THE PHYLOGENETIC PATTERN OF SPECIATION AND WING PATTERN CHANGE IN NEOTROPICAL *ITHOMIA* BUTTERFLIES (LEPIDOPTERA: NYMPHALIDAE)

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Abstract.—Species level phylogenetic hypotheses can be used to explore patterns of divergence and speciation. In the tropics, speciation is commonly attributed to either vicariance, perhaps within climate-induced forest refugia, or ecological speciation caused by niche adaptation. Mimetic butterflies have been used to identify forest refugia as well as in studies of ecological speciation, so they are ideal for discriminating between these two models. The genus *Ithomia* contains 24 species of warningly colored mimetic butterflies found in South and Central America, and here we use a phylogenetic hypothesis based on seven genes for 23 species to investigate speciation in this group. The history of wing color pattern evolution in the genus was reconstructed using both parsimony and likelihood. The ancestral pattern for the group was almost certainly a transparent butterfly, and there is strong evidence for convergent evolution due to mimicry. A punctuationist model of pattern evolution was a significantly better fit to the data than a gradualist model, demonstrating that pattern changes above the species level were associated with cladogenesis and supporting a model of ecological speciation driven by mimicry adaptation. However, there was only one case of sister species unambiguously differing in pattern, suggesting that some recent speciation events have occurred without pattern shifts. The pattern of geographic overlap between clades over time shows that closely related species are mostly sympatric or, in one case, parapatric. This is consistent with modes of speciation with ongoing gene flow, although rapid range changes following allopatric speciation could give a similar pattern. Patterns of lineage accumulation through time differed significantly from that expected at random, and show that most of the extant species were present by the beginning of the Pleistocene at the latest. Hence Pleistocene refugia are unlikely to have played a major role in *Ithomia* diversification.

Key words.—Comparative analysis, Lepidoptera, mimicry, Pleistocene refugia, speciation.

Speciation in the tropics remains especially poorly understood, despite the fact that the world's most diverse ecosystems are tropical (Moritz et al. 2000). One long-standing hypothesis is that diversification occurred due to vicariance in Pleistocene forest refugia (Brown 1982; Haffer 1977). An alternative is that ecological adaptation to novel ecotopes or niches drives diversification, without a necessary requirement for allopatry (Benson 1982; Endler 1977; Fjeldså 1994; Graham et al. 2004; Schneider et al. 1999). Such ecological shifts might occur more readily in tropical ecosystems, if ecological niche diversity were greater in more diverse communities, such that species richness could promote further speciation (Emerson and Kolm 2005). Thus, the relative importance of different modes of speciation needs to be tested empirically, especially in tropical regions.

Molecular sequence data can be used to investigate the tempo and mode of speciation (Nee et al. 1992). Phylogenies provide information on the timing and pattern of diversification, and concordant area cladograms in sympatric but unrelated taxa are seen as good evidence for vicariant diversification (Cracraft and Prum 1988; Hall and Harvey 2002). However, in the tropics, recent evidence has suggested that many taxa diverged before the Pleistocene, casting doubt on the Pleistocene refugia hypothesis (Moritz et al. 2000). Furthermore, many butterfly taxa occurring across a concordant hybrid zone in eastern Peru showed very different degrees of molecular divergence, arguing against a common vicariant event having caused their diversification (Whinnett et al.

2005). Species-level phylogenetic hypotheses have also been used to investigate whether ecological shifts are associated with speciation events (Barraclough et al. 1999) and whether particular traits tend to be associated with clades of greater species diversity (Isaac et al. 2005). Nevertheless, the majority of ecological speciation studies focus on closely related groups of species or incipient species (Schluter 2000); thus, it is often difficult to gauge the broader role of adaptation in the diversification of entire clades.

Here we use molecular phylogenetic data to investigate whether ecological adaptation is likely to have been important in the diversification of a genus of mimetic Neotropical butterflies. Mimetic butterflies have been used both in the identification of putative forest refugia (Brown 1979) where vicariant speciation might have occurred, as well as in studies of ecological speciation (Jiggins et al. 2004a). Butterfly wing patterns are obvious phenotypic traits that show rapid evolution and play a role in reproductive isolation (Vane-Wright 1978; Jiggins et al. 2001; Lukhtanov et al. 2005), being used in sexual signaling and mate choice (Stride 1957, 1958; Brower 1959; Lederhouse and Scriber 1996; Deering and Scriber 2002; Fordyce et al. 2002), as well as in predator defence. A number of butterflies in Neotropical forests are unpalatable to predators and bear warning color patterns, and sympatric species tend to converge in pattern through mimicry to facilitate predator recognition (Müller 1879). In the genus *Heliconius*, it has long been known that rather distantly related species tend to mimic one another, whereas closely

related species commonly have very different color patterns (Turner 1976). These pattern differences between closely related species contribute to reproductive isolation and therefore play a direct role in speciation (Jiggins et al. 2004b). Furthermore, hybrid individuals with intermediate wing patterns are commonly nonmimetic, causing selection against hybrids (Naisbit et al. 2003). Phylogenetic analysis of the heliconiines shows that sister species almost invariably differ in mimetic pattern (Brower and Egan 1997) and demonstrates a correlation between color pattern and species diversity (M. S. Beltrán, N. Isaak, J. Mallet, and C. D. Jiggins, unpubl. ms.). Mimicry is therefore an excellent example of the kind of adaptation that is likely to promote speciation in tropical organisms. However, to investigate the generality of color pattern as a causal factor in speciation it is necessary to test these hypotheses in other mimetic butterflies.

Here we use a recent and taxonomically almost complete molecular phylogeny for the genus *Ithomia* to investigate whether ecological speciation is likely to have been important in diversification. *Ithomia* are abundant, widespread species often involved in mimicry with other genera of ithomiines (Beccaloni 1997). We investigate three aspects of *Ithomia* evolution that might provide support for ecological versus vicariant speciation. First, we investigate whether changes in color pattern are associated with speciation events. Second, since speciation driven by adaptation is not so strongly retarded by gene flow and is thus likely to occur in parapatry and perhaps also sympatry (Maynard Smith 1966; Endler 1977), we also investigate the geographical context of species divergence using a plot of geographic overlap against clade age. Finally, we use the phylogeny to investigate the time course of diversification during the history of the genus, to test the prediction of the Pleistocene refugia hypothesis that most speciation has occurred in the recent past.

