Mitochondrial DNA provides an insight into the mechanisms driving diversification in the ithomiine butterfly *Hyposcada anchiala* (Lepidoptera: Nymphalidae: Ithomiinae)

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**Keywords.** Mitochondrial DNA, Andes, diversification, Ithomiinae, *Hyposcada*, mimicry, refuge hypothesis

**Abstract.** Geographic subspecies of several ithomiine butterflies on the lower east Andean slopes display a black and orange “melanic tiger” aposematic wing pattern that occurs from Colombia to Bolivia, while geographically adjacent lowland subspecies typically bear a coloured, “tiger” pattern. However, it is not clear whether subspecies with similar wing patterns in different regions have arisen through independent events of convergent adaptation, possibly through parapatric differentiation, or result from allopatric differentiation, as proposed by the refuge hypothesis. Here, we examine geographic patterns of divergence in the widespread and common ithomiine butterfly *Hyposcada anchiala*. We present phylogenetic hypotheses for 5 subspecies of *H. anchiala*, based on 1567 bp mitochondrial DNA. All topologies indicated that a single switch in mimetic pattern best explained the wing patterning of the *H. anchiala* studied here. This finding suggests that the subspecies of *H. anchiala* studied here result from at least two stages of differentiation, and is consistent with a single colonisation into a novel altitudinal zone coincident with a wing pattern switch, followed by subsequent divergence within, rather than across altitudinal zones. The subspecies divergences indicated diversifications were consistent with the Pleistocene. Furthermore, the lowland subspecies were more recently derived than the montane taxa, in contrast to predictions of the “Andean species pump” hypothesis.

**INTRODUCTION**

Butterfly species richness reaches a global peak at the western edge of the Amazon basin and adjacent east Andean foothills (Robbins & Opler, 1996). To date, however, there is little agreement on the main causes for this high diversity, and a number of competing hypotheses have been proposed. The Pleistocene forest refuge hypothesis (Haffer, 1969) has perhaps received the most attention (Brown, 1979, 1982, 1987; Hall & Harvey, 2002; Sheppard et al., 1985), and indeed a number of east Andean lower elevation putative forest refugia have been identified (Brown, 1979, 1982). However, critics argue that parapatric differentiation across environmental gradients could produce similar biogeographic patterns (Benson, 1982; Endler, 1982). In particular, the rapid changes in both biotic and abiotic environments at the base of the eastern Andes could have helped generate the high species diversity in this region. Fjeldså (1994) went on to propose that Andean slopes act as a “species pump” for birds, where new species originate before subsequent dispersal into the Amazon lowlands.

The first studies of the effects of putative forest refugia on neotropical butterflies were based principally on two nymphalid subfamilies, the Heliconiinae and the Ithomiinae (Brown, 1982, 1987). Both groups are notable for their members being unpalatable to predators and aposematically coloured, with often remarkable geographic differentiation in warning colour pattern. Heliconiines and ithomiines are extensively involved in Müllerian mimicry, in which two or more distasteful species converge on a common warning signal which is more easily learnt and remembered by predators (Müller, 1879). Theory predicts that Müllerian mimicry increases in value as the number of individuals displaying the warning signal increases, due to a lower rate of attack per individual.

Brown (1982) suggested that such species were likely to differentiate in isolated forest refugia because they are relatively sedentary and because random fluctuations in abundance of co-mimics could drive mimicry pattern change (Brown & Benson, 1974; Joron et al., 1999). However, mimetic species are also expected to be highly susceptible to parapatric differentiation. Mimetic colour patterns also serve as mating cues (Jiggins et al., 2001), so shifts in mimicry pattern, which might be driven by geographic variation in abundance of co-mimics, are likely to disrupt gene flow. In the east Andean foothills mimetic butterfly faunas change rapidly over elevations of several hundred metres and distances of only a few
kilometres (KRW pers. obs.), so the potential for colonisation of new mimetic environments is high.

