

1 Out of the Andes: patterns of diversification in clearwing butterflies

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Abstract

Global biodiversity peaks in the tropical forests of the Andes, a striking geological feature that has likely been instrumental in generating biodiversity by providing opportunities for both vicariant and ecological speciation. However, the role of these mountains in the diversification of insects, which dominate biodiversity, has been poorly explored using phylogenetic methods. Here we study the role of the Andes in the evolution of a diverse Neotropical insect group, the clearwing butterflies. We used dated species-level phylogenies to investigate the time course of speciation and to infer ancestral elevation ranges for two diverse genera. We show that both genera likely originated at middle elevations in the Andes in the Middle Miocene, contrasting with most published results in vertebrates that point to a lowland origin. Although we detected a signature of vicariance caused by the uplift of the Andes at the Miocene–Pliocene boundary, most sister species were parapatric without any obvious vicariant barrier. Combined with an overall decelerating speciation rate, these results suggest an important role for ecological speciation and adaptive radiation, rather than simple vicariance.

Keywords: Andes uplift, ecological speciation, Ithomiinae, phylogeny, vicariance

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Introduction

Neotropical forests are by far the most diverse ecosystems on the planet (Myers *et al.* 2000). In tropical South America, the Andes are a striking geological feature. Obviously, the uplift of the Andes and its acceleration from the Miocene–Pliocene (Gregory-Wodzicki 2000; Garzzone *et al.* 2008) may have triggered diversification by isolating populations on either side (vicariant speciation, e.g. Chapman 1917). However, the Andes could also have contributed to vicariant diversification in more subtle ways, such as by influencing the lowland Amazon River network

(Hooghiemstra & van der Hammen 1998; Lundberg *et al.* 1998) or providing forest refugia during Pleistocene climatic fluctuations (Hooghiemstra & Van der Hammen 2004; Weir 2006). Importantly, the altitudinal gradient and intricate topography of the Andes also provide a considerable range of ecological conditions, from warm rainforests to dry and cold deserts, offering many opportunities for ecological diversification and speciation (Chapman 1917; Bush 1994). Fjeldså (1995) proposed that climatically stable regions in the Andes have been the source for many lowland Neotropical bird species, and that the Andes have thus played a crucial role in explaining why the Amazon basin is so much richer than other tropical regions. Of particular interest therefore is whether there is evidence for multiple colonizations between the Andes and adjacent lowlands,

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suggesting one of these regions is an important source of species diversity in its neighbour (Chapman 1917; Fjeldså 1995), or whether colonizations are rare and tend to be followed by bursts of diversification (e.g. Willmott *et al.* 2001).

The possibility of generating and calibrating comprehensive species-level molecular phylogenies now permits a deeper exploration of evolutionary patterns of diversification. Most recent phylogenetic studies of Andean taxa have focused on vertebrates, especially birds (e.g. Garcia-Moreno *et al.* 1998; Perez-Eman 2005; Weir 2006; Brumfield & Edwards 2007; Ribas *et al.* 2007; Dacosta & Klicka 2008; Miller *et al.* 2008; see also Haag *et al.* 2007 for rodents; or Roberts *et al.* 2006 and Koscinski *et al.* 2008 for frogs). The most common pattern among vertebrate groups of Miocene age is a lowland origin with dispersal into the highlands (Roberts *et al.* 2006; Brumfield & Edwards 2007; Ribas *et al.* 2007). It has also been argued that vicariance separating eastern and western lineages and Pleistocene climatic fluctuations have played a role in divergence (Perez-Eman 2005; Weir 2006; Brumfield & Edwards 2007; Chaves *et al.* 2007; Ribas *et al.* 2007; Koscinski *et al.* 2008). More rarely, some taxa have originated in the highlands and later colonized lowland areas (Haag *et al.* 2007; Torres-Carvajal 2007).

By contrast, there have been fewer molecular phylogenetic studies investigating spatial and temporal patterns of diversification in Neotropical insects (Brower 1996; Jiggins *et al.* 2006; de Paula *et al.* 2007; Wahlberg & Freitas 2007; Silva-Brandão *et al.* 2008). Insects represent an overwhelming proportion of biodiversity, and understanding the origins of tropical insect diversity poses one of the most exciting challenges in evolutionary ecology (Godfray *et al.* 1999). Insects tend to have much narrower ecological niches than vertebrates, suggesting that ecological adaptation across relatively small-scale environmental gradients might be of greater importance in driving divergence. The few available studies of Neotropical insects inhabiting both lowland and montane regions, including those based on morphological data, point out the importance of the Andes in causing vicariance (Brower 1996; de Paula *et al.* 2007), and in providing ecological gradients along which species can diversify (Willmott *et al.* 2001; Hall 2005; Whinnett *et al.* 2005; Wahlberg & Freitas 2007). To date, however, no study has employed comprehensive molecular species-level phylogenetic hypotheses to investigate the role of the Andes in insect diversification. We here compare both the pattern and timing of insect diversification in the Andean region in two diverse 'clearwing' butterfly genera, *Ithomia* and *Napeogenes*, building on a previous study of speciation in the genus *Ithomia* (Jiggins *et al.* 2006).

Clearwing butterflies (Nymphalidae: Ithomiinae, or ithomiines) represent an excellent study group for this purpose. They form an exclusively Neotropical subfamily, comprising over 360 species and more than 1500 differen-

tiated races (Lamas 2004). They numerically dominate understorey butterfly communities in lowland and Andean forests. All species are engaged in Müllerian mimicry, where unpalatable species converge in wing pattern to better advertise their toxicity to predators (Müller 1879). Due to their abundance, ithomiines also likely drive the evolution of local mimicry complexes, with important implications for unrelated insect groups (Bates 1862; Brown & Benson 1974; Beccaloni 1997; Joron *et al.* 1999). Finally, because ithomiines are one of the best-studied butterfly groups, knowledge of species geographical ranges and other ecological traits is relatively detailed. The genera *Napeogenes* and *Ithomia* are two of the most diverse and widespread ithomiine genera (Lamas 2004), and belong to sister tribes (Brower *et al.* 2006; Willmott & Freitas 2006). They occur in both montane and lowland habitats and share a similar distribution range across South and Central America, with centres of diversity in the upper Amazon and eastern Andes.

Previously, we have shown that diversification in the genus *Ithomia* shows an association with colour pattern evolution, and that analysis of geographical ranges suggests mixed patterns of geographical modes of speciation (Jiggins *et al.* 2006). We here re-analyse *Ithomia* phylogeny, and present a comprehensive phylogenetic hypothesis for the genus *Napeogenes*, revising the existing species taxonomy where appropriate based on our results. We used a recent estimate of divergence time among Nymphalidae lineages (Wahlberg *et al.* 2008) to calibrate both phylogenies in order to infer patterns of diversification through time in these genera. Rather than study geographical ranges, which are highly labile (Losos & Glor 2003), we instead inferred the ancestral elevation range of the study genera – a trait that is likely to be more conservative given its links to both physiological (e.g. oxygen intake, thermoregulation, larval development) and ecological (e.g. hostplant distribution, mimicry) traits. This comparative analysis of two diverse genera highlights common patterns in the diversification of these butterflies.

