restricted to the western side of the Andes (Brown 1979).[9]

H. cydno appears to be one of the more phenotypically labile *Heliconius* species, exhibiting not only geographical variation in wing patterns, but also sympatric wing pattern polymorphism in several different areas: *H. cydno chioneus* is polymorphic in western Ecuador (Kapan 2001); *H. cydno weymeri* has at least three morphs in the upper Cauca valley on Colombia (Linares 1997b); *H. timareta* is polymorphic in eastern Ecuador (Brown 1979). Let us imagine that peripheral populations of *H. cydno* spilled over onto the eastern slopes of the Andes during Pleistocene warm periods, where they came into contact with more abundant red-and-yellow members of the *H. erato-H. melpomene* mimicry ring. Under such circumstances, mutations that resulted in red wing color patterns would confer a selective advantage (at least against less acute predators than jacamars), and might provide the initial impetus to switch mimicry rings. Recall that since *H. cydno* is descended from *H. melpomene*, such mutations would not represent evolutionary novelties, but rather reversals to an ancestral phenotype, and could be accomplished by switching on or off alleles already present in the underlying genetic architecture of wing pattern.

But doesn't this seem implausible and ad hoc, given the apparent rarity of wing pattern mutants? Gilbert (2003) suggested a spontaneous wing pattern mutation rate of 1:4,000, which is not very different from Mallet et al.'s (2007) estimate of the rate of hybridization between *H. melpomene* and *H. cydno*, 1:2,000. Since the red forewing patch allele B is dominant in *H. heurippa* and other "red" alleles are usually dominant in *H. melpomene* (Sheppard et al. 1985), we can hypothesize that novel red-producing mutations would also be dominant in *H. cydno* and therefore expressed in heterozygotes. If such alleles were beneficial in a new mimetic environment, they would rapidly increase in frequency in the population. And the individuals bearing them would not suffer a loss in fitness due to hybrid sterility because they would not need to hybridize with *H. melpomene*. Furthermore, if a number of the aberrant specimens interpreted by Mallet et al. (2007) to be interspecific hybrids actually were mutants, that would imply that the mutation rate could be higher, while the inferred rate of interspecific hybridization between *H. melpomene* and *H. cydno* is concomitantly lower.

The evolution of mimetic diversity despite frequency-dependent selection for a common aposematic pattern remains a paradox, and no hypothesis explaining the origin of novel forms is immune from criticism. However, *H. heurippa*, *H. florencia*, *H. timareta* and *H. tristero* have all been shown by available data to be mainly reproductively compatible with/related to/descended from *H. cydno*; those alleles shared between *H. heurippa* and *H. melpomene* appear to be also widely shared among other members of the *H. cydno-H. melpomene* clade; and the underlying genetic architecture to produce red wing pattern elements is plesiomorphic for the entire genus. The HHS hypothesis has evolved to accommodate the lack of evidence of *H. heurippa's* hybrid genome. The current version, "hybrid trait speciation" (Jiggins et al. 2008), requires only the genes controlling adaptive traits to move across species boundaries, while neutral genetic variation is either assorted by species or shared among all members of the clade (turning the Turner et al. 1979 "contrasted modes" hypothesis on its head). It may come to pass that incontrovertible *H. melpomene* alleles responsible for mate discrimination and selective divergence are discovered in *H. heurippa* and its red-winged cousins, but until that happy day, the alternative scenario for the origin of these species presented here seems a somewhat less onerous alternative.

Postscript: 3′ *kinesin*, smoking gun, or magic bullet?

While this manuscript was in review, a recent, highly relevant article by Salazar et al. (2010) came to my attention. In that paper, the authors announced the discovery of a

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[9] Of course, there are black-and-white *Heliconius* species, such as *H. congener* Weymer, and *H. antiochus* (L.) that occur east of the Andes and may be involved in mimicry with *H. cydno cordula* and *H. cydno gadouae* Brown & Fernandez-Yepez but perhaps these species are too uncommon to exert a mimetic gravitational pull, or do not overlap in elevational distribution with other *H. cydno* cognates.