This debate still remains today partly because of the difficulties in testing between vicariance or ecological adaptation as causes of divergence. Here, we examine the possible roles of these two causes in explaining divergence in a single west Amazonian ithomiine species, *Hyposcada anchiala*. The Ithomiinae (Lepidoptera: Nymphalidae) is an exclusively Neotropical subfamily which includes ~355 species (Lamas, 2004), all of which are thought to be highly unpalatable. These butterflies form part of multiple, diverse mimicry rings in a single location (Beccaloni, 1997a,b; Joron & Mallet, 1998).

*Hyposcada anchiala* occurs from eastern Panama to Bolivia and western Brazil, and contains 12 recognised subspecies (Lamas, 2004). These subspecies display wing patterns of two distinct mimicry complexes, the orange and black tiger (melanic tiger) complex and the tiger complex. These complexes are strongly altitudinally zoned, constrained to high altitude (submontane) and low altitude sites respectively. In this paper we focus on relationships between the submontane melanic tigers, *H. a. mendax* Fox from N. Peru and *H. a. fallax* (Staudinger) from S. Peru and Bolivia, and the geographically adjacent, lowland tiger-pattern subspecies, *H. a. interrupta* Tessmann in the north and *H. a. richardsi* Fox, in the south (Fig. 1.).

If individuals cluster by subspecies, there are 26 possible rooted cladograms for *H. a. mendax*, *H. a. fallax*, *H. a. interrupta* and *H. a. richardsi*. Of those, 6 are not informative about the associated mimetic-altitudinal shifts, for example an unresolved quadchotomy, 7 support just one mimetic-altitudinal shift, and 13 support two mimetic-altitudinal shifts. An example of the 7 topologies which support just a single shift in mimicry and altitude is a bifurcating topology, with both montane tiger melanic subspecies in one clade, and both non-melanic tiger, lowland subspecies in the other clade (as depicted in Fig. 1), this can best be explained by a vicariant diversification of two previously widespread ancestral populations. The other 13 topologies support two shifts of mimicry and altitude, and thus ecological diversification (Benson, 1982; Endler, 1982), an example is a sister pairing of the...
two subspecies from N. Peru (one montane, melanic and one lowland, non-melanic tiger), and a sister pairing of the two subspecies from S. Peru (one montane, melanic and one lowland, non-melanic tiger) (as depicted in Fig. 1.). Under this scenario, local processes drove the diversification of an ancestral N. Peru

H. anchiala interrupta

H. anchiala interrupta

H. anchiala interrupta

H. anchiala mendax

H. anchiala mendax

H. anchiala mendax

H. anchiala mendax

H. anchiala mendax

H. anchiala mendax

H. anchiala mendax

H. anchiala mendax

H. anchiala mendax

H. anchiala ecuadorina

H. anchiala ecuadorina

H. anchiala richardsi

H. virginiana

H. zarepha flexibilis

Table 1. Specimen information.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Voucher number</th>
<th>Collection locality</th>
<th>Approximate collection altitude (m)</th>
<th>Genbank accession</th>
</tr>
</thead>
<tbody>
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<tr>
<td>H. anchiala interrupta</td>
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<td>150</td>
<td>DQ078356</td>
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<tr>
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<td>ECUADOR: Napo: Jatun Sacha. 01°04'S, 77°36'W</td>
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<td>270</td>
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<tr>
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<td>8283</td>
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Genomic DNA was analysed from the four described Hyposcada species with two, independent mtDNA datasets, one consisting of two melanic subspecies, plus an additional non-melanic tiger pattern subspecies, H. a. ecuadorina Bryk. Outgroup taxa were chosen to represent two closely related species, H. virginiana (Hewitson) and H. zarepha (Hewitson), as determined by the sequencing of five Hyposcada species with two, independent nuclear regions, wingless and elongation factor 1-a (data not shown) as well as mtDNA data (Table 1).