MATERIALS AND METHODS

Study Group and Phylogeny

Butterflies in the nymphalid subfamily Ithomiinae are distasteful to predators and often brightly colored. Similar to *Heliconius*, they are involved in mimicry and show extreme intraspecific geographic diversity of wing patterns. However the group is far more diverse (360 spp.) than the heliconiines, offering greater opportunity for comparative analysis (Fox 1940; Lamas 2004). Our analysis is based on a phylogeny obtained for the genus *Ithomia* based on seven genes, the mitochondrial *CoI, CoII* and the tRNA-leucine, and the nuclear genes *Ef1* α, *Tektin*, *Wg*, and *RpL5* (Mallarino et al. 2005). The phylogeny derived from a Bayesian analysis of the concatenated sequences of six of these genes (all except *RpL5*) was consistent with the separate analysis of the individual gene trees and was proposed as a most likely phylogenetic hypothesis for the genus. Likelihood ratio tests implied that both individual gene alignments and the complete dataset were consistent with the assumption of a strict molecular clock (Mallarino et al. 2005). The phylogeny is generally well resolved, with the exception of the relationships of four species in the *I. agnosia* clade and two branches in the *I. eleonora* and *I. iphianassa* clades that lacked strong support. The implications of phylogenetic uncertainty on our

results are discussed where appropriate. The original dataset included two or more individuals for each species, and the consensus phylogeny (Mallarino et al. 2005) is presented here as Figure 1. Since variation within species was minimal, individuals were randomly excluded so as to include just one representative of each species for all analyses presented in this article. Phylogenetic hypotheses were then recalculated with these reduced data using MrBayes to generate a species-level phylogeny (methods as described in Mallarino et al. 2005).

The phylogeny includes 23 of the 24 known species of *Ithomia*. This species total takes into account some revisions of the most recent alpha taxonomy (Lamas 2004) based on genetic and field data (Mallarino et al. 2005). For the purposes of the present analysis we split *I. terra* into two genetically divergent species. We refer to *I.* (*terra*) sp. *nov* as the eastern form of *I. terra*, although we note that at this stage it is not clear whether the name *terra* should correctly apply to the eastern or western form, or even whether the two are truly distinct species (Mallarino et al. 2005). The authors favor either the biological species concept or the genotypic cluster species definition, both of which lead us to recognize the same species in *Ithomia*.

Testing for Diversification Associated with Mimicry Change

To test for the role of color pattern change in cladogenesis we require a classification of color patterns. In most cases, pattern classes are easily distinguished and unambiguous but are nonetheless based on subjective human judgment. Previous studies have used a similar approach (Beccaloni 1997; Lukhtanov et al. 2005), the validity of which is supported by the fact that bird predators are unable to distinguish species that humans perceive to be mimetic (Turner 1977) and that birds perceive similarity between species in a similar manner to humans (Dittrich et al. 1993). A classification of wing patterns, termed the *mimicry classification,* was devised based on mimicry pattern variation throughout the Ithomiinae, including previously published studies of mimicry within ithomiine populations (Beccaloni 1997; Haber 1978). Similar patterns were treated as distinct complexes if they occurred in sympatry, separated by a consistent phenotypic gap from other such complexes, while concordant minor geographic variation in pattern was used to group allopatric patterns into the same mimicry complex (K. R. Willmott, unpubl. data; Table 1). The patterns of all geographic races of every species of *Ithomia* were also classified into broader classes based on appearance, without reference to other comimetic species, in a manner similar to that proposed for the heliconiines (Brown 1981). This led to a more coarse-scale classification in which several mimicry classes were grouped together. This was termed the *pattern classification.* There were six pattern classes recognized: *Clearwing (C), Yellow and Black (YB), Tiger (T), Black Mosaic (BM), Colored (Co),* and *Orange (O)* (Table 1 and Fig. 2). The two classifications, which were independently derived by two of the authors (KRW and CDJ), were complementary to one another, with the latter constituting a coarser scale grouping of classes recognized by the former. The two are presented as alternative methods of classifying the mimicry evolution of the
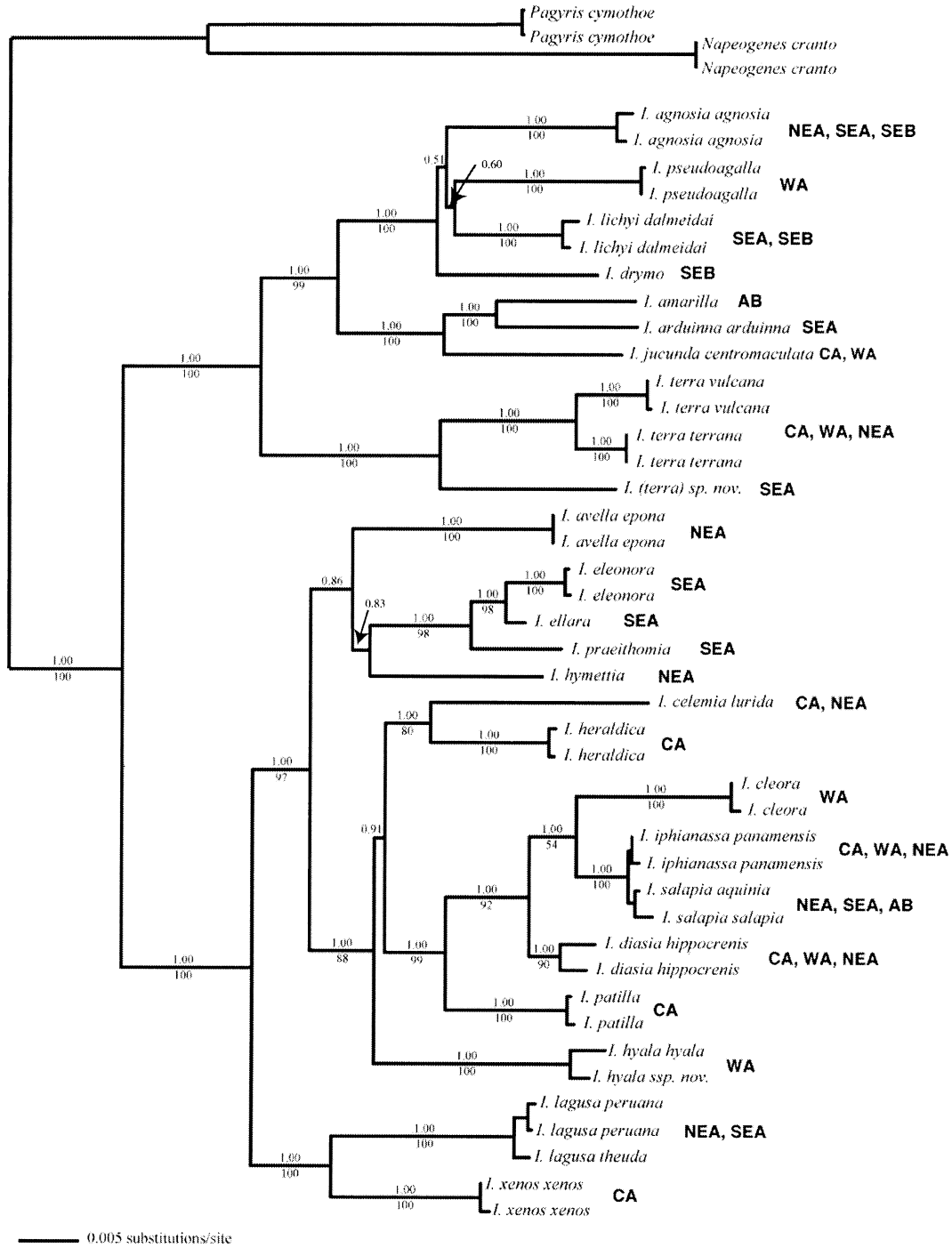
FIG. 1. Combined evidence phylogeny of the genus *Ithomia*. Biogeographic regions are shown for each species: CA, Central America; NEA, northeastern Andes; SEA, southeastern Andes; WA, western slopes of the Andes; AB, Amazon Basin; SEB, southeastern Brazil. Methods used in tree reconstruction are as described by Mallarino et al. (2005). Numbers above branches represent Bayesian support values, while numbers below are parsimony bootstrap support for the same node. Absence of a value indicates either that the node was absent in the parsimony tree, or that suppport was less than 0.5 (Bayesian analysis) or 50 (boostrap analysis).