Materials and methods

Specimens and genes sequenced

One hundred and seventy-two (172) *Napeogenes* specimens representing all known extant species (23 according to Lamas (2004), 24 after revision in the light of our data), and 59 (42%) of the 140 known subspecies were collected by the authors or received from colleagues from several locations (Table 1, Table S1, Supporting information). Particular efforts were made to obtain morphologically divergent taxa from the Andes. Species from the related genera *Hypothyris* and *Ithomia* (Brower *et al.* 2006; Willmott & Freitas 2006) were used as outgroups (Table S1). DNA was extracted using the

Table 1 Species and subspecies included in the analyses, with origin of specimens, reliable elevation range limits and mean (*m*) of all recorded localities. Detailed information on specimens and GenBank Accession numbers are given in Table S1, as well as elevation range limits and mean for *Ithomia* species. Taxonomy is updated according to our findings, with the former taxonomy (Lamas 2004) given in brackets

Species and elevation range (metres)	Subspecies included in the analyses	Origins of specimens
<i>N. aethra</i> (150–600, <i>m</i> = 301)	<i>N. aethra aethra</i> (<i>N. larina aethra</i>)	Colombia
<i>N. apulia</i> (1100–1700, <i>m</i> = 1200)	<i>N. apulia ocnita</i>	Ecuador
<i>N. benigna</i> (1550; 1800)*	<i>N. benigna sandra</i> (<i>N. harbona benigna</i>)	Colombia
<i>N. cranto</i> (750–1400, <i>m</i> = 950)	<i>N. cranto paedaretus</i>	Panama
<i>N. duessa</i> (100–1250, <i>m</i> = 399)	<i>N. duessa incas</i>	Peru
	<i>N. duessa jamariensis</i>	Brazil
	<i>N. duessa orellana</i>	Ecuador
	<i>N. duessa</i> n ssp. 1	Brazil
<i>N. flossina</i> (1325–2400, <i>m</i> = 1844)	<i>N. flossina flossina</i>	Ecuador
	<i>N. flossina</i> n ssp.	Peru
<i>N. glycera</i> (1250–2100, <i>m</i> = 1587)	<i>N. glycera ellariformis</i>	Peru
	<i>N. glycera eunomia</i>	Ecuador
	<i>N. glycera glycera</i>	Ecuador
	<i>N. glycera olyrina</i>	Peru
<i>N. gracilis</i> (800–1550, <i>m</i> = 1113)	<i>N. gracilis gracilis</i>	Peru
<i>N. harbona</i> (1500–2400, <i>m</i> = 1813)	<i>N. harbona podocarpus</i>	Ecuador
	<i>N. harbona</i> n ssp. 1	Peru
<i>N. inachia</i> (100–1000, <i>m</i> = 443)	<i>N. inachia inachia</i>	Brazil
	<i>N. inachia johnsoni</i>	Colombia
	<i>N. inachia juanjuensis</i> (<i>N. juanjuensis</i>)	Peru
	<i>N. inachia patientia</i>	Peru, Bolivia, Brazil
	<i>N. inachia pozziana</i>	Ecuador, Peru, Colombia
	<i>N. inachia pyrois</i>	Brazil
	<i>N. inachia sulphurina</i>	Brazil
	<i>N. inachia</i> n ssp.	Peru
<i>N. larilla</i> (2000–2700, <i>m</i> = 2119)	<i>N. larilla larilla</i>	Ecuador
	<i>N. larilla reventador</i>	Ecuador
<i>N. larina</i> (100–750, <i>m</i> = 482)	<i>N. larina deucalion</i>	Peru, Brazil
	<i>N. larina otaxes</i>	Peru
	<i>N. larina pyrrho</i>	Peru
	<i>N. larina quadrilis</i> (<i>N. quadrilis</i>)	Ecuador
	<i>N. larina zurippa</i>	Bolivia
<i>N. lycora</i> (1100–1900, <i>m</i> = 1572)	<i>N. lycora attali</i>	Ecuador, Peru
	<i>N. lycora lycora</i>	Ecuador
<i>N. peridia</i> (0–1200, <i>m</i> = 516)	<i>N. peridia hoppi</i>	Ecuador
	<i>N. peridia peridia</i>	Colombia
<i>N. pharo</i> (90–1100, <i>m</i> = 453)	<i>N. pharo acreana</i>	Brazil
	<i>N. pharo lamia</i>	Peru
	<i>N. pharo pharo</i>	Ecuador, Peru, Brazil
<i>N. rhezia</i> (100–900, <i>m</i> = 411)	<i>N. rhezia achaea</i> (<i>N. achaea achaea</i>)	Ecuador
	<i>N. rhezia adelphe</i>	Brazil
	<i>N. rhezia adulta</i>	Venezuela
	<i>N. rhezia cyrianassa</i>	Brazil
	<i>N. rhezia rhezia</i>	Brazil
	<i>N. rhezia xanthone</i>	Brazil
<i>N. sodalis</i> (1700)†	<i>N. sodalis</i>	Peru
<i>N. stella</i> (0–1000, <i>m</i> = 481)	<i>N. stella opacella</i>	Ecuador
	<i>N. stella</i> n ssp.	Panama
<i>N. sulphureophila</i> (1100–1300, <i>m</i> = 1050)	<i>N. sulphureophila</i>	Ecuador
<i>N. sylphis</i> (100–1250, <i>m</i> = 430)	<i>N. sylphis caucayaensis</i> (<i>N. sylphis corena</i>)	Ecuador, Colombia
	<i>N. sylphis corena</i>	Peru
	<i>N. sylphis rindgei</i>	Peru
	<i>N. sylphis sylphis</i>	Peru
	<i>N. sylphis thira</i>	Peru

Table 1 Continued

Species and elevation range (metres)	Subspecies included in the analyses	Origins of specimens
<i>N. tolosa</i> (230–1250, $m = 869$)	<i>N. tolosa amara</i>	Panama
<i>N. verticilla</i> (635–1425, $m = 1114$)	<i>N. verticilla</i>	Peru
<i>N. species 1</i> (1525)†	<i>N. species 1</i>	Peru
<i>N. species 2</i> (1200–1650, $m = 1406$)	<i>N. species 2</i> ssp. 1 (<i>N. juanjuensis</i> n ssp. 1)	Peru
	<i>N. species 2</i> ssp. 2 (<i>N. juanjuensis</i> n ssp. 2)	Peru

*Only these two reliable elevation records are available for *Napeogenes benigna*; other known localities for museum specimens only have general labels, but other specimens with these labels are all of species that are either from low or mid-elevation. Together with elevational stratification of mimicry complexes, this suggests that *N. benigna* should be classified as a mid-elevation species.

