

THE SPECIES BOUNDARY IS A CONTINUUM:
NATURAL HYBRIDIZATION IN HELICONIINE BUTTERFLIES

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Abstract – Interspecific hybrids occur regularly in *Heliconius* and *Eueides* (Lepidoptera: Nymphalidae) in the wild: 24% of the 68 species of Heliconiiti are involved. Hybridization is, however, rare on a per-individual basis. For species in parapatric contact, phenotypically detectable hybrids may be 10% of the population, but for species in sympatry, hybrids usually form less than 0.05% of individuals. In well-studied cases, backcrossing occurs in the field and fertile backcrosses have been verified in insectaries, which indicates that introgression is likely. The evolutionary importance of such hybridization is as yet unknown; it may potentially contribute to adaptive evolution and speciation, as is known from other taxonomic groups. Hybridization between species of *Heliconius* appears to be a natural phenomenon, and there is no evidence that it has been enhanced by recent human habitat disturbance. Nor is there any convincing reason to believe that hybridization is always a result of secondary contact; it could as easily be a natural outcome of gradual divergence in parapatry or sympatry. The rate of hybridization decreases approximately exponentially with genetic distance between species. Species that coexist in sympatry therefore form part of a natural continuum with geographic races in terms of hybridization rates or probability of gene flow, the only difference being that genetic distances between species are usually somewhat greater and their rates of hybridization are usually somewhat lower. This finding implies that processes leading to speciation are continuous, rather than sudden, and that they are the same as those operating within species, rather than requiring special punctuated effects or complete allopatry. The perception, as under biological or some versions of phylogenetic species concepts, that species of sexual organisms are qualitatively distinct from geographic races and “real” in terms of phylogeny or a lack of hybridization and gene flow is illusory. Instead the probability of hybridization and gene flow declines continuously and approximately

exponentially with genetic distance across the species boundary, as in prokaryotes. In spite of these apparent philosophical problems, named species in heliconiines, as in other groups, remain useful for predicting ecological, behavioural and genetic characteristics of biological diversity.

Since the Modern Synthesis, the predominant definition of species has been the so-called “biological species concept” (Mayr, 1942, 1963, 1982). Under this concept, members of the same species “actually or potentially interbreed” (Mayr, 1963), whereas members of different species cannot interbreed. Most recent theories about hybridization and speciation claim to have been elaborated and tested using this idea (e.g. Mayr, 1942, 1970; Barton and Hewitt, 1989; Coyne, 1994; King, 1993; Harrison, 1993; Futuyma, 1998, Howard and Berlocher, 1998). However, “... taxa that remain distinct despite gene exchange have in fact been classified as separate species even by the originators of the biological species concept. Thus there is a clash between two views of species; one is based on the pattern of gene flow, and the other on the maintenance of a cluster of phenotypes ... stable to invasion by foreign genes” (Barton and Hewitt, 1989). To define and study the evolution of species, we need to acknowledge and understand facts about interspecific hybridization and gene flow in nature.

The biological species concept was chiefly intended to be applicable to animals, because plants were well known to hybridize much more readily than animals (Mayr, 1942:122, 1963:129). Eukaryotes and most prokaryotes share DNA as a genetic material and undergo similar evolutionary forces. We might therefore expect species to differ in degree, perhaps, across the tree of life, rather than in kind. It would be most parsimonious to expect that a single idea of species will suffice for all organisms, and at least for all sexual organisms. However,

botanists have frequently rejected the biological species concept, both because hybridization rates are higher in plants (Stace, 1991), often resulting in speciation (Otto and Whitton, 2000) and because plant populations seem much more subdivided than those of animals (Raven, 1976). More recently, Mayr (1992) investigated whether the biological species concept applies even to most plants. He surveyed the local flora in 27 square miles around Concord, Massachusetts, and suggested that even plants largely conform to the biological species concept. This work has been criticized (Mitchell et al., 1993) because a lack of evidence for hybridization in a small area is not necessarily good evidence for a general absence of hybridization over the entire range of each species.

Much more is now known about the genetics and ecology of the species boundary in animals than at the time of the modern synthesis. The discreteness, and “reality” of species is being eroded both below and above the level of species. Below the species level, forms are now known which can apparently remain distinct in spite of potential or actual gene flow.

Examples are: host races in phytophagous insects (Bush, 1993, 1994; Claridge et al., 1997; Feder et al., 1998; Via, 1999; Emelianov et al., 2001) and other parasites (e.g. Gibbs et al., 2000; Anderson 1993, 1995) or ecologically or sexually divergent coexisting forms of animals as diverse as sea urchins (Quicke et al., 1983), cicadas (Simon et al., 2000), fish (Schluter, 1995; Seehausen et al., 1997; Lu and Bernatchez, 1999), dolphins (Wang et al., 1999) and killer whales (Ford et al., 1998). There is perpetual doubt about the status of related forms which replace one another geographically. New molecular evidence, coupled with revised species concepts has led to many readily identifiable taxa that were formerly regarded as geographic subspecies becoming upgraded to full species (Cracraft, 1997; Zink, 1996), even though hybridization in contact zones has been documented in many such cases. Above the

species level, we are beginning to appreciate that hybridization is not at all rare. Data collected over several centuries on birds and butterflies shows that animal hybridization can quite common per species, even though rare on a per individual basis. Across the globe, 9% of the world's bird species are known to hybridize with at least one other species (E.N. Panov in Grant and Grant, 1992). The figure is similar for European butterflies as a whole (11%; Guillaumin and Descimon, 1976), and North American Papilionidae (Lepidoptera) hybridize at very much of the same order of magnitude (6%; Sperling, 1990). Known natural hybridization between European mammal species totals 5.7% in 175 species (extracted from Gray, 1972; MacDonald and Barrett, 1995; we exclude hybridization between forms normally considered subspecies such as *Mus musculus musculus* and *M. m. domesticus*). A well-known case of hybridization occurs between the mountain hare *Lepus timidus* and common hare *L. europaeus*. In Scandinavia, this hybridization has apparently led to a flow of mitochondrial genomes (Thulin et al., 1997). Even the biggest animal that has ever existed on this planet, the blue whale *Balaenoptera musculus*, hybridizes with its close relative the fin whale *B. physalus*: there is also good genetic evidence for successful backcrossing in this pair of species (Arnason et al., 1991). In *Drosophila*, hybridization has been documented between 8 pairs of species (Gupta et al., 1980). Because hybridization is known from the three species groups most studied by geneticists (the hybridizing pairs are: *heteroneura-sylvestris*, *melanogaster-simulans*, and *pseudoobscura-persimilis*), and given that most *Drosophila* are very poorly known, it seems likely that hybridization in *Drosophila* is commoner than these few records indicate. Recent evidence has suggested that post-speciation gene flow between members of the *pseudoobscura* group and *melanogaster* of *Drosophila* has resulted in divergent genealogies of different genes in the same species (Wang et al., 1997, Ting et al., 2000; Wu, 2001).