butterflies and to demonstrate that the results are not strongly dependent on the degree to which pattern classes are ''split''. The classes recognized were used as character states for the species in all subsequent analyses, with divergent geographic races included as within-species polymorphisms. The only species whose classification was somewhat ambiguous was

I. pseudoagalla, which has a smoky brown pattern that is somewhat intermediate between classes. This species was initially classified in the *Clearwing* pattern class by CJ, but in the Dilucida (*Colored*) mimicry class by KRW. The pattern, although largely transparent, is more closely mimetic to darker species found sympatrically in western Ecuador

such as *I. iphianassa* than to other clearwing species. Hence the change in pattern is slight, but arguably sufficient to represent a change in mimicry ring. This uncertainty is taken into account by repeating all analyses with both possible classifications of *I. pseudoagalla* (*Clearwing* or *Colored*; see below).

In butterfly mate recognition, color patterns are primarily used as a means by which males recognize females as potential mates (Fordyce et al. 2002; Jiggins et al. 2004b). Hence, where there is sexual dimorphism it is the female color patterns that will most likely generate reproductive isolation. Therefore, in cases of sexual dimorphism, results were compared both using male and female patterns classified as a polymorphism, and also including just the female patterns. This only affected the classifications for the two species *I. ellara* and *I. eleonora*. To visualize the distribution of pattern changes on the tree, color pattern change was reconstructed by parsimony using MacClade 4.0 (Maddison and Maddison 1997).

We focused on testing the hypothesis that changes in color pattern are associated with speciation events. Likelihood models of ancestral character state reconstruction can be used to compare alternative evolutionary scenarios, such as gradual evolution along branches of a phylogeny versus punctuationist evolution in which character states change at nodes. If color pattern change is associated with speciation, then we expect the punctuationist model to be a better fit to the data. We therefore used likelihood to compare punctuationist versus gradualist models of color pattern evolution using the program Bayes-MultiState version 1.0 (Pagel 1994; Pagel et al. 2004). This program has the advantage that multiple character states can be used, such that species polymorphisms can be scored as polymorphic character states on terminal branches. Under a gradualist evolutionary model, the probability of color pattern change is expected to be in direct proportion to branch length. For the purpose of this analysis it is assumed that genetic distance measured from our sequence data is representative of the opportunity for phenotypic change along any particular branch. In contrast, under a punctuationist model, color pattern changes occur during cladogenesis. A simple likelihood model of character evolution can be used to test between these hypotheses by altering the branch length scaling parameter, κ (Pagel 1994). A gradualist model is represented by a κ value of one, whereby pattern change occurs in direct proportion to genetic distance. Here we compare this simple model with a more complex model in which κ is allowed to vary. Under this scenario, as κ tends to zero the model becomes more punctuationist. The significance of alternative hypotheses can be investigated using likelihood ratio tests (LRT), where the model in which κ is allowed to vary has one extra parameter, such that the LRT ($-2\Delta\ln L$, equivalent to G) has one degree of freedom (Edwards 1972). The effect of phylogenetic uncertainty on our results was investigated by calculating the likelihood ratio for every tree sampled in our Bayesian Markov chain Monte Carlo (MCMC) reconstruction of the species-level tree using the program MrBayes (Huelsenbeck and Ronquist 2001). The first 10,000 iterations of the chain were discarded and stationarity of the remaining samples confirmed by plotting the log-likelihood values of the remaining trees (data not shown). After stationarity had been achieved, 9900 trees were sampled, which should be representative of uncertainty in the phylogenetic hypothesis. The difference in log likelihood between the puntuationist and gradualist model was calculated for all 9900 trees.

Determining the Geographic Pattern of Speciation

We investigated the relative importance of allopatric and sympatric speciation using the methodology of Barraclough and Vogler (2000). The degree of range overlap was plotted against node age for all nodes in the tree. If speciation is allopatric, recently diverged sister species are expected to display little or no overlap in geographic ranges. If speciation is predominantly sympatric, recently diverged sister species are expected to display a high degree of range overlap.