†Only one reliable elevation record is available for these species, but additional information (general labels, mimicry) suggest these species should be classified as a mid-elevation species.

QIAGEN DNeasy Kit, according to the manufacturer's protocol. We sequenced the mitochondrial region spanning *CoI* and *CoII* genes (2228 bp), and fragments of the nuclear genes *EF1 α* (1255 bp) and *tektin* (738 bp). Primers, polymerase chain reaction (PCR) and sequencing reaction conditions are similar to those used in previous studies (Mallarino *et al.* 2005; Elias *et al.* 2007) and are detailed in Table S2, Supporting information. Museum specimens (23 specimens, Table S1) required the sequencing of more, shorter fragments (Table S2). Sequences for the genus *Ithomia* (24 out of 26 extant species) were downloaded from GenBank (Mallarino *et al.* 2005; Table S1). All available genes except *RpL5* were used: a mitochondrial region (*CoI-CoII*, 1599 bp) and three nuclear genes (*EF1 α* , 1028 bp, *tektin*, 715 bp, *wingless*, 386 bp).

Phylogenetic analyses of *Napeogenes*

Phylogenetic analyses of *Napeogenes* were conducted using maximum parsimony, maximum likelihood and Bayesian inference. All analyses were performed on the entire data set as well as for each region separately (*CoI-CoII*, *EF1 α* , *tektin*). *Ithomia agnosia*, *Hypothyris anastasia*, *H. cantabrica* and *H. moebiusi* were used as outgroups (Table S1; Brower *et al.* 2006).

Maximum parsimony analyses were performed using the New Technology Search implemented in TNT, employing all four search methods — ratchet, tree-fusing, tree-drifting and sectorial (Goloboff 1999), followed by traditional search using tree-bisection-reconnection (TBR) branch swapping, with all characters equally weighted. A strict consensus tree was computed whenever multiple equally parsimonious trees were obtained, using the program WinClada (Nixon 1999). The consistency index (CI) and the retention index (RI) were also calculated in WinClada. The stability of each branch was determined using the nonparametric bootstrap test (Felsenstein 1985), with 1000 replicates and 100 random taxon additions. Bremer support and partitioned Bremer support values (to obtain the contribution of each data set

to the Bremer support values of the combined analysis; reviewed in Brower 2006) were calculated using the scripting feature of TNT (Peña *et al.* 2006). The analysis was conducted with 100 random taxon addition replicates, TBR branch swapping and 100 trees held in each replicate.

Maximum likelihood analyses were performed using RAxML from Vital-IT and Cipres cluster Web servers (Stamatakis *et al.* 2008) with a partition by codon position within each region, each of them following the implemented GTR + Γ substitution model. Branch stability was estimated with 100 bootstrap replicates.

Bayesian analyses were conducted in MrBayes 3.1 (Huelsenbeck & Ronquist 2001), with a partition similar to that used in the maximum likelihood analyses. Since MrBayes allows substitution models simpler than the GTR model, we first selected the best model for each partition element using MrModelTest version 2 (Nylander 2004). Details are shown in Table S3, Supporting information. We performed two runs of four simultaneous Markov chains each for 10 000 000 generations, sampled a tree every 1000 generations and applied a 10% burn-in after checking that the chains had converged. The consensus tree and posterior probability of nodes were calculated after confirming that both runs converged to the same topology.

Calibrated species-level phylogenies

After updating the taxonomy in light of our results, we used one individual to represent each *Napeogenes* species. We generated species-level phylogenies for *Napeogenes* and *Ithomia* using a Bayesian uncorrelated lognormal relaxed clock model in BEAST version 1.4.7 (Drummond & Rambaut 2007). The data set was partitioned in three and four regions for *Napeogenes* and *Ithomia*, respectively, corresponding to the mitochondrial region and each of the nuclear genes. Each region followed a GTR + Γ model of substitution implemented in BEAST, and two Markov chain Monte Carlo were run for 100 000 000 generations (Yule speciation model, sampling every 10 000 generations, 10% burn-in).

The analysis for *Napeogenes* resulted in a consensus topology slightly different from that based on all specimens (position of *N. species 2*, which is poorly supported in most analyses), but this did not affect the results.

Two sets of phylogenetic analyses were conducted for each genus. First, we generated an uncalibrated phylogeny, such that the variation in the posterior distribution of trees was only due to topology and branch length uncertainty. These trees were used in the analyses detailed in the following paragraphs (diversification through time and evolution of elevation range). Second, to date splits in the trees we ran a single analysis that included both genera and imposed normal priors on ages of the splits between *Ithomia* and *Napeogenes* [21.54 ± 1.26 million years ago (Ma)] and between *Napeogenes* and *Hypothyris* (17.47 ± 1.27 Ma), based on a recent calibration for the entire Nymphalidae family with six butterfly fossils and the ages of six larval food plant families (Wahlberg *et al.* 2008). The latter analysis takes into account both phylogenetic uncertainty (as in the former analyses) and uncertainty in the dates of the calibration points, as estimated in Wahlberg *et al.*'s (2008) analysis.

In both sets of analyses, the posterior distribution of trees was summarized with TreeAnnotator version 1.4.7 (Drummond & Rambaut 2007), computing the maximum clade credibility tree with average branch lengths.

Patterns of diversification through time

To investigate patterns of diversification through time, we generated lineage-through-time plots (LTT plots) by plotting the number of lineages against node heights. The gamma parameter (γ), which measures the deviation from diversification under a pure birth process (Pybus & Harvey 2000), was calculated with TreeStat version 1.1 (Rambaut 2007). A positive value of γ indicates an increasing speciation rate, whereas a negative value points to a decreasing speciation rate. As γ might be affected by incomplete sampling of extant species (Pybus & Harvey 2000), we simulated 10 000 phylogenies under a pure birth process using PhyloGen version 1.1 (Rambaut 2002) and generated the corresponding distribution of γ when 10%, 25% and 50% of the extant species were missing, such that significance of the observed values of γ under each scenario could be assessed as the proportion of simulated values of γ that were more extreme than that observed (Pybus & Harvey 2000). A similar analysis has already been published for *Ithomia* under a strict-clock model of evolution (Jiggins *et al.* 2006), and we here repeat this under a relaxed-clock model of evolution to provide a direct comparison with *Napeogenes*.