Mayr (1942, etc.) was a pioneer in documenting hybridization in a modern evolutionary context, and has estimated that one in ten species of North American land birds are involved in hybridization (Mayr and Short, 1970). However, Mayr also pointed out that rates of hybridization per individual are very low in animals (Mayr, 1963: 114). Mayr (1942, 1963, 1970) therefore viewed hybrids and intermediates between species as due to “breakdown in isolating mechanisms” primarily brought about by disturbance caused by humans, and more rarely due to natural catastrophes. To Mayr, hybridization was a secondary phenomenon of little or no evolutionary importance (e.g. Mayr, 1963: 133), rather than a natural stage of a continuous speciation process. Associated with this view was the idea that, because hybrids were unnatural, actual intermediate stages of speciation should be seen only rarely in nature. Hybrid zones between differentiated parapatric species or subspecies were therefore interpreted as zones of “secondary contact”: differentiation was assumed to have occurred in allopatry. Hybridization was even defined by Mayr as “the crossing of individuals belonging to two natural populations that have secondarily come into contact” (Mayr, 1963: 110).

Mayr’s views led to an emphasis on allopatric speciation, and especially rapid “founder event” (Mayr, 1954, 1963) or “punctuated equilibrium” (Gould and Eldredge, 1977) models that explained the apparent “reality” of species, that is the perceived lack of coexistence of intermediate stages of speciation. Criticisms of these interpretations of divergence and speciation have been thoroughly dealt with by Endler (1977), Barton and Charlesworth (1984); see also Mallet (1993), Turner and Mallet (1996), and Mallet and Turner (1998) for critiques of allopatric refugium models in the evolution of *Heliconius* and neotropical biotas. Perhaps because hybrids and other “aberrations” were viewed, under Mayr’s influential

paradigm, as unnatural, it is hard to find hybrids figured in field guides dating from the 1930s to the 1970s. Upon being asked why his vast collection of European butterflies contained not a single aberration, Lionel Higgins, author of the famous European field guide (Higgins and Riley, 1970) replied “Well – they’re not perfect, are they?” (Salmon, 2000: 221). In its defence, the Mayr viewpoint was an understandable reaction to decades of typological “variety-hunting” which had had disastrous effects on nomenclature from about 1890-1950 (Salmon, 2000), but it also suppressed an interest in the continuity between species, geographic races, and varieties that had followed the Darwinian revolution. Conservation policies dating from the 1960s similarly discriminated against hybrids or hybridizing populations: hybrids were viewed as improper, impure entities not suitable for conservation, even though this legislation was later regarded as too extreme by supporters of the biological species concept (O’Brien and Mayr, 1991). The biological species concept, originating in the late 1930s and 1940s, seemed to encourage a view of “good species” similar to the eugenic ideas of the time as applied to human races, and hybridization between species was regarded as an unimportant anomaly to be ignored when discussing the “true nature” or “essence” of species and their origins (Mallet, 1995, 2001a; Mallet et al., 1998a).

Alternatively, hybrids and hybridization are a natural outcome of speciation; they may be a primary expression of the progress of differentiation, rather than an unnatural secondary phenomenon. An encyclopaedic knowledge of the many intermediates between populations across vast geographic regions led Darwin (1859) and Wallace (1865) to form their own more pragmatic view of species as assemblages of individuals between which few intermediates existed. In their view, speciation resulted when intermediates were out-competed by extreme forms; indeed it was this view that led to their theory of speciation by natural selection.

Today, there is a revival of interest in biodiversity in all its variety, including a strong interest among biologists in hybrid zones and hybridization from an evolutionary point of view, and modern theories of speciation by natural and sexual selection against intermediates are close to Darwin's own views about speciation (e.g. Higashi et al., 1999; Dieckmann and Doebeli, 1999; Kondrashov and Kondrashov, 1999; Kirkpatrick and Ravigné, 2001). Furthermore, there is renewed interest in reinforcement, whereby selection to avoid hybridization leads to divergence in mate choice (Howard, 1993; Liou and Price, 1994; Butlin, 1995; Kelly and Noor, 1996). Hybrids and discussions about them are once again appearing in the more complete field guides, and the overall prevalence of hybrids is beginning to be appreciated (e.g. Grant and Grant, 1992). Hybridization is becoming increasingly important in discussions of the nature of animal species and their evolution (Templeton, 1989; Barton and Hewitt, 1989; Rieseberg and Brouillet, 1994; Arnold, 1997; Mallet, 1995, 2001a; Jiggins and Mallet, 2000), as well as in applied fields such as conservation (Rojas, 1992; Allendorf et al., 2001) and in safety assessments for the release of transgenic organisms (Mikkelsen et al., 1996). Hybridization is especially important in conservation for two opposing reasons. Firstly, hybridization with an introduced alien can often dilute endemic rarities, particularly on islands (Levin et al., 1996; Rhymer and Simberloff, 1996). Secondly, endangered species legislation typically views hybrids as much less valuable than "pure" species; however, if hybrids are natural outcomes of divergence, there is no reason why populations which include natural intermediates should not be valued as highly as populations which are naturally "pure" (Allendorf et al., 2001). Indeed, hybrid zones between parapatric forms might could become viewed as endangered phenomena that are worth conserving because of their restricted range of distribution.