Locality data were obtained from specimens in the Natural History Museum (NHM), London, the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima (MUSM), and unpublished Ecuadorian records of K. R. Willmott and J. Hall (unpubl. data). Localities were georeferenced using a variety of sources, but mainly (Brown 1979), the National Imagery and Mapping Agency (http://www.nima.mil) and G. Lamas (pers. comm.). Species ranges were inferred from dot map coordinates on equal-area maps using ArcView 3.2 (ESRI, Redlands, CA). A sinusoidal projection with a central meridian of 75°W was used. For each species, circles of 350 km diameter were drawn around each dot to generate a single continuous distribution. This diameter is arbitrary and was chosen based on the density of collection sites, aiming to generate continuous distribution areas. In our experience in the field, gaps between collecting sites almost invariably represent information gaps rather than genuinely disjunct distributions so it is more likely to be correct to infer a continuous distribution from such disjunct points. Altitudinal range information was incorporated by excluding regions above and below the altitudinal limits of each species, as estimated from reliable collection locality data. An area map was generated for each 500-m contour, and these were then used to subtract the upper and, in the case of montane species, also the lower altitudinal limit from each species range. Range maps were then trimmed by hand according to known geographic barriers—most notably where circles had crossed known barriers such as the Andes. These range maps are crude, but are likely to be the best achievable with the relatively sparse collection data available for many species and are consistent with our field experience.

The degree of range overlap was then calculated for each node in the tree (Barraclough and Vogler 2000; Fitzpatrick and Turelli 2006). For terminal species pairs, the area of range overlap was calculated and this value divided by the area of the smallest species range. For deeper nodes in the tree we present various alternative methods. The method originally proposed was to sum ranges for each clade and use these total clade ranges for calculating overlaps at each node (Lynch 1989; Barraclough and Vogler 2000). As has been highlighted by Berlocher and Feder (2002), this method is problematic because range areas are necessarily larger at deeper nodes in the tree, thus the absolute size of ranges being compared is larger at older nodes. This means that there

TABLE 1. Classification of color patterns of *Ithomia* species. Brackets indicate sexually dimorphic characters, with braces showing patterns restricted to males, and parentheses those restricted to females. The mimicry classification shown is based on groups of co-occurring mimetic species separated by phenotypic gaps from other co-occurring species. The names for the different classes are derived from ithomiine species or races. The pattern classification shown has only six classes that represent a grouping of similar mimicry classes: *Clearwing (C), Yellow and Black (YB), Tiger (T), Black Mosaic (BM), Colored (Co)*, and *Orange (O)*.

| | Mimicry classification | | | | | | | | | |
	AGNOSIA	AMALDA	BANJANA-M	CONFUSA	DERASA	DILUCIDA	EURIMEDIA	EXCELSA	HERMIAS	HEWITSONI
Pattern group	C	O	C	C	YB	Co	YB	Co	T	C
Ithomia species										
agnosia	X									
amarilla							X			
arduinna	X						X			
avella			X							X
celemia						X			X	
cleora									(X)	
diasia		X								
drymo										
eleonora			{X}							
ellara			{X}							
heraldica										
hyala	X									
hymettia			X							
iphianassa						X			X	
jucunda		X								
lagusa			{X}	X						
leila										
lichyi	X									
patilla										
praeithomia			X							
pseudoagalla						X				
salapia	X				X		X			
sp. nov.			X							
terra	X		X							
xenos						X		X		

is a higher probability of sympatry in comparisons deeper in the tree topology. Thus an increase in the degree of sympatry through time (i.e., older nodes are more likely to be sympatric) is generated in part as an artifact of the way the method treats sister clades (Berlocher and Feder 2002). Hence, we also present an alternative method that has recently been proposed, using independent contrasts between pairs of taxa (Fitzpatrick and Turelli 2006). Average pairwise overlaps are calculated using a method developed for the study of reproductive isolation (Fitzpatrick 2002). Third, we present maximum pairwise overlap between any pair of species in the two clades (Fitzpatrick and Turelli 2006). Changes in average overlap could simply be an artifact of increasing variance in overlap as ranges move over time, so maximum pairwise overlap might overcome this problem. Maximum overlaps might be expected to start low and gradually increase for allopatric speciation, but start high and remain high under sympatric speciation. The overlap values calculated using all three methods described above were plotted against node age, estimated from a tree reconstructed under the assumption of a strict molecular clock.

Estimating significance of such plots is problematic. The distribution of overlap values is clearly different at deeper nodes compared to recent splits, so parametric methods are hard to justify (Barraclough and Vogler 2000). Alternatives such as permuting ranges among species are also problematic, since there is a clear phylogenetic signal in geographic ranges across our phylogeny (Fitzpatrick and Turelli 2006). In other

words, closely related species are more likely to share a biogeographic region than expected at random. For descriptive purposes and for comparison with previous results, we used linear regression on overlap values to estimate intercepts and slopes (Barraclough and Vogler 2000) and nonparametric Spearman's rank tests to investigate the significance of the slope.

Speciation Rates

We also focused on the time course of speciation. The Pleistocene refugia hypothesis would predict high speciation rates during the Pleistocene and perhaps a significant increase in speciation rates during this period. We therefore investigated patterns of lineage accumulation during the history of the genus and then related this to geological epochs by comparison with published insect molecular clocks (see Discussion). The accumulation of species in *Ithomia* was investigated by plotting the log of the number of lineages against the scaled branch-length distances of the clock-constrained maximum-likelihood tree. Log(diversity) is expected to show a straight line relationship with time under a constant net speciation model. Deviation from a constant speciation model (Barraclough and Vogler 2002) was tested using the γ-statistic of Pybus and Harvey (2000). A positive value of γ indicates that the speciation rate increases toward the tips of the cladogram, whereas a negative value is expected if the speciation rate decreases through time. The hypothesis of a

TABLE 1. Extended.