We then used the R package Laser (Rabosky 2006) to determine whether the observed patterns of diversification through time could be explained by a simple model of a constant speciation rate, or by a more complex model with

variable speciation rates with time. Tests were performed by computing the Akaike information criterion corrected for small sample sizes or AICc (Burnham & Anderson 2002) of different models (one, two or three speciation rates), after confirming that a pure birth model was a better fit than a birth-death model (Rabosky & Lovette 2008b). A two-unit difference in AICc indicates a good support for the most likely hypothesis (Burnham & Anderson 2002).

Evolution of elevation range

Napeogenes and *Ithomia* range from sea level up to 3000 m in Central America and the Andes, with a mean elevational range per species of 800 m and 1200 m, respectively. Information on elevation (Table 1, Table S1) and geographical ranges (Fig. S1, Supporting information) was obtained from more than 7000 specimen locality data points, collated from public collections, our own field records, colleagues and the literature (Jiggins *et al.* 2006). Based on this information, we classified *Napeogenes* species among three elevation categories, that reflect both the relatively discrete distribution of elevation values and the elevational stratification of mimicry complexes: low elevation (0–1200 m), mid-elevation (500–1700 m) and high elevation (> 1300 m). *Ithomia* species tended to have broader elevation ranges than *Napeogenes* species and were classified into two categories: low-to-mid elevation (0–1500 m) and mid-to-high elevation (> 1000 m). Only one species, *Ithomia 'lagusa' theuda* had an elevation range that apparently spans both categories (230–1900 m) and was considered polymorphic.

Ancestral elevation ranges were reconstructed on the sets of trees using parsimony and maximum likelihood with Mesquite 2.5 (Maddison & Maddison 2008) and BayesTraits version 1.0 (Pagel *et al.* 2004), respectively.

We wanted to test whether elevation range evolved gradually, implying that the probability of a range shift depends on the branch length, or punctationally, implying that shifts in range are associated with speciation and are independent of branch length. We used BayesTraits version 1.0 to compare models where the branch scaling parameter kappa (κ) was fixed to one (gradual evolution) to models where kappa (κ) was fixed to zero (punctational evolution), by computing the AICc in each case. This information was taken into account in the reconstruction of ancestral elevation range. Alternative hypotheses for the elevational origin of the genera were tested by fixing the root to different states and computing the respective AICc.

Results

Napeogenes phylogenetic relationships

Napeogenes was monophyletic with respect to its closest extant relative, *Hypothyris*. The phylogeny of *Napeogenes*

was in general well supported and congruent among the three methods (Fig. 1), supporting the highland *Napeogenes larilla* as sister to all remaining species. The main uncertainties concerned the positions of *Napeogenes verticilla*, *N. peridia*, and *N. species 1*, and the relationships among *Napeogenes inachia*, *N. species 2*, *N. apulia* and *N. gracilis*. The relationships between *N. benigna* and *N. sodalis* were unresolved, but this was possibly due to missing data caused by poor quality nuclear DNA obtained from the museum specimen of *N. sodalis* set13. Mitochondrial DNA as well as morphological characters support the monophyly of *N. sodalis* with respect to *N. benigna* (K. Willmott, unpublished). There was evidence of introgression between *N. lycora* and *N. harbora*. *Napeogenes harbora* sspn1 05–1148, which had nuclear sequences and morphological characteristics typical of *N. harbora*, possessed *N. lycora* mitochondrial DNA (Fig. S2, Supporting information), meaning that a diagnosis based solely on the DNA barcoding method (Hebert *et al.* 2003) would have lead to erroneous identification of this specimen (Dasmahapatra & Mallet 2006).

Our results prompted a re-examination of morphological, ecological and distributional information for certain taxa, which led to a number of taxonomical revisions (detailed in another manuscript; Willmott *et al.* in preparation, as well as in Willmott & Vitale 2008). In this paper, we use the revised taxonomy (see Table 1 for information on previous and revised taxonomy).

Calibration and time course of diversification

Based on the calibration proposed by Wahlberg *et al.* (2008), *Napeogenes* and *Ithomia* both separated from their extant sister group in the Middle Miocene, c. 17.6 Ma (Fig. 2, Fig. S3, Supporting information). *Ithomia* started diversifying 14.4 ± 1.4 Ma, while the first detectable split in *Napeogenes* occurred slightly later, 12.7 ± 1.1 Ma.

The model of a constant speciation rate was rejected in both cases (*Ithomia*: $\Delta\text{AICc}_{2 \text{ rates vs. } 1 \text{ rate}} = 3.40 \pm 1.87$ across trees; *Napeogenes*: $\Delta\text{AICc}_{2 \text{ rates vs. } 1 \text{ rate}} = 3.40 \pm 1.62$). The models with two and three speciation rates resulted in a better fit for the two-rate model in *Napeogenes* ($\Delta\text{AICc}_{2 \text{ rates vs. } 3 \text{ rates}} = 2.96 \pm 3.26$), and a marginally but not significantly better fit for the two-rate model in *Ithomia* ($\Delta\text{AICc}_{2 \text{ rates vs. } 3 \text{ rates}} = 1.00 \pm 4.18$). Under the two-rate models, both genera show a slow-down in the last 3–4 million years: *Ithomia* diversified at a rate of 0.239 ± 0.031 speciation/lineage/million years until 3.37 ± 0.69 Ma, after which the diversification rate dropped to 0.063 ± 0.012 . Similarly, *Napeogenes* diversified at a rate of 0.213 ± 0.023 until 3.87 ± 0.85 Ma, after which the diversification rate dropped to 0.054 ± 0.012 . Under the three-rate model, *Ithomia* showed an acceleration of the diversification rate at the Miocene–Pliocene boundary, just before the final deceleration (Fig. 3a; speciation rates: 0.159 ± 0.606 until 5.50 ± 2.07 Ma, then 0.510 ± 23.87 until

3.76 ± 0.84 Ma, and 0.069 ± 0.020 until present; note the large variance among trees in the second rate, due to the very short period of acceleration).

The overall decrease in diversification rate in *Napeogenes* is reflected in the distribution of gamma ($\gamma = -2.34 \pm 0.27$, $P = 0.010$ assuming complete sampling), which is unlikely to be due to missing species ($P = 0.013$, 0.025, and 0.064 for 10%, 25% and 50% missing species, respectively). The overall diversification rate of *Ithomia* marginally decreased ($\gamma = -1.61 \pm 0.38$, $P = 0.067$ assuming that all but two extant species were sampled), although a similar value of γ could be obtained under a pure birth diversification model if the number of missing species were much higher ($P = 0.071$, 0.126, and 0.225 for 10%, 25% and 50% missing species, respectively).