As a part of this movement, many such studies are now being done on hybrid zones (Barton and Hewitt, 1989; Harrison, 1993; Butlin, 1998) and on host races (Feder et al., 1994; Via, 1999; Emelianov et al., 2001). However these studies have concentrated mainly on zones or cases of sympatric hybridization where hybrids are abundant enough to sample easily. Under the biological species concept, hybridization in most hybrid zones is between geographic races, and arguably demonstrates a failure to complete speciation, rather than giving many clues to speciation or species maintenance. Although difficult to carry out, it would now be useful to have more studies of natural hybridization between taxa generally recognized as species (i.e. between which natural hybrids are rare compared to parental forms from the same area).

Here, we review natural interspecific hybridization in a particularly well studied group, the heliconiine butterflies. Our survey contributes to a reappraisal of the nature of species and speciation such that sexual and dioecious animals, as well as plants and bacteria, can all be seen to obey the same fundamental laws of gene flow and introgression. Building on a firm base of biological work on *Heliconius* and its relatives, these data give unrivalled information on the continuum between polymorphisms, races, and species.

Natural hybridization between species of Heliconius and Eueides

Heliconius and related genera are currently classified as subtribe Heliconiiti in the Heliconiinae, a subfamily of Nymphalidae (Brown, 1981; Harvey, 1991; Brower and Egan, 1997; Penz, 1999). Their bright colours have led to the species becoming well-represented and highly prized in collections (Brown, 1979). Detailed studies on ecology, behaviour, systematics, coevolution, mimicry, and speciation of this group have been carried out

(reviewed by Brown, 1981; Turner, 1981; Gilbert, 1991; Mallet, 1993; Mallet et al., 1998a; Jiggins et al., 2001b); studies of the inheritance of racial colour pattern differences (Sheppard et al., 1985) considerably aid the detection and understanding of interspecific hybrids. Scattered reports of natural hybrids between *Heliconius* species have been published (e.g. Ackery and Smiles, 1976; Brown, 1976, 1979; Descimon and Mast de Maeght, 1984; Brown and Fernandez-Yepe, 1985; Holzinger and Holzinger, 1994; Jiggins et al., 1996; Mallet et al., 1998a), but this is the first attempt to collate and analyse interspecific hybridization across the Heliconiini. We here review hybridization for the whole subtribe, and report many new hybrids, including previously undocumented examples within the genus *Eueides*.

We put the hybrids into their phylogenetic context within the genus. According to both morphological (Brown, 1981; Penz, 1999) and molecular (Brower, 1994; Brower and Egan, 1997) evidence for the phylogeny of *Heliconius*, the sub-tribe can for our purposes be divided into a number of phylogenetic sub-groups (Fig. 1). There is a basal group of small genera, currently of unclear affinities (*Philaethria*, *Agraulis*, *Dione*, *Podotricha*, *Dryadula*, *Dryas*). The genus *Heliconius* and allies form the bulk of the group, consisting of *Eueides* and *Heliconius sensu lato* as sister taxa. *Heliconius sensu lato* consists of three major groups. First there is a probably paraphyletic “basal group” that contains two small segregate genera (*Neruda*, *Laparus*), as well as the *wallacei/burneyi* and *xanthocles/hecuba* groups within *Heliconius sensu stricto*. The second group is the monophyletic *melpomene*-silvaniform group, consisting of two probably monophyletic parts: (i) a “silvaniform” subgroup, in which *atthis*, *hecale*, *ethilla*, *ismenius*, *numata*, and *pardalinus* are mainly Müllerian mimics of the yellow and brown “tiger pattern” Ithomiinae, while *besccke* and *elevatus* have red and yellow more typically heliconiine mimicry patterns; (ii) a *melpomene* subgroup containing *Heliconius*

melpomene and *H. cydno*, as well as a handful of segregate “species” – *timareta*, *tristero*, *heurippa*, and *pachinus* – which are most closely related to *cydno* and are considered here as geographically disjunct subspecies of *cydno*. The final group also consists of two parts – the *erato* sub-group and the *sara/sapho* sub-group – and is also monophyletic (Fig. 1).

MATERIALS AND METHODS

Detection and definition of “hybrids”

Data and photographs of specimens noted here from literature records, museums, and private collections were collated into a database (Mallet et al., 2001). There may be many other existing specimens of interspecific *Heliconius* hybrids in public and private collections not visited by us, but we believe our coverage is adequate for the purpose of documenting the extent of hybridization across the genus.

Closely related species of *Heliconius* almost always belong to distinct mimicry rings (Turner, 1976), suggesting that a shift in mimicry plays a role in speciation and the maintenance of specific distinctness thereafter (Turner, 1981; Mallet et al., 1998a; Jiggins et al., 2001b).

Therefore, putative hybrids between such species are mostly easy to identify. Having located potential hybrid specimens, we must decide whether they constitute hybrids or intraspecific variants. This is not always easy. Hybridization or introgression between species creates difficulties in defining the species themselves, let alone their hybrids and intergrades. We here define the term “hybrids” and “pure species” operationally via morphology and knowledge of genetics: “pure species” are usually known from hundreds of individuals, and, in heliconiines, their biology will usually be known. Even if rare, a pure species is often numerous in some areas, and only rarely is polymorphic (exceptions to this rule exist: for example in *H. numata*

and *H. cydno* [Brown and Benson, 1974; Joron et al., 2001; Kapan, 2001; Mallet, 2001b]). “Hybrids” are highly unusual phenotypes from well outside the normal range of variation of known species that are most easily interpreted as progeny of crosses between two known species because of a combination of traits from each. First generation (F_1) hybrids are usually easy to distinguish providing the parents are sufficiently distinct in morphology. However, if there are backcrosses, and if the colour pattern differences depend on relatively few loci (as is the case between *H. erato* and *H. himera*; see Jiggins et al., 1996; Jiggins and McMillan, 1997; Mallet et al., 1998a; and between *H. melpomene* and *H. cydno*: see Gilbert, 2001; Naisbit, 2001), backcross and F_2 progeny can potentially recreate the full range from parental phenotypes to F_1 -like. Therefore, when we use the term “ F_1 ”, we mean that the phenotype could have been produced as a first generation cross (though it may sometimes actually have been produced by a backcross or F_2), and by “backcross” we mean all other hybrids that do not have the F_1 phenotype (see also Mallet et al., 1998b). Since hybridization is usually very rare for any pair of species, it is likely that almost all “ F_1 s” are actually first generation hybrids, and most “backcrosses” are offspring of actual F_1 s backcrossed to a parental species (although some backcrosses will be missed among “ F_1 s” and among “pure” specimens).