Mimicry classification										Pattern classification
IDAE	LERIDA	MAMERCUS	MANTINEUS	MESTRA	PANTHYALE	PARALLELIS	PRAXILLA	TICIDA-M	SUSIANA	
T	C	Co	Co	C	C	T	C	C	BM	
										C
										YB
										C/YB
					X			X		C
X		X				X				Co/T
			X							Co
	X									C/O
	X									C
									(X)	C/BM
									(X)	C/BM
		X								Co
	X									C
										C
X										Co/T
	X									C/O
		X		X			X			C/Co
	X									C
	X									C
	X									C
									(X)	C/BM
										C
										C/YB
										C
	X									C
										Co

constant speciation rate is rejected if γ is less than −1.645 or greater than +1.645 (Pybus and Harvey 2000). Pybus and Harvey (2000) proposed a method of correcting for incomplete sampling, which results in lower γ values as internal nodes tend to leave more descendants than terminal nodes. We therefore used the Monte Carlo constant rates (MCCR) test of Pybus and Harvey (2000) to adjust our expected values, taking into account the proportion of sampled taxa. This test simulates the distribution of γ under the pure birth process when only a specified proportion of the extant species are sampled. The program APE (E. Paradis; available via http://www.isem.univ-montp2.fr//ppp/pppphylogenie/paradishome.php) was used to run the simulations (10,000 simulations, 23 sampled taxa of 24 extant taxa) and obtain the γ-statistic (Paradis et al. 2004).

RESULTS

Color Pattern Change

Parsimony reconstruction of color patterns in *Ithomia* using the pattern-based classification showed that the ancestral pattern was most likely to have been a clearwing butterfly (Fig. 3). This pattern is found in species distributed widely across the *Ithomia* tree and is shared with the closely related genus *Pagyris*, although *Placidina*, which is probably sister to *Pagyris* (K. R. Willmott and A. Freitas pers. comm..) has a *Tiger* pattern. The reconstruction of color pattern change was generally unambiguous under the accelerated changes method. A reconstruction using decelerated changes showed far more ambiguous character states on internal branches (not shown), but showed the same ancestral state for the genus as a whole. However, whichever method was used, there were relatively few switches in color pattern between closely related species (Fig. 3). There was an unambiguous switch between the sister species, *I. salapia* and *I. iphianassa*, and possibly another between *I. pseudoagalla* and its sister *I. lichyi*. A reconstruction using the second, mimicry-based classification was virtually identical, with the added mimicry classes mostly representing polymorphisms within species rather than switches between species (Table 1; reconstruction not shown). These reconstructions were not affected by phylogenetic uncertainty, because poorly supported branches were not in regions of the tree that would affect reconstruction of ancestral patterns.

Likelihood analysis showed an association of color pattern change with cladogenesis. Using the pattern classification to assign character states, the gradualist model gave a probability of a clearwing ancestor of 0.95, and a rate parameter for pattern change of 2.76 (Ln L = −25.07). In contrast, when κ was estimated, the most likely model had a rate parameter two orders of magnitude lower of 0.026, a κ value of 0.000, and a probability for a clearwing ancestor of 0.99 and was a better fit to the data (LnL = −20.71; comparison of the two hypotheses gives −2ΔLn L = 8.72, $P < 0.01$). Thus, the punctuationist model with κ = 0 was the best fit to the data. This result was robust to different methods of

FIG. 2. *Ithomia* species and their color pattern classes. A single specimen of each species is shown; thus, all geographic variation is not necessarily represented. The species name (italics, top) and both mimicry-based (capitals, center) and pattern-based (capitals, bottom) classifications are shown. The *Tiger* pattern is distinguished from the *Colored* pattern in being entirely opaque (all *Colored* patterns are somewhat transparent). The *Black Mosaic* pattern is distinguished from the *Clearwing* patterns by the heavy black scaling on the veins, giving a spotted appearance. The *Yellow and Black* and *Orange* patterns are easily recognized by the yellow background color and orange hindwing, respectively.

scoring character states, such as when *I. pseudoagalla* was classified as a *Co* pattern, or when only female color patterns were included (data not shown). Similarly, analysis using the mimicry-based classification, with 20 character states, also gave strong support for the punctuationist model (-2ΔLn L $= 17.46$, $P < 0.01$). When the difference in log likelihood was calculated across the 9900 trees sampled during the Bayesian MCMC analysis of the sequence data, all trees had κ estimates of zero and significant support for the more complex model (Fig. 4).

The Role of Geography in Speciation

The pattern of change in geographic overlap through time was heavily dependent on the method used to compare overlap at ancestral nodes. Nonetheless, all methods showed that many of the most recently separated species pairs had some degree of geographical range overlap (Figs. 1 and 5). When the range summing method was used, the intercept fitted line was $0.239 + 9.04 \times$ age and there was no significant slope (Spearman's $\rho = 0.32$, ns). Under the independent contrasts method, the intercept was 0.434, the slope was -3.589, and
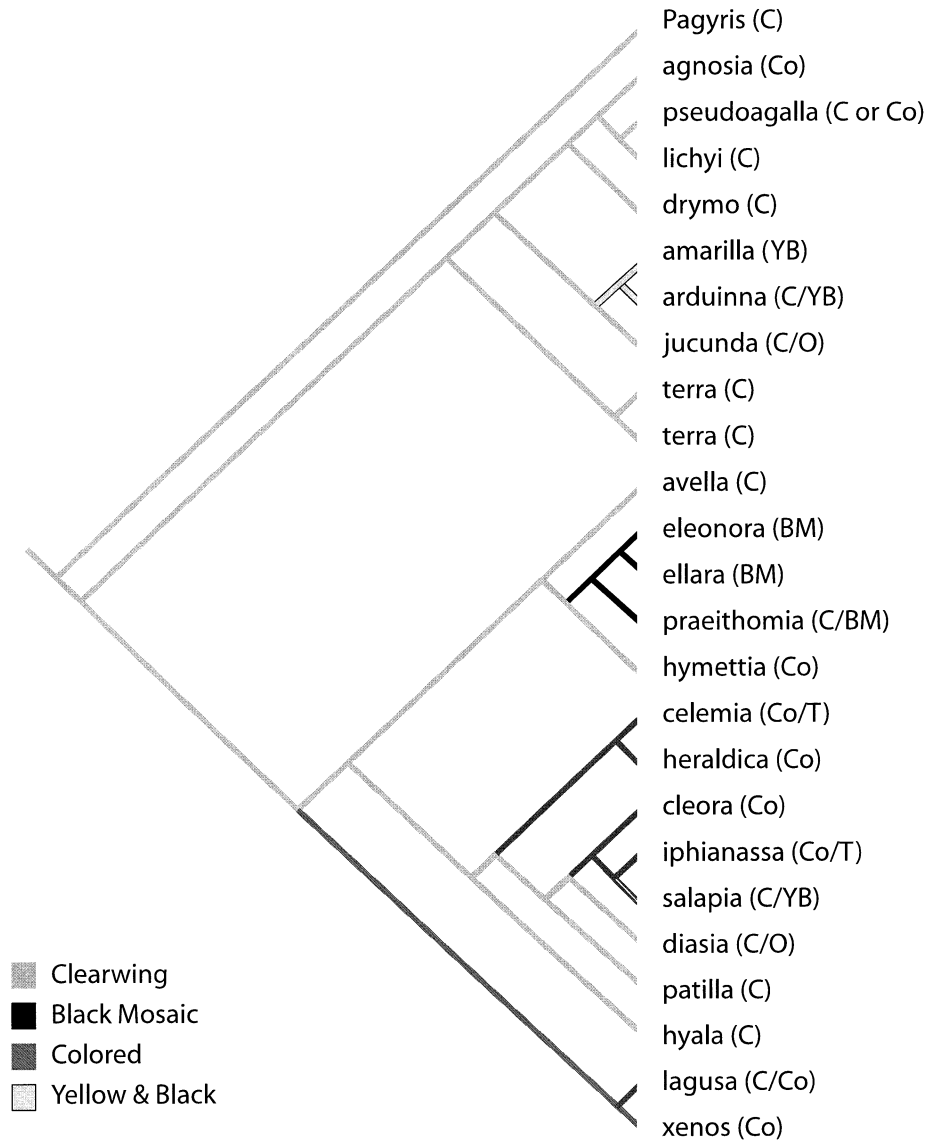
FIG. 3. Reconstruction of ancestral color patterns in the genus *Ithomia*. Estimation of ancestral states was carried out using parsimony with ACCTRAN (accelerated changes), implemented in MacClade 4.0. Character states are assigned to terminal taxa based on the pattern classification of all pattern variants found in *Ithomia*. Polymorphisms represent either different geographic races within a species or sexual dimorphism. Color pattern state abbreviations are as given in Table 1. The *Orange* and *Tiger* mimicry rings are represented at terminal nodes but are not reconstructed at any of the internal nodes, so are not shown in the legend.