Evolution of elevation range

When the branch scaling parameter κ was estimated, its values were 0 in more than 97% of the trees for *Ithomia* and in over 99% of the trees for *Napeogenes*, indicating a punctuational evolution of elevation range in both genera. In other words, changes in elevational range were just as likely to occur on short branches as on long branches and are therefore associated with cladogenesis events rather than branch length. This was further supported by comparing likelihoods of the data under a punctuational evolution of elevation range ($\kappa = 0$) and a gradual model in which $\kappa = 1$ ($\Delta\text{AICc}_{\kappa=0 \text{ vs. } \kappa=1} = 2.25 \pm 0.68$ for *Ithomia* and $\Delta\text{AICc}_{\kappa=0 \text{ vs. } \kappa=1} = 3.38 \pm 0.74$ for *Napeogenes*). The parameter κ was therefore fixed to 0 in the subsequent maximum likelihood analyses.

Both parsimony and maximum likelihood inference of ancestral elevation ranges indicate that *Ithomia* and *Napeogenes* originated at middle elevations (Fig. 2, Fig. S4, Supporting information). For *Ithomia*, an origin and primary diversification at mid-to-high elevation (likelihood comparison of mid-to-high vs. low-to-mid elevation origin: $\Delta\text{AICc}_{\text{mid-high vs. low-mid}} = 6.33 \pm 0.14$) was followed by two radiations into lowland areas, one primarily east of the Andes (*drymo* clade, 7.9 ± 1.7 Ma) and the other primarily west of the Andes (*iphianassa* clade, 5.7 ± 1.3 Ma). The sister genera *Pagyris* and *Placidina* also occur at mid-to-high elevations. In *Napeogenes*, a low elevation origin was clearly not supported ($\Delta\text{AICc}_{\text{mid vs. low}} = 4.04 \pm 0.35$ and $\Delta\text{AICc}_{\text{high vs. low}} = 5.59 \pm 0.37$), but although a high-elevation origin was more likely, it was only weakly supported over mid-elevation ($\Delta\text{AICc}_{\text{high vs. mid}} = 1.55 \pm 0.70$). This uncertainty remains throughout the tree such that range shifts between mid and high elevation cannot be reliably tracked (Fig. 2b). However, these implied shifts are illogical for older nodes because the Andes were lower than today, and a mid-elevation origin therefore seems most plausible. In *Napeogenes* one clade (*cranto*) remained at middle elevations whereas the two other main clades (*tolosa* and *larina*) diversified

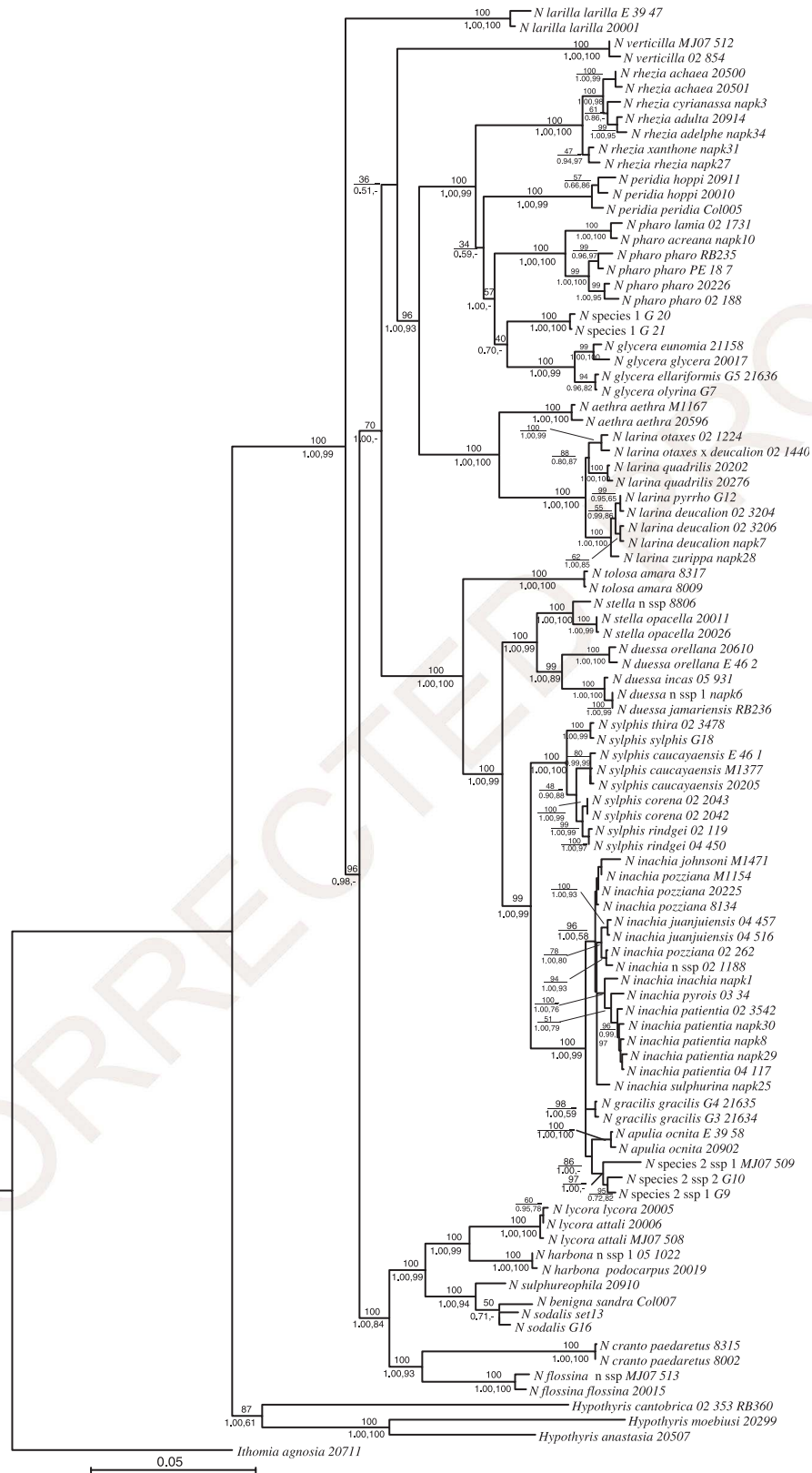


Fig. 1 Maximum likelihood (ML) tree for the genus *Napeogenes*. This tree is based on mitochondrial (*Col-CoII*) and nuclear (*EF1a*, *tektin*) genes, and shows ML bootstraps (above), Bayesian posterior probabilities (bottom left) and parsimony bootstraps (bottom right). Details of trees obtained with each genomic region are presented in Fig. S2.