A specimen of H. virginiana in 20% dimethylsulphoxide, 0.25M EDTA and saturated NaCl solution (DMSO solution) was identified and provided by Chris Jiggins (University of Edinburgh) and a dried specimen of H. a. richardsi was provided by GL. All other specimens were collected by the authors, preserved in DMSO solution, and identified to subspecies by GL and KRW. DNA was extracted from one third of the abdomen (H. a. richardsi) or one third of the thorax (all other specimens) using the DNAeasy kit (Qiagen, West Sussex, UK), according to the manufacturer’s instructions, with an initial 3 h incubation at 55°C, and a final elution volume of 300 µl. Genomic DNA extracts were preserved at –20°C. Dried wings were retained as vouchers at University College London, except for the H. a. richardsi and H. virginiana which were retained by the donors. PCRs were performed using primers Jerry and Pat (COI), or George and Imelda (COII) (Simon et al., 1994), in a 25 µl volume, using 2 µl template DNA under the following conditions: 1 × PCR buffer, 0.6 mM dNTPs, 4 mM MgCl2, 0.5 µM each primer, 0.025 U/µl Taq, and ddH2O, with an amplification profile of 94°C for 2 min, followed by [COI: 4 cycles of (94°C for 45 s, 51°C for 45 s, 72°C for 60 s) then 29 cycles of (94°C for 45 s, 51°C for 45 s, 72°C for 90 s)] or [COII: 33 cycles of (94°C for 45 s, 55.5°C for 45 s, 72°C for 90 s)] and a final 5 min extension at 72°C. The H. a. richardsi sample proved difficult to amplify, probably due to its preservation with paralliclorobenzene (PDB), a chemical known to form covalent bonds to DNA. Weak H. a. richardsi PCR products were therefore excised from an agarose gel and incubated at 70°C for 10 min in 1.5 ml ddH2O. 2 µl of this suspension was used for reamplification following the above protocol. Final PCR products were purified using the QIAquick PCR purification kit (Qiagen, West Sussex, UK), according to the manufacturer’s protocol and sent to a commercial facility for cycle sequencing using the PCR primers, precipitation and sequencing.

Sequences were edited using SeqEd v1.0.3 (Applied Biosystems, Inc., Foster City, USA), PAUP version 4.0b 10 (Swofford, 2000) was used to calculate the numbers of variable and parsimony informative sites, and absolute and Hasegawa-Kishino-
or (senbeck & Ronquist, 2001), with Bayesian hypothesis was generated using MrBayes 3.0 (Huelto). Absolute pairwise distances (Table 2). For comparison, Neighbour Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were performed in PAUP, and a Bayesian hypothesis was generated using MrBayes 3.0 (Huelto & Ronquist, 2001), with H. virginiana, or H. zarepha, or (H. zarepha + H. virginiana) as the outgroup taxa. NJ trees were generated using HKY85 distances with random break ties, confidence in each node was assessed by performing 1000 rounds of NJ bootstrapping. MP topologies were conducted using a 10 replicate heuristic search with trees-bisection-reconnection (TBR) branch swapping, with random stepwise addition of sequences, support for clades was assessed by 1000 rounds of full heuristic bootstrapping with TBR branch swapping. ML trees were generated starting from random trees, using a heuristic search with TBR, random sequence addition, with the best fit model of sequence evolution (HKY+G, by hLRT) and parameter estimates as identified by Modeltest 3.04 (Posada & Crandall, 1998). Bayesian analyses were performed with parameters identified by Modeltest 3.04 (nst = 2, rates = invgamma), with four simultaneous chains run for 1,000,000 generations, sampling a tree every 100 generations. Consensus trees with branch support in the form of posterior probabilities were derived from the final 9,000 trees (representing the final 900,000 generations), after confirmation that likelihood values had stabilised after the first 100,000 generations. All possible unrooted trees were generated and corresponded minimum evolution (ME) scores calculated, for one representative of each H. anchiala subspecies plus a H. virginiana outgroup, using distance settings.