there was no significant change in degree of sympatry over time (Spearman's $\rho = -0.14$, ns). The only overlap value that gave a significant slope was the maximum overlap, which showed a significant increase with time ($0.277 + 10.384 \times$ age; Spearman's $\rho = 0.45$, $P < 0.05$).

Speciation Rates

The pattern of accumulation of phylogenetic diversity in *Ithomia* differed significantly from that expected at random (Fig. 6). The γ-statistic was -2.447 ($P = 0.0027$) for the tree derived from all genes, and -2.8203 ($P = 0.0019$) for the mitochondrial DNA tree alone. These results indicate a significant slowing down of speciation rates in the latter part of the history of the genus. Topological uncertainty is un-

likely to affect this analysis, because it is dependent on branch lengths rather than exact topology.

DISCUSSION

Color Pattern Change and Speciation

The species-level phylogeny of the genus *Ithomia* provides evidence for an association of color pattern change and speciation at deeper nodes in the tree, supporting the hypothesis that changes in mimicry are associated with speciation events in mimetic butterflies, as first suggested by Bates (1862). The genus *Ithomia* has clearly undergone considerable evolutionary change in color pattern, with several examples of convergent evolution leading to mimicry between distinct line-
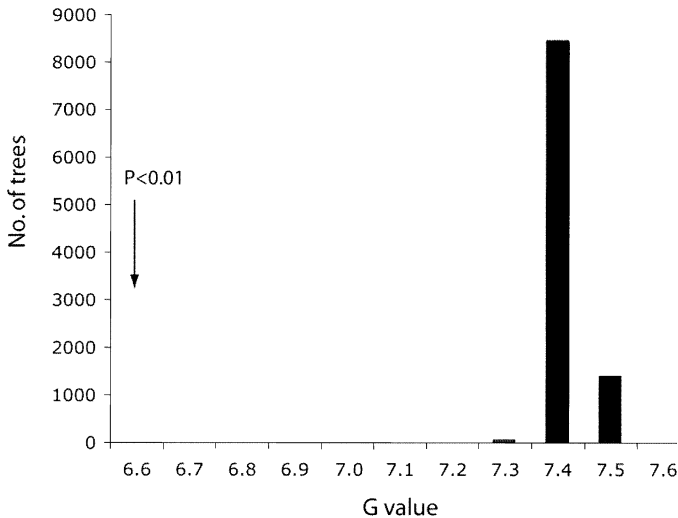
FIG. 4. Difference in log-likelihood values between punctuationist and gradualist hypotheses in *Ithomia*. Data shown are for 9900 trees sampled during Bayesian MCMC analysis of combined sequence data. The *G*-value was calculated for each tree by comparing the punctuationist (κ allowed to vary) and gradualist models (κ = 1). The observation that all values are significant indicates that there is support for the punctuationist model across all of the plausible tree space suggested by our sequence data. The right hand tail of the distribution is truncated for clarity.
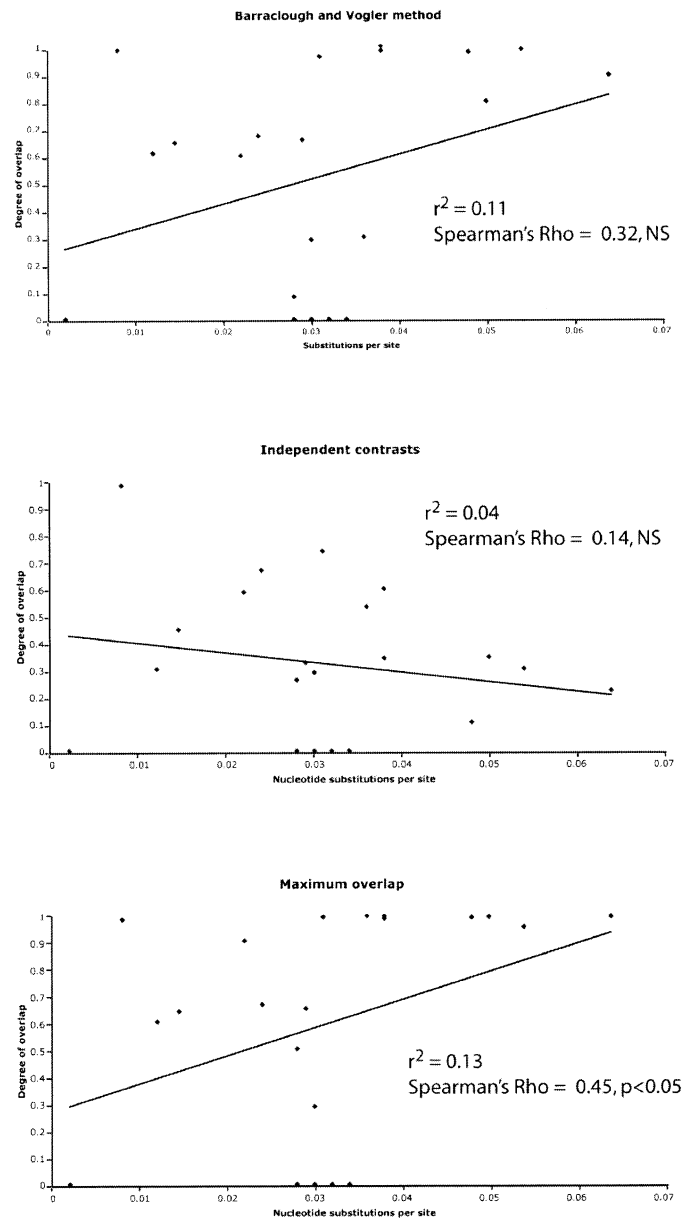