Speciation requires genetic divergence, but there is always the possibility that alleles now common in one species have remained as polymorphisms at low frequency in a sister species. It is therefore hard to differentiate rare ancestral polymorphism, potentially augmented by mutation, from polymorphisms introduced by introgression (i.e. hybridization and backcrossing). We used two major criteria for hybridization. First, specimens showing two or more presumably independently intermediate characters strongly suggest hybridization as a cause. If rare ancestral alleles or mutation-derived phenocopies of genetic traits in another

species are present in the absence of hybridization, it is very unlikely that two or more such traits will be found in the same individual provided that genetic loci coding for the variation are independent; for example, if each putatively hybrid trait has frequency 0.1% (a generous estimate for the frequency of the commonest hybrid phenotypes, for example the frequency of red forewing bands putatively from *Heliconius melpomene* within *Heliconius cydno* – see below), two such traits should be found at a frequency of one in a million, and three traits at a frequency of only one in a billion. In true hybrids, on the other hand, hybrid traits should normally be found together.

As well as the correlation of hybrid phenotypes within individuals, we also used correlations between the location of capture of hybrids and the geographic distributions of putative parental species and races. For instance, a putative hybrid between *Heliconius cydno* and *H. melpomene* would be highly unlikely in the Cauca valley of Colombia or in Brazil because only *cydno* is present in the Cauca, only *melpomene* in Brazil. The existence of red *melpomene*-like forewing bands in specimens otherwise similar to *H. cydno* might be due to ancestral polymorphism, because the two species are sister taxa (Fig. 1). If due to hybridization, such phenotypes should be present only from areas where *H. melpomene* is present and has a red forewing band (as they are). This geographic aid to hybrid identification is considerably enhanced because the species acting as parents of hybrids consist of as many as 30 very strongly divergent geographic races distinguished by colour pattern. Normally, we identified hybrids by means of their external phenotype alone, but in some of the commonest cases of interspecific hybridization we have genetic evidence of hybrids, as detailed in Appendix 1.

The potential for fraudulent hybrids manufactured in captivity

A possible consequence of the interest that these hybrids now generate on the international butterfly market is that there is a financial incentive to offer captive-bred hybrid specimens for sale with fraudulent locality labels. One of us (WN) has identified probable insectary-reared hybrid specimens for sale being passed off as wild-caught, which he declined to buy.

Fraudulent hybrids seem most likely from the late 1980s onwards, when “butterfly houses” and commercial breeding facilities in the tropics supplying livestock became more widespread. The specimens tabulated and figured here were largely collected before this time.

We can be certain that the older specimens are genuine, since multiple-generation *Heliconius* culture was unknown before the 1950s, and practised only by a handful of academic *Heliconius* biologists before the 1980s. Post-1980s specimens could be more dubious, and we have used only specimens whose provenances seem impeccable; we have visited key sites in Colombia, Costa Rica, and Panama, and have personally communicated with some of their collectors (León Denhez, Diego Torres, and Rodrigo Torres in the Cali area, Ernesto Schmidt-Mumm in Bogotá, José Urbina in Otanche, and Adolfo Ibarra in México).

Mitochondrial DNA divergence

DNA sequences have been obtained for many of the species of Heliconiiti (Brower, 1994; Brower and Egan, 1997). In this paper we use data from 942 bp of mitochondrial DNA of the genes COI, leu-tRNA, and COII obtained in these studies, supplemented by some new data obtained by Beltrán et al. (2001). Mitochondrial sequences in Lepidoptera are a particularly useful standard for genetic divergence both within and between species, for two reasons. Firstly, there is thought to be no recombination between mitochondria, due to unisexual inheritance; thus genetic divergence is unlikely to be affected by occasional introgression.

Secondly, in *Heliconius*, as in many Lepidoptera (Jiggins et al., 2001a; Naisbit et al., 2001; Sperling, 1990), hybrid females are often sterile, an example of Haldane's Rule. Haldane's rule will ensure that introgression of maternally inherited mitochondria is prevented at an earlier stage of speciation than for nuclear loci; the latter may be transferred between species by backcrossing of male hybrids.

RESULTS

Hybrid specimens examined are databased in Table 1. We figure examples of specimens either not previously published or little-known (Fig. 2). Colour photographs of specimens figured in Fig. 2 as well as most of the rest of those in Table 1 are illustrated elsewhere (Mallet et al., 2001). Detailed lists of known hybrid specimens, discussions of the specimens, laboratory evidence for hybridization, and estimates of frequency in the most abundant forms are given in Appendix 1. To save space, we present only hybrids; pure forms are illustrated in several useful books which cover the genus (Smart, 1976; D'Abrera, 1984; Holzinger and Holzinger, 1994).

Hybrids are unknown from the basal genera of the Heliconiini, and from *Neruda*, *Laparus* and the basal group of *Heliconius*, all of which consist of distantly related species highly divergent at mtDNA (Appendix 2). Hybrids are known only within the three recent radiations: *Eueides*, the *melpomene*-*silvaniform* group, and the *erato-sara-sapho* group.

DISCUSSION

Existence and geographic relations of hybridizing species

It is clear from our data that interspecific hybridization regularly occurs within *Eueides* and *Heliconius*. A few cases of hybridization are in doubt, particularly some of those involving *H. elevatus*. In other cases, the parents of obvious hybrids are in doubt, for example within *Eueides* or the silvaniforms (Appendix 1). It is even possible that a few of the more recent hybrids are “manufactured” in captivity. Yet most of the specimens we cite here are natural interspecific hybrids of known parentage. We have good evidence for this from many different collectors, and from a large geographic range. Although we have uncovered a substantial number of previously unknown hybrids, previous authors have come to similar conclusions about many of the earlier specimens (see references in Table 1).