RESULTS

Sequences up to 1611 bp in length were successfully obtained from 13 individuals, representing five H. anchiala subspecies and two outgroup species (GenBank accession numbers DQ078312, DQ078355–62, and DQ078474–77). To avoid problems associated with missing characters, sequences were edited to a final alignment covering the 1567 bp region amplified in all specimens. The 1567 bp span 805 bp COI (alignment positions 1–805), 62 bp leucine-tRNA (806–867) and 700 bp COII (868–1567). Excluding the outgroup taxa, just 19 (1.21%) sites were variable, 10 in COI and nine in COII, of which 11 (0.70%) were parsimony informative. Of these 19 polymorphic sites, 16 differed in just one species (4 fixed and 5 polymorphic in H. a. mendax, 3 in H. a. fallax, 3 in H. a. richardsi, and 1 in H. a. ecuadorina), and three polymorphisms were shared; C in H. a. mendax + fallax, and T in H. a. ecuadorina + richardsi + interrupta; G in H. a. fallax + richardsi + interrupta, A/G in H. a. mendax, and A in H. a. ecuadorina; and A in H. a. ecuadorina + richardsi + interrupta, A/T in H. a. mendax, and T in H. a. fallax.

Absolute H. anchiala pairwise divergences (Table 2) ranged from 0, within the H. a. interrupta or between H. a. mendax 02-1644, 02-1645 and 02-2141, to 12 between H. a. ecuadorina or H. a. richardsi and H. a. mendax 02-1644, 02-1645, 02-2141 and 02-1602. There was no significant rate heterogeneity among lineages of the clock enforced [ln likelihood 2487.286] and non-clock enforced [ln likelihood 2482.988] trees, suggesting the data conform to a molecular clock. Mean between subspecies divergence times were estimated using a mitochondrial clock (Brower, 1994) to between 55,000 and 320,000 years ago, for (H. a. interrupta + H. a. ecuadorina) and (H. a. mendax + H. a. richardsi) respectively.

Overall, the H. anchiala phylogenies had short internal branches and thus were quite poorly resolved. The clearest case of phylogenetic agreement between all reconstructions was the well supported recovery of the five H. a. mendax specimens as a monophyletic clade, for

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<th>interrupta</th>
<th>mendax</th>
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TABLE 2. Absolute pairwise distances for all H. anchiala individuals (below diagonal), mean HKY85 subspecies pairwise distances (in %, above diagonal) and mean estimated time since divergence of subspecies [in years (y), to the nearest 5,000, above diagonal].
example, in analyses using a H. virginiana + H. zarepha
outgroup by bootstrap values of 94 (NJ) and 92 (MP),
and a Bayesian posterior probability of 0.72. Although
the arrangements of H. anchiala differed both with outgroup
used and reconstruction method, the topologies could be
categorised into three, repeatedly generated types. NJ and
MP analyses, using H. zarepha and (H. zarepha + H. vir-
giniana) as the outgroup, shared a similar topology (as
in Fig 2). All ML analyses and MP with a H. virginiana
outgroup recovered the lowland H. anchiala as paraphyletic
with regard to a sister pairing of H. a. mendax and H. a.
fallax. The Bayesian analysis with a (H. zarepha + H. vir-
giniana) outgroup shared a topology with NJ, MP and
Bayesian analyses using just a H. virginiana outgroup (as
in Fig 3). In addition, a fourth arrangement of a (H. a.
mendax – H. a. fallax) and (H. a. richardi – H. a. inter-
rupta – H. a. ecuadorina) sister pairing was recovered
once, by Bayesian analyses with a H. zarepha outgroup.
In summary, of the four obtained topologies, two sup-
ported the lowland non-melanic H. anchiala having
evolved from their montane melanic counterparts, one
supported the vicariant hypotheses depicted in Fig 1.,
and the other paired the montane melanic butterflies,
but did not indicate their relationship to the non-melanic low-
land H. anchiala. Although these arrangements differed
in the direction of the wing pattern switch and altitudinal
shift, all four topology types support the main finding that
H. anchiala wing patterns can be most parsimoniously
explained by a single switch in mimetic pattern and altitu-
dinal range.
105 different trees were generated using distance set-
tings, to represent all possible topologies. The mean ME
score for those trees most parsimoniously explained by
one mimetic pattern shift was 0.0429 (n = 21, range
0.0419–0.0445, median 0.0427) (the ME score of the
topology which mirrored that recovered by NJ analysis
was 0.0429), and the mean ME score for those topologies
most parsimoniously explained by two shifts was 0.0439
(n = 84 range 0.0424–0.0454, median 0.0439).