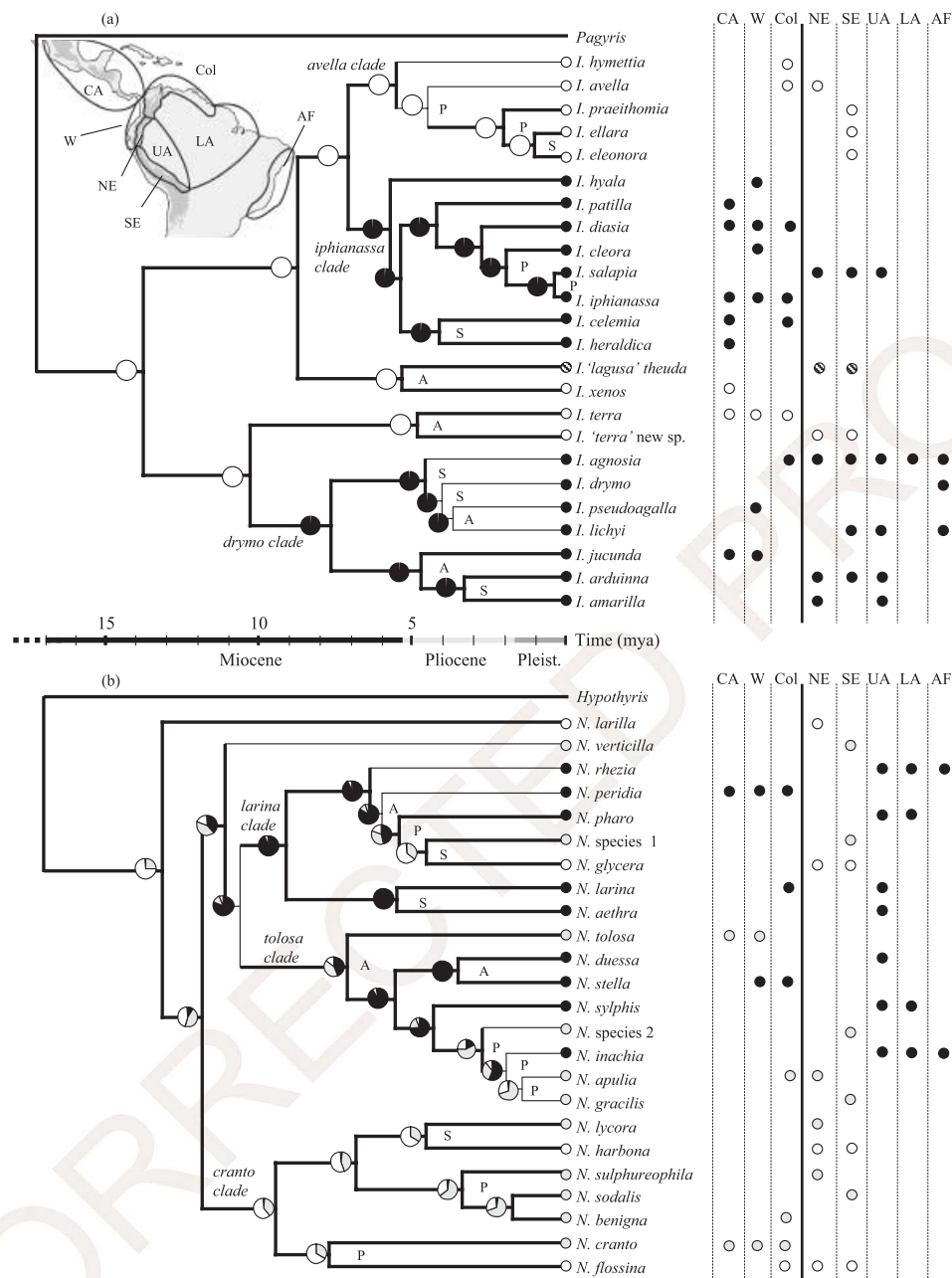


Fig. 2 Bayesian-dated relaxed-clock maximum credibility clade tree for (a) *Ithomia* and (b) *Napeogenes*. Thinner branches have a Bayesian posterior probability lower than 0.90, and also represent species whose position on the tree is poorly supported. Names of clades cited in the text are written above the relevant branches. Current elevation ranges are shown by coloured circles at the tips of the trees (*Ithomia*: black, low-to-mid elevation; white, mid-to-high elevation; striped, low-through-high elevation; *Napeogenes*: black, low elevation; grey, mid-elevation; white, high elevation), and maximum likelihood inferred ancestral ranges are shown for each node (same colour code, the proportion of the pie occupied by a state represents the probability of this state). Parsimony reconstruction of ancestral states is shown in Fig. S4. Rough geographical distribution is indicated on the right: CA, Central America; W, west slope and coast of the Andes; Col, Colombian (Cordilleras and Magdalena Valley, which extends to northern Venezuela for *Ithomia iphianassa* and *I. agnosia*); NE, northeastern slopes of the Andes (excluding Colombian and Venezuelan Andes); SE, southeastern slopes of the Andes; UA, upper Amazon; LA, lower Amazon (which extends to the Guianas for *Napeogenes rhezia*, *N. sylphis* and *N. inachia*); AF, Atlantic Forest. The Andes separate regions on either side of the thicker line between Col and NE on the right of the Figure. Letters A, P and S near internal nodes refer to allopatric (with respect to the Andes), parapatric and sympatric distribution of species or clades, respectively. Detailed geographical ranges are given in Fig. S1. Differences in elevation within geographical range are indicated by circle colour, as above. Note that we consider *Ithomia 'lagusa' theuda* a species different from *Ithomia lagusa lagusa*, which is not represented in our phylogeny but appears related to *Ithomia hymettia* (K. Willmott, personal observation).

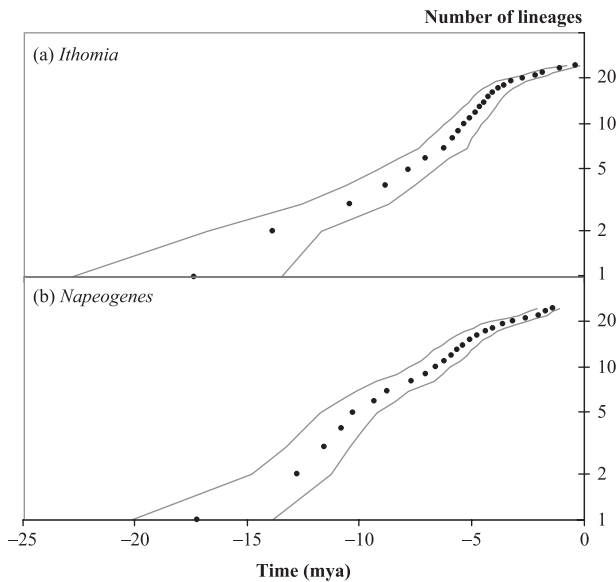


Fig. 3 Lineages-through-time (LTT) plots showing the accumulation of lineages through time in (a) *Ithomia* and (b) *Napeogenes*. Dotted lines represent the 95% credibility interval.

mostly at low elevations, with a few instances of reversal towards montane habitats (Fig. 2b).