Most hybrids recorded here are between distinct forms that overlap substantially in their distributions, and which are therefore generally considered to be different species. In two cases, *H. erato* x *H. himera*, *H. charithonia* x *H. peruvianus*, the forms were traditionally considered to be subspecies (e.g. Brown, 1979). We consider these to be species operationally, on the grounds that, in areas of contact intermediates are rare compared with parental forms (Appendix 1; Jiggins et al., 1997; Jiggins and Davies, 1998; Mallet et al., 1998b; Jiggins and Mallet, 2000). In a third case, *H. cydno* x *H. pachinus*, the existence of hybrids between forms traditionally regarded as species almost certainly represents occasional contact between otherwise disjunct subspecies across the central cordillera in Costa Rica. The destruction of suitable habitat by agriculture and urbanization has now made hybridization between these taxa almost impossible to detect except for occasional aberrant specimens on either side (Appendix 1; Gilbert, 2001). Although listed for completeness in Table 1, these *cydno* x *pachinus* hybrids are not considered as interspecific hybridization in the analysis.

The most abundant hybridizations are between sister taxa, for example between *H. melpomene* and *H. cydno*. However, there is plenty of evidence for hybridization between non-sister species, for example between *H. numata* and *H. melpomene*. Hybridization of *ismenius*, *hecale*, *atthis*, *melpomene* and *cydno* in insectaries by Gilbert (2001) and Jean-Pierre Vesco (pers. comm.; see also Appendix 1) confirm that non-sister hybridization is possible. Similarly, *H. erato* hybridizes with its sister species *H. himera* wherever the two meet, but also with *H. charithonia*, which is distantly related (Fig. 1). The two types of *Eueides* hybrids involving *isabella* must logically involve at least one non-sister hybridization.

Hybridization and introgression between species is often associated with rapid adaptive radiation on islands; for example in the Darwin's finches on the Galapagos, the Hawaiian silverswords (Compositae) or Hawaiian *Drosophila*, the birds of paradise in New Guinea, cichlids in African lakes, or fish colonists of glacial lakes in the Northern Hemisphere (see Introduction). This study shows that hybridization is not just a feature of island radiations: *Heliconius* is a highly successful genus in the mainland and lowlands of the continent with the most diverse biota on earth. However, hybridizing species are all within *Eueides* and the two major "non-basal" groups of *Heliconius*. These are the three monophyletic groups that appear to be radiating most rapidly compared with sister groups within the subtribe (Fig. 1); thus, hybridization in *Heliconius* does seem to be a feature of relatively recent radiations, even though not necessarily on islands.

Frequency of hybridization as a fraction of the population

It is clear that the frequency of hybridization is low on a per-individual basis, as is the case in birds: “Hybrids form in only a very minute percentage of the individuals in all the species mentioned, and I know of no case in which the occurrence of hybrids has resulted in a blurring of the border line between these species” (Mayr, 1942: 262). On the other hand, as Mayr admitted, such statements contains a tautology: “The definition of hybridization as ‘the crossing of individuals belonging to two different species’ results in circular argument because the decision whether or not to include two populations in the same or in two different species may depend on the occurrence of hybridization” (Mayr, 1963: 111). Obviously, hybrids must be rare whether the biological species concept or even a character-based criterion of species is used, because a total “blurring of the border line” would result in a single species being recognized. Although hybrids must be rare, it is not circular to estimate *how* rare they are. Mayr (1963: 114) estimated that only one out of 60,000 specimens of birds (across all species) was a true interspecific hybrid. In the birds of paradise, about 30 hybrids were found in 100,000 skins (Mayr, 1942), or 0.03%. These values seem about right for *Heliconius* as well. We have estimated that morphologically detectable hybrids between *H. erato* and *H. himera* form 9.8% of the population in centre of the best-studied hybrid zone (Mallet et al., 1998b), but this is unusually high, and occurs only between two species that replace one another across an extremely restricted hybrid zone. For the closest pair of sympatric hybridizing species, *Heliconius melpomene* and *H. cydno*, the fraction of hybrids in natural sympatric populations is of the order of 0.05% (Appendix 1).

Frequency of hybridization as a fraction of species

On the other hand, the frequency of hybridization per species is high. In all 16 recognized species out of 46 *Heliconius sensu lato* (including *Laparus* and *Neruda*) are involved in

hybridization, or 35%. As discussed above, the *cydno* group of species, and *hortense-clysonymus* may each be considered a single species. If these changes are made, hybridization involves 13 species of a total of 41, giving 32% of species hybridizing. For *Eueides*, three out of 12 species are involved in hybridization, a fraction of 25%. Overall, there are 68 species of Heliconiiti, of which 16 species hybridize (after lumping *cydno* and *clysonymus* group species: otherwise, 19/73 species hybridize). Thus, about 24% of all Heliconiiti species are involved in hybridization.

Factors affecting rates of hybridization

It is often said that hybridization between species is distributed patchily among taxonomic groups. According to Mayr (1942: 260-263, 1963:126-127), hybrids in birds are more commonly found in highly dimorphic species such as ducks, game birds, and birds of paradise, that are commonly polygamous or have lekking sexual behaviour. Mayr argued that the short contact period between mates led to more “mistakes”. Prager and Wilson (1975) used a molecular clock argument to propose that amphibians and birds could remain compatible enough to hybridize for over 20 million years, whereas mammals lose their capacity for hybridization after only 2-3 million years. These authors argue that regulatory gene evolution preventing hybridization has occurred more rapidly in mammals than in birds or amphibia. Whether or not this is true, the natural fractions of species that hybridize seem not very different between the birds and mammals (9% vs. 6%; see Introduction).

It would be rather surprising if there were no heterogeneity in hybridization among phylogenetic lineages. However, a number of biases that affect estimates of hybridization rate may inflate the apparent heterogeneity. Firstly, sexually-selected colours often differ strongly

between species in both sexes, so that hybrids could merely be more detectable than between sexually monomorphic birds. As an example, many hybrids are known from the American warblers (Parulidae); about 24% of a total of 116 species are involved (Curson et al., 1994). These birds are pair-formers, but are often brightly coloured (and also sexually dimorphic) so that hybrids can be detected easily. On the other hand, in a recent treatment, no hybrids were reported between members of the ecologically similar Eurasian sylviid warblers (Parmenter and Byers, 1991). Given the difficulty of identifying many of the species, let alone their hybrids, this is perhaps not so surprising. The apparently lower hybridization rates in mammals, which are mostly small, dowdy, and nocturnal, could be due to a similar bias. Secondly, if polygynous mating systems and sexual selection affect rates of speciation, it could be that a greater rate of hybridization is caused merely by the fact that taxa with gaudy, sexually dimorphic colour have younger and more compatible species. In the Heliconiiti, the lower rates of hybridization in the phenotypically homogeneous *Eueides* and tiger silvaniforms could be due to a similar bias.