Fig. 10 Phylogenetic relationships of 3′ *kinesin* alleles. Strict consensus of 3,488 trees; length = 1,852 steps; CIx = 0.4649; RI = 0.8723. Note that although the branch support (sensu Brower 2006) = 9 for the node leading to *H. melpomene* and *H. heurippa* alleles, that the *H. heurippa* alleles form a polytomy with and are not embedded within the *H. melpomene* alleles, contra Salazar et al. (2010)
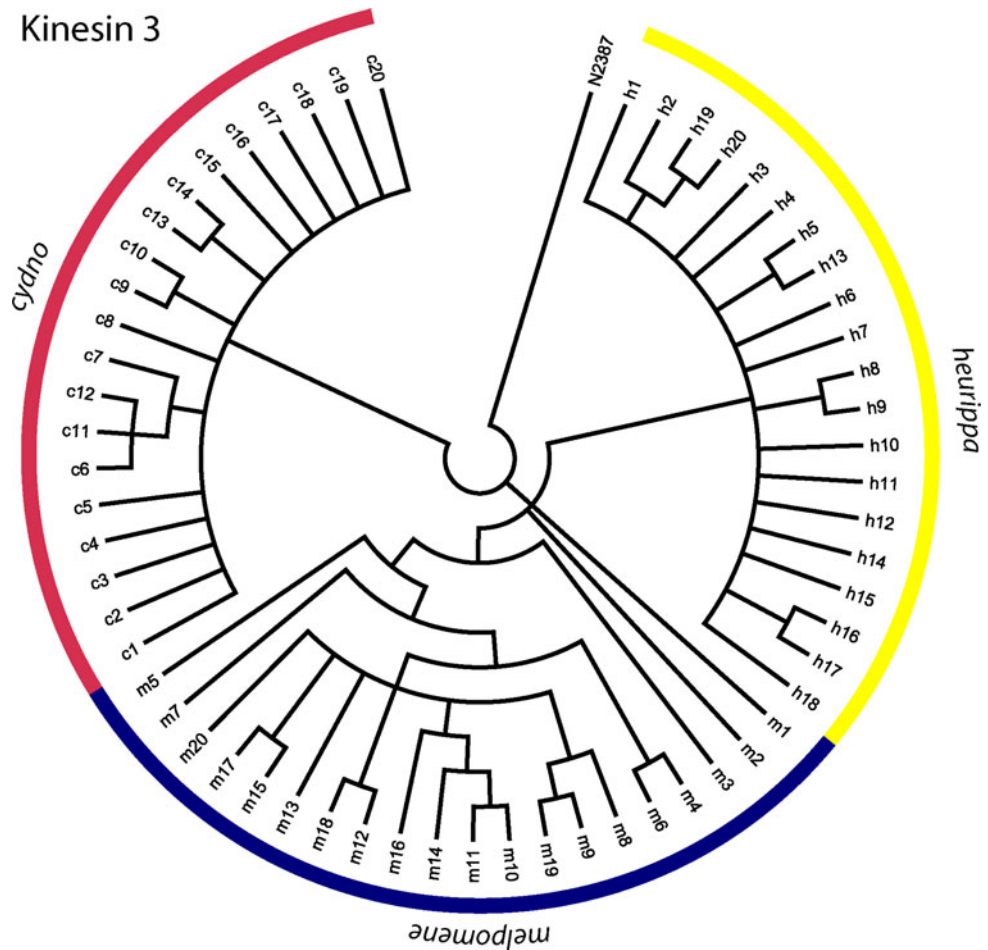
region at the 3′ end of the *kinesin* gene with 14 single nucleotide polymorphisms (SNPs) that are shared between *H. melpomene* and *H. heurippa,* which the authors argue represents conclusive evidence of hybridization between those taxa. They describe this pattern as a "magic trait" (Gavrilets, 2004): a feature that promotes speciation by both causing assortative mating and experiencing divergent natural selection in a polymorphic population. One reviewer of my manuscript suggested that I withdraw the paper in light of this "molecular evidence for adaptive introgression during hybrid speciation" (Salazar et al. 2010). I have manifestly not obliged the reviewer's desire, but instead will briefly take stock of the new evidence here.

Salazar et al. (2010) is an example of several disturbing emergent trends in genomic-era publications. The paper alludes to analysis of an enormous amount of data: nearly 45 kb of sequence from 30 individuals, representing nearly 3,000 individual GenBank accessions. The sheer quantity of data entails a somewhat telegraphic description of analytical methods, that makes results difficult to evaluate critically. For example, ten specimens each of *H. heurippa,* *H. m. melpomene* and *H. cydno cordula* were processed to produce a total of sixty terminal entities ("alleles") for

each locus, but it is never explained how the genomic DNA of individual butterflies was segregated for amplification and sequencing of two separate alleles from each diploid individual. Nor are these "alleles" labeled so that the composite genotypes of individual butterflies may be reassembled.