DISCUSSION AND CONCLUSIONS
Despite the rapid substitution rate of mtDNA compared
to most nuclear regions, sequence results yielded only
low levels of divergence among H. anchiala subspecies.
Tree reconstruction was compromised both by sequence
invariance and possibly by the outgroup taxa assuming a
position rather distant from the ingroup. Although tree
reconstruction method, and use of H. virginiana, H. zare
pha, or (H. zarepha + H. virginiana) as the outgroup
affected ingroup arrangements, all the phylogenetic
hypotheses were most parsimoniously explained by a
single colour shift between melanic and nonmelanic taxa,
thus the most recent radiations appear to be within altitu-
dinal zones rather than between them. Taken along with
the estimated subspecies divergences times, this finding is
consistent with geographic diversification during the
Pleistocene (11,000 – 1.8 Mya). This result of geographic

Fig. 2. Phylogenetic hypothesis inferred with maximum parsimony. Maximum parsimony bootstrap support values are given along branches. Subspecies symbols are as in Fig. 1., with filled symbols representing melanic butterflies.

Fig. 3. Phylogenetic hypothesis inferred with Bayesian methods. 3 clades have Bayesian probabilities over 0.5: (1644, 1645 and 2141), (1644, 1645, 2141 and 1602) and (1644, 1645, 2141, 1602 and 716) are supported with respective probabilities of 0.64, 0.85 and 0.72. Subspecies symbols are as in Fig. 1., with filled symbols representing melanic butterflies.
divergence within an altitudinal zone parallels the findings by Willmott (2001) and Willmott et al. (2001) based on morphological phylogenies of *Hypanartia* Hübner (Nymphalinae) and *Adelpha* Hübner (Limenitidinae). In these genera speciation across elevational gradients occurred, but appeared relatively more difficult, infrequent and more typical of basal diversifications. The most common form of differentiation was speciation within or among montane regions. In contrast, in a recent study on *Ithomiola* Felder & Felder (Riodinidae), Hall (in prep.) found multiple instances of parapatric differentiation up the sides of mountains. In *Ithomiola*, montane species in different regions are most closely related to adjacent species occurring at lower elevations, indicating that speciation across an altitudinal gradient is the rule in these butterflies.

Although theories of Neotropical diversification have tended to concentrate on taxa recognised as species, studies based on subspecies are also of great value as these taxa may often form an early stage in the diversification continuum that eventually leads to complete speciation. The subspecies diversifications here (Table 2) suggest that the lowland taxa are more recently derived (*H. a. richardsi* + *H. a. interrupta* 85,000 y) than the montane taxa (*H. a. mendax* + *H. a. fallax* 310,000 y). Our finding of older montane taxa appears to contrast Fjeldså’s (1994) reports that Andean bird species were, on average, younger than Amazonian species. Fjeldså went on to suggest that the Andes acted as a species “pump” producing novelities that were absorbed into the Amazon, which acted as a species “museum” However, a problem lies in just looking at taxa categorised (maybe incorrectly) as distinct species, and ignoring diversity at other (often lower) levels. For example, if the taxa regarded as young Andean species by Fjeldså were actually old subspecies, and the Amazon contained many, well-differentiated young subspecies (not recognised as being differentiated in species level analysis), the conclusions regarding the location of diversifications would be the exact opposite to Fjeldså’s.