FIG. 5. Degree of sympatry plotted against relative node age, shown as substitutions per site, in *Ithomia*. Three methods of calculating range overlap are shown (see Materials and Methods for more details). (A) Species ranges are merged for each clade before calculating overlap, (B) overlaps are averaged across species pairs using independent contrasts, and (C) the maximum pairwise overlap between each pair of clades is shown. Lines shown are simple linear regressions.

ages in the genus. For example the *Yellow and Black* pattern (*I. salapia* and *I. amarilla*), the *Tiger* pattern (*I. iphianassa* and *I. celemia*) and the *colored* pattern (*I. heraldica*, *I. cleora/iphianassa*, and *I. xenos/lagusa*) have all evolved at least twice in the history of the genus. In spite of this, however, there is little evidence that the most recent speciation events have involved switches in color pattern. Several polymorphic species share at least one pattern with their closest relative. Thus, *I. iphianassa* shares the *Colored* pattern with *I. cleora*, *I. xenos* similarly shares this pattern with *I. lagusa*, and *I. jucunda* shares the *Clearwing* pattern with *I. arduinna*. In these cases, the most parsimonious hypothesis is that the novel derived patterns found in *I. iphianassa*, *I. lagusa*, and *I. jucunda* have evolved subsequent to speciation, although it is also likely that in some cases the shared putative ancestral pattern actually represents recent convergence. Only one sister species pair, *I. salapia* and *I. iphianassa*, has unambiguously diverged in color pattern and is therefore a candidate for color pattern change having occurred at a recent speciation event. Furthermore, there are some clades in which speciation has definitely occurred without any significant color pattern switches, leading to groups of related and sympatric species with similar patterns. Examples include *I. ellara* and *I. eleonora* and the group of *I. agnosia*, *I. lichyi*, and *I. drymo*. This pattern contrasts with that seen in *Heliconius*, in which most sister species differ in pattern. Clearly, the *Ithomia* have undergone speciation via mechanisms that do not involve changes in color pattern.

However, across the genus as a whole there was strong evidence that shifts in color pattern occurred in association with cladogenesis. A punctuationist model was a significantly better fit to the data than a gradualist model, a result that was robust to different color pattern classifications and to

uncertainty in the phylogeny reconstruction. The implication of this result is that the rate of pattern change increases where rates of speciation are higher, rather than being correlated with branch lengths. This accords with our observation that the rate of speciation was higher in the earlier part of the history of the genus and that most pattern shifts were not found at the tips of the tree, but rather on internal branches. Of course we cannot directly infer a causal link between pattern shifts and speciation from these comparative data.
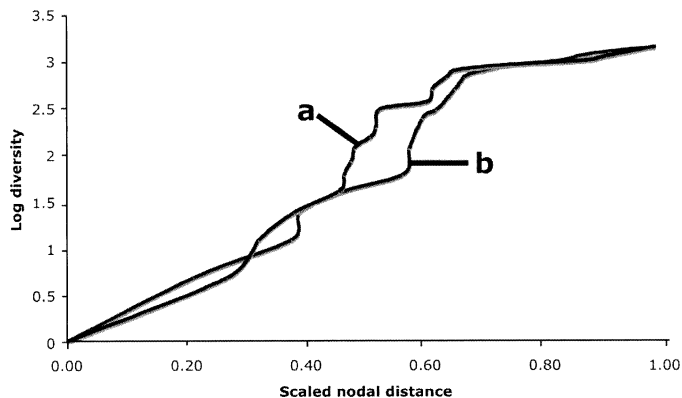
FIG. 6. Lineage-through-time plot for *Ithomia*. log-transformed lineage diversity and scaled branch-length distances under a maximum likelihood clock-constrained tree are shown. Line (a) corresponds to the plot for mtDNA data only, (b) corresponds to the plot for the topology derived from all genes (Mallarino et. al 2005). Analyses were carried out with the mtDNA data alone to confirm that the pattern observed was not an artifact of combining nuclear and mtDNA genes with different rates of evolution.

However, this problem of demonstrating causation applies to all comparative analysis of speciation. In the case of color pattern, such a link does seem likely given the direct evidence for reproductive isolation caused by pattern differences in other mimetic butterflies (Jiggins et al. 2001). Hence, the results show that, although many recent speciation events have not involved color pattern shifts, where color pattern changes have occurred in the history of the genus they were associated with speciation.