It would be tangential to the aim of this paper to conduct a comprehensive critical reappraisal of the diversity of analyses performed in Salazar et al. (2010). Perhaps most salient to the data analyses presented above are several phylogenetic trees (their Fig. 4) representing inferred relationships among "alleles" for three gene segments linked to the putative site of the gene encoding the red forewing band of *H. melpomene* and other red-banded *Heliconius* (Baxter et al. 2010): sorting nexin, 5′ *kinesin*, and 3′ *kinesin*. The authors state that their rooted 3′ *kinesin* tree "shows *H. heurippa* forming a well-supported and derived clade within *H. melpomene*" (note that the other two gene trees presented were not rooted with an outgroup). Reanalysis of the 3′ *kinesin* data (generously provided by C. Salazar) shows that the strict consensus MP tree (Fig. 10) is not the same as that shown in Salazar et al.'s Fig. 4. Unfortunately, it is not possible to directly

compare the topologies from these two analyses, because Salazar et al. did not label their terminals, but it is evident from Fig. 10 that rather than being a "derived clade within *H. melpomene*," the *H. heurippa* "alleles" form a basal polytomy with two *H. melpomene* "alleles" and another clade containing the other 18 *H. melpomene* "alleles." The branch support for the *melpomene + heurippa* clade is 9, a relatively high value, but it is important to bear in mind that the gene region analyzed (6,493 bp) was specifically circumscribed because it contained a number of fixed character states supporting a *heurippa + melpomene* relationship.

As noted, the 3′ *kinesin* data set includes an outgroup sequence from the closely-related silvaniform *H. numata*. While one outgroup is certainly better than none (as in Salazar et al.'s sorting nexin and 5′ *kinesin* trees), it is evident from recent analyses of other *Heliconius* genes that inclusion or exclusion of relevant additional taxa can impact phylogenetic relationships among members of the ingroup (cf. Brower 1996a vs. Beltrán et al. 2002 vs. Fig. 3, above). Nor is it clear that samples of the ingroup taxa from a single locality (and collection date?—this is not specified) adequately represent the genetic diversity present in the entire distributions of the three species. For example, it would be very interesting to compare the current data to the 3′ *kinesin* sequence from a form of *H. melpomene* lacking a red forewing band, and also to other putative *H. cydno* cognates with red wing pattern elements, such as *H. timareta* and *H. tristero*.

Stepping back from these details, it is valuable to recall the original *H. heurippa* hybrid speciation hypothesis (Mavárez et al. 2006) and consider how this evidence bears upon it. It would seem that the traditional mosaic hybrid genome idea is soundly refuted: as Salazar et al. (2010) state of most of their compared gene regions, "these data do not strongly support a hybrid speciation scenario, but are more consistent with either recent gene flow among the three species or shared ancestral polymorphism." By contrast, they argue, the 3′ *kinesin* region, with its 14 shared SNPs between *H. heurippa* and *H. melpomene*, is "the most convincing molecular evidence to date for homoploid hybrid speciation in animals." However, they also note that only one of the SNPs represents a nonsynonymous site, and state that "there was no significant evidence for selection on the locus." "Magic traits," as stated above, are supposed to facilitate speciation by promoting selective differentiation of polymorphic lineages. It is hard to envision how 13 silent polymorphisms and a neutral amino acid substitution could accomplish that.

Thus, while the shared 3′ kinesin SNPs are consistent with the introgression interpretation, Salazar et al. (2010) present no evidence either that these SNPs have anything to do with color pattern (other that they are located in a region of a chromosome to which a wing pattern gene has been

mapped), nor that they are under selection, both fundamental criteria for "hybrid trait speciation" in this system. Interpretations of the various sources of evidence evaluated above are enthusiastically cited as though they were facts in overwhelming support of the HHS scenario, rather than a confection of equivocal, irrelevant or even contradictory data, as I have shown. The efforts of these authors are truly heroic, but it is unfortunate that their enthusiasm to confirm a hypothesis conceived in the Gilbert lab in the 1980s appears to have blinkered them to the consideration of alternative interpretations of the evidence.

> "What characterizes the empirical method is its manner of exposing to falsification, in every conceivable way, the system to be tested. Its aim is not to save the lives of untenable systems, but, on the contrary, to select the one which is by comparison the fittest, by exposing them all to the fiercest struggle for survival." (Popper 1965).

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