However, the data and phylogenetic hypotheses presented here must be interpreted with caution. Only the *H. a. mendax* clade is repeatedly well supported. Additionally, the topologies represent the mtDNA tree, which is not necessarily equivalent to the species tree, due to processes including horizontal transfer, introgression and ancestral polymorphism (Brower et al., 1996). Given that the five *H. anchiala* subspecies here diverged moderately to rapidly on an evolutionary scale (within the last 300,000 years) the internodal branches are short, increasing the chance that ancestral copies failed to coalesce before diversification and therefore reducing the likelihood that mtDNA reflects the true organismal phylogeny (Maddison, 1997). Additionally, there is evidence that introgressive gene flow may have considerably shaped the *H. anchiala* mtDNA topologies. Although distinguished by a mean pairwise divergence of 0.5% (corresponding to a radiation 220,000 years ago), intermediates are known between *H. a. fallax* and *H. a. richardsi* (Lamas, pers. obs.). One intermediate is also known between *H. a. fallax* and *H. a. interrupta*. That these subspecies are capable of interbreeding introduces potential for introgressive gene flow. Even rare events can have a large impact, for example, an advantageous mutation could lead to a selective mtDNA sweep. Such gene flow can make two taxa appear to have diverged more recently than their actual original divergence time, creating a particular problem in studies, such as this, when the timing of the diversification event is critical for making inferences (Barraclough & Nee, 2001).

To date, few studies have taken a phylogeographic approach to investigate the diversification of mimetic Neotropical butterflies. Despite 10 years since publication, Brower’s (1994, 1996) mtDNA data for *Heliconius* butterflies remains the most significant contribution to this field. Our estimates of subspecies divergence dates are largely consistent with Brower’s findings, but in contrast we found no evidence that similarly appearing mimetic races were mainly the result of convergent evolution. Brower (1994, 1996) found that the *Heliconius* mtDNA topology largely mirrored the biogeographic distributions, but that there was a lack of concordance between geography and wing pattern. The dissimilarity between *Hypopscada anchiala* and *Heliconius* might simply be an artefact of the chosen loci, and given the above concerns that mtDNA might not be a reliable foundation for subsequent inferences, it would be of value to corroborate both data sets with independent nuclear loci. Unfortunately such research is currently hampered as few molecular markers are available which evolve rapidly enough to allow reliable phylogenetic reconstruction of closely related groups of Lepidoptera. This study could also be improved by sequencing additional *H. anchiala* subspecies, but sufficiently preserved samples are typically hard to obtain. It would also be of interest to investigate other species with parallel upland-lowland distribution patterns. Notwithstanding these concerns, it seems likely that differences in ithomiine and heliconine mimicry evolution reflect genuinely different evolutionary histories. Such differences have previously been reported, for example, in the ithomiine genus *Ithomia* Hübner, mimicry shifts between species occur rarely (Mallarino et al., 2005), in sharp contrast to *Heliconius* (Brower, 1996).

Although offering detailed insights into the mechanisms contributing to Neotropical diversification, it is likely that different taxa have experienced highly heterogeneous modes of diversification and thus individual examples are poor at elucidating the general patterns which account for total taxon diversity. Reliable distribution data and robust phylogenies are needed to further investigate geographic patterns of diversification (Willmott et al., 2001). Progress is being made with mtDNA and nuclear loci in the ithomines, at both the genus level and species level, but, it can be argued that it is the more recently diverged taxa that most accurately retain the signatures of the factors that drove the diversifications. Subspecies within ithomiine species are often at least partly reproductively isolated, for example by mimetic alle-
giance or habitat adaptations, therefore, to test the predictions of distinct mechanisms of diversification we may need to look closely at subspecies level phylogenies.

In summary, mtDNA sequence data revealed that the diversification of 5 subspecies of H. anchiala was more consistent with allopatric divergence than ecological adaptation, and occurred largely during the Pleistocene. Evidence suggests that, at least in this group, the divergent forms here assigned to subspecies are evolving most rapidly in the Amazon basin.

REFERENCES


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