Geographic Pattern of Speciation

The geographic pattern of speciation in *Ithomia* appears to be a mixed one. There were some clear cases of allopatric divergence, such as the sister species *I. xenos* (Central America) and *I. lagusa* (Andes). Other clades, such as that containing the species *I. ellara*, *I. eleonora*, and *I. praeithomia*, are composed of sympatric species that show considerable range overlap within a geographic region, in this case the southern Andes, suggesting within-region diversification. Over the genus as a whole, the pattern of overlap clearly showed that the younger species in the genus were predominantly sympatric to some degree with the exception of one species pair, *I. iphianassa* and *I. salapia* (Fig. 5), although the latter are parapatric rather than allopatric. However, as has been highlighted by others, species ranges are likely to move relatively rapidly subsequent to speciation (Losos and Glor 2003). This is especially true in butterflies in which where considerable range movements are known to have occurred over the last century due to climatic warming (Hill et al. 2001; Thomas et al. 2001). The data are therefore consistent with either rapid range movement, or a mixed model of sympatric and allopatric speciation. Comparative analyses alone are unlikely to convincingly differentiate these hypotheses.

The pattern of change in geographic overlap with time was heavily dependent on the method used to reconstruct ancestral range overlaps. The original method (Barraclough and Vogler

2000) involved summing ranges for each clade and showed increased overlap at older nodes as expected following allopatric speciation. However, as noted by Berlocher and Feder (2002), this method leads to older nodes having larger ranges. Thus, there is an increase in the absolute range sizes being compared at deep versus shallow nodes. This leads to an increased chance of sympatry at deeper nodes, giving an allopatric speciation pattern that is partly an artifact of the methodology. Calculating range overlaps by averaging pairwise range overlaps between clades, gives a very different pattern. As has been noted previously (Berlocher and Feder 2002), range overlaps show far less tendency to increase with time. In the present data set, deeper nodes show overlaps of around 20% rather than 100% (Fig. 5) and the general trend is of decreasing rather than increasing sympatry. However, given the different pattern seen under different methods, and the range movement that is likely to have occurred, we caution against putting too much emphasis on the pattern of overlap between the deeper nodes in the tree. The most recent nodes in a phylogeny are likely to be the most informative.

The *Ithomia* pattern contrasts with that shown in many taxa that have been studied previously (Barraclough and Vogler 2000; Lukhtanov et al. 2005). Four bird taxa, swordtail fish (*Xiphophorus*) and *Agrodiaetus* butterflies all showed closely related species to be allopatric, as expected under allopatric speciation. Only two groups, fruitflies (*Rhagoletis*) and tiger beetles (*Elipsoptera*) showed a tendency toward sympatry in closely related species. The intercept value of 0.239 in our data (using the same methodology as Barraclough and Vogler 2000) was greater than that for fruitflies (0.17) and tiger beetles (0.21), both groups in which it was suggested that sympatric speciation may have occurred (Barraclough and Vogler 2000). This is suggestive of nonallopatric speciation in *Ithomia*, although as already noted rapid range movement could generate the same pattern.

Time Course of Divergence

To test the Pleiostocene refuge hypothesis we need to establish the time course of diversification and relate this to actual time by comparison with a molecular clock. Unfortunately, dating divergence times is problematic due to the lack of an agreed molecular clock for the Lepidoptera. The deepest mitochondrial DNA divergence in the genus is around 12.5% (*I. patilla* to *I. jucunda*, ML-corrected distance). Under the standard insect molecular clock, based largely on dated nodes in the genus *Drosophila* (2.3% pairwise sequence divergence per million years; Brower 1994), this would suggest a divergence time of approximately 5–6 million years before present. However, a more recent molecular clock proposed for *Papilio* (0.7–1.0% pairwise sequence divergence per million years (Zakharov et al. 2004) would suggest much older divergence times of 12–16 million years before present for the base of the genus. It does seem likely that evolutionary rates are slower in the Lepidoptera as compared to *Drosophila*, given fossil evidence for the age of the clade (Hall et al. 2004; Vane-Wright 2004). However, estimated dates must remain somewhat tentative until better estimates of evolutionary rates can be obtained from multiple lepidopteran taxa.

Nonetheless, analysis of lineage accumulation through time clearly shows a significant slowing of diversification in the most recent history of the genus, which argues strongly against any Pleistocene speciation model. Most of the extant species were already present throughout the last 30% of the time since the most recent common ancestor. As described above, different molecular clock estimates give dates of either 5–6 or 12–16 million years before present for the most recent common ancestor of the whole clade. Thus, depending on the molecular clock used, most speciation was complete by between 1.5 and 5 million years before present, and certainly by the very early Pleistocene at the latest. The data therefore suggest that forest fragmentation during the Pleistocene has not played a major role in species diversification and are in agreement with results from other tropical and temperate studies, which have similarly shown that most extant species formed significantly before this period (Hewitt 2000; Moritz et al. 2000; Taberlet and Cheddadi 2002). It seems more probable that diversification is associated with the latter stages of the uplift of the Andes and the rise of Central America. In particular, it is thought that the northern and central Andes were only half their present height at 10 million years ago, such that the diversification of these butterflies most likely corresponds to a period of rapid uplift of the Andes (Gregory-Wodzicki 2000). The diversification of microclimates and habitats that must have resulted from this geological activity may have triggered the diversification of the *Ithomia*.

Conclusions

We aimed to investigate the hypothesis that mimicry adaptation has caused speciation in *Ithomia* butterflies, and contrast this with the Pleistocene refuge hypothesis, which has been commonly cited as an explanation for recent diversification in the neotropics. A likelihood analysis of color pattern clearly shows an association between pattern change and cladogenesis. However, there are two caveats to this. First, speciation has also occurred without changes in pattern, especially between the most recent sympatric species, showing that color pattern does not explain all speciation events. Second, these comparative data show only an association and do not provide direct evidence for a causal relationship between pattern change and speciation. Nonetheless, color pattern was chosen a priori as a trait with a known role in reproductive isolation in butterflies, so a causal relationship seems likely. This would imply a direct role for adaptive change in speciation in *Ithomia*. Analysis of geographic overlaps through time show that the most closely related species are sympatric or parapatric, consistent with divergence driven by ecological change with ongoing gene flow, although again an alternative explanation is that range movements have been very rapid subsequent to speciation, obscuring the speciation signal. Finally, the temporal pattern of diversification shows that most speciation happened before the Pleiostocene, thus ruling out a strict Pleistocene refuge model. In summary, the data provide support for a model of speciation involving ecological adaptation, with most diversification having occurred significantly before the Pleistocene.

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