Is hybridization natural?

Mayr (1963, etc.) argued forcefully that hybridization in the wild was normally due to a “breakdown in isolating mechanisms”, particularly after human disturbance of the species’ normal habitat. Although this view arises from a somewhat dated view that “isolating mechanisms” are traits beneficial to the species as a whole (e.g. Mayr, 1988), the argument that hybridization is less intense in pristine habitats is still prevalent today. Clearly, humans can alter habitats in ways which could increase levels of hybridization (or decrease them; cf. *cydno* x *pachinus*, see above). Today there are frequent conservation problems when introduced taxa hybridize with native relatives (Rhymer and Simberloff, 1996; Roush, 1997).

In *Heliconius*, most hybrids are so rare that we cannot for certain say whether they are becoming commoner. However, many of the hybrid specimens recorded here were collected in the last century or early this century, long before the major episode of rainforest destruction caused by the invention of the chainsaw. Human activities in rainforests can alter the growth of *Passiflora* foodplants, and can greatly change the densities of *Heliconius*, and have probably done so since prehistoric times. The ecology of the two pairs of species from which we have the most hybrids is well known. In the first pair, even though the species overlap extensively, *H. cydno* is normally found in small lightgaps or in the understory of lowland tropical forest, and is commoner in uplands to about 1800m than *melpomene*. *Heliconius melpomene*, on the other hand, is commoner at lower altitudes and in more open habitats, such as at the margins of rivers, in savannahs, or scrubby second growth (Smiley, 1978; Mallet and Gilbert, 1995). Forest destruction might therefore tend to improve life for *melpomene*, while causing *H. cydno* to retreat. However, while there will have been changes of distribution, and possibly even a temporary increase in contact due to invasion of *melpomene* into habitat with declining populations of *cydno*, there should always have been contact between the two species in Central America, western Colombia and Ecuador, and in the valleys and slopes of the Andes. An increase in patchy “edge” habitat may have caused hybridization rates between the two species to have changed, but overlap and resultant hybridization almost certainly occurred regularly without human intervention.

The second pair hybridize in a very different way. *Heliconius himera* and *H. erato* are found together only in very narrow zones of overlap. Once again, there are habitat differences between the species: *H. himera* is found in mainly higher and drier environments than its close relative *erato* in southern Ecuador and northern Peru (Jiggins et al., 1996). There are

contacts in three areas (Appendix 1; Mallet, 1993). In the one of the contact zones, we do not know the exact source of the *H. himera* that hybridizes with the commoner *H. erato* near Rodriguez de Mendoza in N. Peru, so it is unclear whether habitat disturbance has been to blame. In the other two contact zones, in gallery forests in southern Ecuador and along the Río Marañon in northern Peru, it is easier to imagine that contact was more, rather than less extensive before dry forests were felled for agriculture away from the steep ravines to which both *erato* and *himera* are now restricted.

In most other cases of hybridization in the heliconiines there is no obvious way in which hybridization can be blamed solely on human interference, even though human-wrought changes in the neotropics have been extensive over the last century. In summary, there is nothing in the ecology or distribution of any of these species that would lead one to believe that such hybridization started only recently, solely as a result of human habitat disturbance.

A general law of speciation: the non-linear species boundary

Is there any evidence for a well-demarcated species boundary in these butterflies? If species have a discrete “reality” of reproductive isolation, we might expect a sharp discontinuity in reproductive isolation between geographic races and species. In Fig. 3, we plot the numbers of hybrids known between pairs of species against amount of mtDNA divergence. Clearly, rates of hybridization are negatively correlated with the degree of genetic divergence.

Assuming that molecular evolution is relatively clock-like, this implies that the frequency of hybridization is related to the time since divergence, rather than undergoing a hiatus at the species boundary. This relationship even extends to intraspecific levels. Divergence between members of the same species is less than about 2% for this region of mtDNA (Fig. 3), and the

fitted line therefore predicts that tens to hundreds of hybrids between geographic races should be found in collections (Fig. 3), as observed (Mallet, 1993). In heliconiine butterflies, “reproductive isolation” between populations and species is not only about as continuously distributed as genetic distance, the former is also predicted well by the latter.

Although the exact form of the relationship between genetic distance and hybridization probability is not clear from the scanty data available in Fig. 3, the curve is more or less continuous (if somewhat noisy). An increasing failure to hybridize with genetic divergence might be expected to follow an “exponential failure law”, as plotted in the fitted curve of Fig. 3. A similar log-linear decline in gene flow occurs in transformation experiments with bacteria (Roberts and Cohan, 1993). Rather than demonstrating a special law applying only to eukaryotic, sexual species for which reproductive isolation has some meaning, our data shows that heliconiines follow a log-linear failure law similar to that found in normally asexual prokaryotes. The chief difference is slope: *Bacillus* still exchange genes at a thousandth of the within-strain rate even when their chromosomal DNA differs by as much as 20%; in heliconiines, gene flow and hybridization becomes rare (i.e. falls below the single-hybrid “veil line”) beyond about 7% mtDNA divergence. The difference in slope is not surprising in view of the differences in biology: failure of bacterial transformation may be due to a lack of uptake of foreign DNA by the bacterial cell wall (although not in *Bacillus*), or to a failure of the DNA to integrate into the host genome. In heliconiines, failure to produce hybrids depends on behaviour and the probability of mating, and on the fitness of hybrids. Nonetheless, the similarity of the species boundary, in terms of overall shape and continuity in these very different taxa is evident.

Evolutionary importance of hybridization

In *Heliconius* interspecific hybrids that have been studied, females are often sterile (Gilbert, 2001; Naisbit et al., 2001; J.P. Vesco, pers. comm.; *H. erato* x *H. himera* is an exception, see McMillan et al., 1997). This is an example of Haldane's Rule, in which the heterogametic sex (the female in Lepidoptera) suffers greater inviability and sterility than the homogametic sex (the male in Lepidoptera). Although female sterility is a characteristic of hybrids between species such as *H. cydno* and *H. melpomene* (Naisbit et al., 2001), Haldane's Rule Sterility has recently been found between geographic populations considered to belong to the same species and even subspecies (*Heliconius melpomene melpomene*; Jiggins et al., 2001a), indicating that even hybrid intersterility is not an infallible species characteristic (cf. Darwin, 1859).