Vicariance in the Andes

The Andes clearly act as a strong physical barrier in these genera. The only species to occur on both slopes, *N. harbona*, is found west of the Andes only in southern Ecuador and northern Peru (Fig. S1), where it has apparently colonized across the low (2400 m) passes of this region (also traversed by a few other ithomiine and some *Heliconius* butterflies; Descimon & Mast de Maeght 1983). The Andes separate sister species or sister clades in four and three instances in *Ithomia* and *Napeogenes*, respectively (Fig. 2, Fig. S1). In two additional pairs of sister species, *Ithomia salapia* and *I. iphianassa*, and *Napeogenes flossina* and *N. cranto*, the Andes represent a barrier throughout most of their distribution, but these sister species also have a narrow contact zone in Colombia (Fig. S1). As described previously for *Ithomia* (Jiggins *et al.* 2006), sister species or clades are predominantly parapatric with different levels of range overlap, or fully sympatric (Fig. 2, Fig. S1).

Discussion

Ithomia and *Napeogenes* have diversified independently, but their patterns of diversification present a number of similarities. Notably, reconstruction of ancestral ranges clearly implies a mid-elevation origin in the Andes, most likely due to common ancestry of the two genera, followed

by colonization and diversification into the lowlands. Changes in elevation range are rare and associated with speciation, and both genera show a decrease of their diversification rates after 4 Ma.

Origin and expansion of *Ithomia* and *Napeogenes*

Both *Ithomia* and *Napeogenes* started diversifying at middle elevations in the Middle Miocene, when the central Andes were about 30–50% of their present elevation (Gregory-Wodzicki 2000), i.e. already above 1000 m. Although precise areas of origin cannot be reliably inferred from the current distributions of extant taxa (Losos & Glor 2003), for both genera an origin in the South American Andes appears much more likely than in Central American mountainous regions. Only three *Napeogenes* species occur in Central America, and the sister taxon to the genus is also predominantly South American. Apart from a hypothetical landbridge around 34 Ma (Iturralde-Vinent & MacPhee 1999), long before the split between *Napeogenes* and *Ithomia*, Central and South America have remained isolated by seawater until the formation of the Panama Isthmus, which was completed about 3 Ma (Coates & Obando 1996). The observed patterns of diversity in *Napeogenes*, with virtually all diversification taking place in South America, would therefore be highly implausible under a scenario of Central American origin. Given that *Ithomia* and *Napeogenes* lineages diverged about 21 Ma, when Central America was isolated from South America, *Ithomia* is therefore unlikely to have originated in Central America. This is further supported by the fact that the sister genera of *Ithomia*, *Pagyrus* and *Placidina*, occur only in South America.

A highland origin is rather uncommon among the Amazonian–Andean taxa of comparable age studied to date, with the exception in vertebrates of *Calomys* rodents (Haag *et al.* 2007) and *Stenocercus* lizards (Torres-Carvajal 2007). A scarcity of studies precludes any generalization in insects, particularly in Lepidoptera. Wahlberg & Freitas (2007) found that the subtribe Phycodina colonized South America from North America about 34 Ma, when the hypothesized landbridge connected the two subcontinents (Iturralde-Vinent & MacPhee 1999). At least one phycodine group, *Eresia* s.l., diversified in the Andes, in concert with the rising of the mountains. Similarly, the Neotropical Acraeini probably originated in the Andes (Silva-Brandão *et al.* 2008). By contrast, Hall (2005) concluded from the remarkable elevationally parapatric distribution of sister species in the rioidinid butterfly genus *Ithomiola* that the genus originated in the lowlands and diversified upwards.

Napeogenes promptly expanded and diversified down the eastern slopes of the Andes, resulting in the most diverse clade within the genus (*tolosa* + *larina* clade). Three species from this clade (*Napeogenes peridia*, *N. tolosa* and *N. stella*) apparently independently colonized areas west of

the Andes. The *cranto* clade diversified in highland habitats. Inferred ancestral ranges and temporal patterns of diversification suggest that *Ithomia* diversified in the highlands until about 8–6 Ma, when two clades (*iphianassa* clade and *drymo* clade) independently colonized and diversified into the lowlands. Diversification took place at middle-to-high elevations around 5 Ma in three additional independent lineages, one of these further diversifying until the last million years (*avella* clade).

The uplift of the Andes, which accelerated since the Middle–Late Miocene (Gregory-Wodzicki 2000), thus accompanied the diversification of both genera. *Napeogenes* and *Ithomia* colonized higher habitats as those became available, resulting in high elevations as observed in *I. avella* (nearly 2400 m) or *N. larilla* (2700 m). Similarly, speciation upwards from the lowlands occurred in the *Napeogenes inachia* complex and in the case of *Napeogenes* species 1 and *N. glycera*. No such reversal into highland habitats was observed in *Ithomia*.

The lower Amazon contains only one *Ithomia* lineage (*I. agnosia* and related *I. drymo* and *I. lichyi*) and single members from four independent *Napeogenes* lineages (*N. pharo*, *N. inachia*, *N. sylphis* and *N. rhezia*). Three of these five lineages reached the isolated Brazilian Atlantic region. All these dispersal events occurred in the last 7 million years. Marine incursions during the Middle Miocene (Räsänen *et al.* 1995; Gregory-Wodzicki 2000) and the hypothesized presence of lake Pebas in the upper Amazon 23–8 Ma (Wesselingh *et al.* 2002) might have limited earlier expansion in these areas. Despite the large area of the lower Amazon and thus seemingly greater opportunities for speciation, none of these lineages further diversified there (diversification in the *I. agnosia* clade occurred in the Brazilian Atlantic region). Instead, diversification in lower Amazon forests may have been constrained by the scarcity of potential larval hostplant species (Knapp 2002; PBI *Solanum* Project 2008), all belonging to the Solanaceae family (Willmott & Freitas 2006). By contrast, the region spanning the upper Amazon and eastern Andes, and to a lesser extent the Brazilian Atlantic region, are major diversity hotspots for plants in general (Myers *et al.* 2000), and for Solanaceae in particular (Knapp 2002; PBI *Solanum* Project 2008). These hotspots offer a greater potential for ecological speciation driven by hostplant adaptation, a speciation mechanism considered important in Lepidoptera diversification (Janz *et al.* 2006; Nylin & Wahlberg 2008; Peña & Wahlberg 2008).

Central America is most likely to have been colonized by land from northern South America during the closure of Panama Isthmus. In *Napeogenes*, four species apparently independently colonized western Colombia, three of which are also found in Central America. As in the lower Amazon, *Napeogenes* apparently did not undergo further speciation in these regions. By contrast, one *Ithomia* clade experienced significant diversification in the west Andes and in Central

America, resulting in some species endemic to the latter region. This diversification was perhaps facilitated by the high diversity in the west Andes and in Central America of the *Withania* + *Ichroma* + *Physalis* Solanaceae clade (Martinez 1999; PBI *Solanum* Project 2008), on which *Ithomia* larvae feed (Willmott & Freitas 2006).