In spite of female sterility, male hybrids are almost always fertile (Gilbert, 2001; Jiggins et al., 2001a; Naisbit et al., 2001; J.P. Vesco, pers. comm.), and the presence of natural backcross hybrids indicates that introgression may occur. There is clear evidence for natural backcrossing in six pairs of *Heliconius* species (Fig. 3). In the laboratory, backcross broods between *cydno* and *melpomene* and between *erato* and *himera* are fertile, and can be used to introduce genes from one species to another (Gilbert, 2001; Naisbit et al., 2001). In the *melpomene*-silvaniform group, genes from *hecale*, *atthis*, *ismenius*, *melpomene* and *cydno* can apparently be mixed together at will in the laboratory (Appendix 1; Gilbert, 2001). The similarity of allelic frequencies at some loci, and the strong differences at others in *H. himera* and *H. erato* can be explained by selective gene flow at some loci (Jiggins et al., 1997). In plants, introgression has long been suggested to provide variability useful in adaptation (Rieseberg and Wendel, 1993; Arnold, 1997); while in animals, the possibility that introgression could provide potentially adaptive genetic variation has generally been denied

(Mayr, 1963). However, adaptive variation will pass through a hybrid barrier more readily than neutral variation (Barton and Bengtsson, 1986); even a typically low rate of hybridization of 10^{-4} - 10^{-3} would provide potentially adaptive alleles at a rate far higher than provided by beneficial mutations. Recent evidence from Darwin's finches suggests that hybridization contributes strongly to the high heritability of beak shape (Grant and Grant, 1996).

In *Heliconius*, very similar mimetic colour patterns appear in related, non-sister species, even though closest relatives usually differ in their colour pattern (Turner, 1976; Mallet et al., 1998a). For example, apparently homologous "ray" mimicry patterns appear in Amazonian *melpomene*, (*cydno*) *timareta*, and *elevatus*, and also "radiosus" forms of *H. pardalinus*. One possibility is that the rayed pattern is ancestral; but this would require red forewing bands in extra-Amazonian *melpomene*, and in (*cydno*) *heurippa*, (*c.*) *tristero*, and *besckei* to have evolved independently. Multiple independent evolutionary events may be possible on the *Heliconius* genetic background, but it does not seem unlikely that the occasional hybridization and backcrossing we document has led to horizontal transfer of alleles suitable for different mimetic environments. Under this scenario, some of the diversity of mimicry rings achieved by *Heliconius* is due to their ability to hybridize (Linares, 1989, 1998); in Gilbert's metaphor, hybridization supplies *Heliconius* species with an interspecific "shared toolkit" of mimicry genes (Gilbert, 2001).

An important practical consequence of introgression is that conflicts between morphological or molecular characters in phylogenetic reconstruction may sometimes be explained by gene transfer as well as by parallel evolution and errors in phylogeny estimation. A "true" bifurcating phylogeny of closely related species may be unattainable, except as an artificial

consensus of gene genealogies (O'Hara, 1994; Baum and Shaw, 1995; Avise and Wollenberg, 1997; Maddison, 1997). Although individual cases of introgression or horizontal gene transfer among species are now known (Kidwell, 1993; Rieseberg and Wendel, 1993; Syvanen, 1994; della Torre et al., 1997), no multi-locus studies are yet available that cover any taxonomic group. In *Heliconius*, the above prediction that horizontal transfer of adaptive colour pattern genes has occurred will become testable when genes affecting colour pattern are characterized at the molecular level.

In plants, hybrid speciation may involve chromosome doubling (e.g. Otto and Whitton, 2000), or may be due to diploid hybridization (Rieseberg, 1995). Polyploid speciation is virtually absent in higher animals (Otto and Whitton, 2000), but speciation involving hybridization or introgression among diploids remains an untested possibility. Most *Heliconius* have 21 chromosomes, but a few species in the *sapho-sara* group may have as many as 40-60 chromosomes (Brown, 1981), suggesting allopolyploidy or autopolyploidy as a possible cause of speciation. As already mentioned, introgression may explain some puzzling phylogenetic patterns of the distribution of mimicry between species. Because the evolution of novel mimetic patterns very likely contributes both to mate choice and to "post-mating isolation" (Mallet et al., 1998a; Jiggins et al., 2001b), colour pattern transfer between diploids could be important in speciation, as well as in adaptive evolution.

Nihilistic view of species, or post-Modern rebirth?

Perhaps the most important lesson from data on hybridization is that species, or at least the entities to which the term "species" is normally applied, are not completely reproductively isolated, and that speciation does not completely close down gene flow. With time,

reproductive barriers will often become more complete, but they may remain leaky in closely related species, and indeed gene transfer seems likely by hybridization even between non-sister taxa. We could apply a strict interpretation of the biological species concept, and lump all species between which hybrids are known, or at least, between which backcrossing and introgression is possible. However, this radical solution would require uniting virtually the whole *melpomene*-silvaniform clade of *Heliconius*, many of the *Geospiza* Darwin's finches, and many species and even genera of ducks, game birds, birds of paradise, and so on.

Furthermore, if gene flow is our criterion, rather than hybridization, occasional gene flow via horizontal gene transfer is rife across much larger systematic divides, especially at the base of the tree of life where it seems to have triggered important adaptive innovations (Doolittle, 1999). Yet occasionally hybridizing taxa coexist, diversify, radiate and have distinguishable ecologies, sexual behaviour, and genetics, as we expect for species. Instead of adopting a strict concept of species based on reproductive isolation, it would be sensible to allow that hybridization and introgression are natural, biological characteristics of many species. In this revised view, a pair of related species which differ at multiple loci in an area, and a single population polymorphic at multiple loci are merely extremes of the same phenomenon. In a sense, this is a return to a Darwinian view of species (Darwin, 1859; Wallace, 1865) justified by empirical, molecular population genetics findings. Species are then multi-locus polymorphisms in which correlations between loci, or linkage disequilibria, are strong, so that different taxa are recognizable because a complete "blurring of the border line between these species" (Mayr, 1942: 262) does not result.