Patterns of speciation

Our results provide support for vicariant speciation in both genera. All currently allopatric sister lineages distributed across the Andes diverged before or by 3.9 Ma (Fig. 2, Fig. S1), when the Colombian Andes started rising at an accelerated rate (Gregory-Wodzicki 2000) and could have provided a significant barrier to gene flow. Andean vicariance is therefore likely to have played a role only in the middle diversification history of these genera, and apparently accounts for less than 15% of all speciation events. There is little evidence for the role of major rivers acting as a barrier (riverine barrier hypothesis, Capparella 1988; Capparella 1991), as none of the species studied here has its distribution limited by a major river such as the Amazon; or for a role of Pleistocene climatic fluctuations causing speciation through population isolation in refugia (Haffer 1969; Haffer & Prance 2001). In both genera, more than 90% of the lineages, including virtually all montane species, predate the Pleistocene, confirming previous results for *Ithomia* (Jiggins *et al.* 2006). Although major rivers and Pleistocene climatic fluctuation did not result in species formation, they could well have caused diversification at the infraspecific level. Brown (1977) proposed that refugia could explain subspecies formation in Ithomiinae and Heliconiinae. Yet, a recent molecular study found that divergence times between parapatric races of over 20 ithomiine species in the same area were not congruent (Whinnett *et al.* 2005) and thus reflected many independent events of differentiation rather than a limited set of episodes of population isolation.

When there are no obvious physical barriers between sister species, the current distribution of extant species is a poor indicator of their geographical mode of speciation, and a test of the importance of allopatry (Phillimore *et al.* 2008) would require a larger group to achieve enough statistical power. However, our results provide indications that speciation driven by ecological adaptation might have played an important role in the diversification history of both genera. First, a punctuational evolution of elevation range is significantly better supported than a gradual evolution in both genera, implying that shifts in elevation range tend to be associated with speciation. Shifts in elevation might require adaptation in a number of physiological traits. Perhaps more importantly, shifts in elevation tend to result in marked changes in ecological communities, including larval hostplants, parasitoids, predators, and,

in the case of ithomiines, abundance of other comimics. Adaptations caused by any of these changes can have cascading effects on all aspects of the life cycle (Elias *et al.* 2008), and it is therefore no surprise if such changes rapidly lead to speciation. Only one species, *Ithomia 'lagusa' theuda*, apparently occurs in both lowlands and highlands, although it is possible that the lowland and highland forms represent incipient or actual species. Broad elevational ranges are in general rare in Andean butterflies; although a few migratory or secondary growth species may have ranges of over 3000 m, the mean elevational range size for over 2000 east Andean butterfly species in Ecuador is approximately 800 m (K. Willmott & J. Hall, unpublished). Second, Both *Napeogenes* and *Ithomia* experienced a decrease in speciation rate in the last 4 million years, after all new areas where colonized. A pattern of decreasing speciation rate is often interpreted as a signature of adaptive radiation, for instance when a lineage colonizes a new set of niches that promotes diversification until niches are filled (Schluter 2000). However, in adaptive radiations the rate of speciation is a decreasing function of the number of species (e.g. Nee *et al.* 1992; Rabosky & Lovette 2008a), whereas a decline in speciation rate might also be explained by nondensity-dependent processes (Rabosky & Lovette 2008a), by random effects (Phillimore & Price 2008), or by failure to recognize cryptic species. While in *Ithomia* and *Napeogenes*, the colonization of the lowlands could well have driven adaptive radiation due to the availability of entirely new types of habitats and hostplants, this would need to be confirmed by further analyses of the diversification rates combined with comprehensive information on hostplant use when the latter becomes available. In fact, adaptive radiations may have been important throughout the entire Ithomiinae subfamily. The switch to a new family of larval hostplants, the Solanaceae, from ancestral Apocynaceae (Willmott & Freitas 2006), clearly offered a major diversification opportunity for the group which now harbours over 360 species, with subsequent specialization on particular Solanaceae clades or species, and one further switch to the Gesneriaceae plant family (Willmott & Freitas 2006). In the most diverse communities, most Solanaceae species are used as larval hostplants by ithomiine species (K. Willmott & M. Elias, unpublished), suggesting that the hostplant niche is near saturation.

Our results thus suggest that although speciation events between the Andes and adjacent lowlands did occur, they were relatively rare, and therefore, in contrast to Fjelsdå's (1995) hypothesis, the Andes do not serve as an important source for lowland species, or vice versa. Instead, infrequent colonizations between these regions may have provided the ancestors of subsequent apparent adaptive radiations that took place within single elevational zones, contributing significantly to the species diversity of both genera.

Conclusion

We found that the Andes played a major role in the diversification of the 48 species in the two studied ithomiine genera, both by isolating populations on either side of the chain (vicariance), but also by offering a range of ecological conditions. Ecological speciation is probably more common in insects (Willmott *et al.* 2001; Hall 2005; Jiggins *et al.* 2006), because they tend to occupy smaller ecological niches than vertebrates.

New areas were apparently promptly colonized as they became available (neighbouring lowlands, then lower Amazon and Central America), sometimes enabling further diversification. Most major ithomiine groups occur in the Andes (Lamas 2004) as well as in the lowlands on both sides, and the orogenesis is likely to have contributed to the diversification of other major ithomiine lineages. Our phylogenetic analyses clearly show that the ancestors of both *Napeogenes* and *Ithomia* occurred at higher elevations. The early role of the Andes in ithomiine diversification contrasts with most studies on Andean vertebrates of similar age, which often find a lowland origin followed by colonization of the Andes. Whether our findings hold for most insects will require further investigation, since among diverse lowland groups the ithomiines are unusual in maintaining an equal diversity into middle elevations.

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6 Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1 Maps showing the geographical ranges of *Napeogenes* and *Ithomia* species.

Fig. S2 *Napeogenes* trees for each gene region (*Col-ColII*, *EF1 α* , *Tektin*) obtained by maximum likelihood, Bayesian inference and maximum parsimony, with bootstrap values and Bayesian posterior probabilities; and maximum parsimony tree based on all gene regions with bootstrap values, Bremer support and partitioned Bremer support.

Fig. S3 Dated tree (uncorrelated lognormal relaxed clock), showing 95% credibility interval for timing of *Ithomia* and *Napeogenes* lineages.

Fig. S4 Parsimony reconstruction of ancestral elevation ranges in *Ithomia* and *Napeogenes*.

Table S1 List of all specimens used in this study (including *Ithomia*) with GenBank Accession numbers and locality data

Table S2 Primers, PCR and sequencing reaction conditions for the phylogeny of *Napeogenes*

Table S3 Nucleotide substitution model for each gene region for the phylogeny of *Napeogenes*

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