If the above argument from hybridization against a strict isolation concept is accepted, it seems clear also that most variants of the phylogenetic species concept must also fail. Given

the possibility of gene flow between species taxa, genealogies are liable to remain reticulate even with non-sister taxa for some while after speciation. A monophyly-based species concept will not do, nor will a concept based on genealogical concordance between multiple loci apply, at least strictly. Instead we are forced to accept that the taxa we name are “unreal” phylogenetic units whose names are merely useful because we can tell them apart. These taxa have some (but not necessarily all) loci that are more or less genealogically distinct. But even their genealogically distinct alleles may have relationships that differ between loci. Calling these taxonomic units species might seem unsatisfying to a purist. However, heliconiine names such as those of the taxa enumerated in Fig. 1 will continue to be used because the forms they circumscribe remain separate and identifiable even when in contact with related taxa, and because they correctly predict different biological traits of interest.

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Figure legends

FIGURE 1. Phylogenetic relationships of Heliconiiti. The phylogeny pictured is one of 54 equally parsimonious cladograms (Brower and Egan, 1997: 974) obtained using a combination of mtDNA (COI+COII) and a nuclear gene, *wingless*. Changes in placement or additions based on other work are shown using dashed lines. We have added *Heliconius hecalesia*, *H. peruvianus* and *Eueides lineata* in approximate positions suggested by their divergence at mtDNA (Appendix 2; Jiggins and Davies, 1998; Beltrán et al., 2001).

Divergence between species at some mtDNA sites begins to saturate at around $D \geq 7\%$, so the precise arrangements of deeper nodes, in particular, are subject to revision. For example, it is currently uncertain whether the segregate genera *Laparus* and *Neruda* stand outside *Heliconius sensu stricto*. According to molecular data, both fall inside *Heliconius* (Brower and Egan, 1997), but *Neruda* in particular has a number of *Eueides*-like egg, pupal, and adult plesiomorphies which seem very unlikely to be reversals: analysis of morphological characters places both *Laparus* and *Neruda* outside *Heliconius* (Penz, 1999). We therefore represent *Neruda* as a sister genus to *Heliconius*, and *Laparus* as sister to the “basal” and *melpomene*-silvaniform *Heliconius*, although these placements may turn out to be incorrect. Overall, several major features of the phylogeny are generally agreed and accurate enough for our purposes here (see also Brown, 1981; Beltrán et al., 2001). In particular, the monophyly of the *melpomene*-silvaniform and *erato-sara-sapho* groups, and of *Eueides* are well-supported and agreed on by all authors.

FIGURE 2. Examples of hybrids between species of *Heliconius* and *Eueides*. *a. Eueides isabella eva* x *E. vibilia vialis*, hybrid no. 4; *b. Eueides isabella eva* x *E. procula vulgiformis*,

hybrid no. 5; *c. Heliconius numata aurora* x *H. melpomene malleti*, hybrid no. 9; *d. Heliconius hecale zeus* x *H. elevatus perchlorus*, hybrid no. 12; *e. Heliconius ethilla narcaea* x *H. besckei*, hybrid no. 23; *f. Heliconius numata superioris* x *H. melpomene meriana*, hybrid no. 8; *g. Heliconius melpomene cythera* x *H. cydno alithea*, hybrid no. 28; *h. Heliconius melpomene ssp.* x *H. cydno hermogenes*, hybrid no. 49; *i. H. erato petiverana* x *H. charithonia vasquezae*, hybrid no. 133; *j. Heliconius hecalesia octavia* x *H. hortense*, hybrid no. 136. For further details, see Table 1. All hybrids are putative F₁ progeny of interspecies hybridization, except *e* which is interpreted as a backcross to *H. besckei*. Photos: *a,i* – Sandra Knapp; *b,g* – James Mallet; *c,f,j* – Walter Neukirchen; *d,e* – Andrew Brower, *h* – Mauricio Linares.

FIGURE 3. A graphical representation of the species boundary. The numbers of natural hybrids known between pairs of species (from Table 1) are plotted on a logarithmic scale against the uncorrected DNA divergence estimated from data for 942 bp of mtDNA. If backcrossing is also known, points are shown as a pair of concentric circles. The data are from 59 heliconiine taxa sequenced by Brower and Egan (1997) supplemented by data for *Heliconius peruvianus* from Jiggins and Davies (1998), and for *Eueides lineata* and *H. hecalesia* from Beltrán et al. (2001). The comparisons reflect only those species that have zones of sympatry between which hybrids would be geographically possible; distance measures are from Appendix 2. There are no known hybrids between species groups, and no estimates of divergence have been included for intergroup comparisons (*Neruda* and *Laparus* are here treated as part of the *melpomene*-silvaniform group to which they are closest in mtDNA divergence). A least-squares exponential fit to the data is shown. (To display species pairs which lack known hybrids on the log-linear plot, they have been assigned 0.1 hybrids

each, but the fitted line is based on untransformed data). Because the comparisons are non-independent, especially where branches of the same phylogeny or even the same species are used twice, a simple statistical analysis is not appropriate (under an assumption of independence, there is a highly significant negative correlation between in rates of hybridization and genetic distance: $N = 159$, $r = 0.29$, $P < 0.0001$, although the proportion of the variance explained is not high, $r^2 = 8.5\%$, because of the large number of species pairs for which no hybrids are known). Except for the single hybrid between *Heliconius erato* and *H. charithonia* (mtDNA divergence at these 942 bp, $D = 8.82\%$), hybrids are unknown between pairs of species differing by $D > 7\%$, and only three pairs of species with $D > 5\%$ are known to hybridize. The two closest pairs of species from which hybrids are known (*erato/himera*, $D = 3.02\%$ and *melpomene/cydno*, $D = 3.07\%$) are also the only pairs from which more than ten hybrids are known. Intraspecific hybridization also approximately fits this scheme; given mtDNA divergence of $0.55\% \leq D \leq 1.84\%$ (average 1.1%) between geographic races within species, many more hybrids are predicted than for interspecific hybridization. This is in fact observed: many more than ten, and up to many hundreds of natural hybrids are known from areas of overlap between races normally considered members of the same species.