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Timing the diversification of the Amazonian biota: butterfly divergences are consistent with Pleistocene refugia

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ABSTRACT

Rejection of the Pleistocene refugium hypothesis (PRH) as an explanation for the high biodiversity of Neotropical forest is based in part on the assertion that biotic elements of these forests evolved during the Neogene. That argument is justified, in turn, by the ages of crown groups (the age of the most recent common ancestor of extant species of a clade). We consider the use of crown ages as a metric to reject the PRH to be an unfair test, because the circumscription of crown groups of interest is arbitrary, and their ages represent overestimates of the time of species formation. We present divergence times between pairs of sister species (131 pairs), and among pairs of sister species and their closest relative (56 triplets), from 35 genera of Neotropical butterflies. Our aim is to refocus the discussion about the timing of diversification of the Neotropical biota on the time of the formation of extant species, a metric that is consistent and comparable across taxa. Our results show that 72% of speciation events leading to the formation of butterfly sister species occurred within the last 2.6 Myr, a result consistent with the temporal predictions of the PRH, suggesting that the PRH cannot be completely discarded as a driver of Neotropical diversification.

Keywords

Amazonia, biodiversity, crown ages, diversification, Lepidoptera, mitochondrial DNA, Neogene, Neotropics, Pleistocene refugium hypothesis.

INTRODUCTION

The reasons for the enormous numbers of species hosted by Neotropical forests intrigued 19th-century naturalists, and still puzzle systematists, ecologists, geologists and palaeontologists today. There are two contrasting positions regarding the patterns and timing of biotic diversification in the Neotropics in evolutionary time: one that emphasizes Neogene (23–2.6 Ma) vicariance events as a result of major rearrangements of the Amazonian landscape, and a second that points to Pleistocene (< 2.6 Ma) climatic cycles as an engine of diversification. While controversy over timing may not seem to be a biogeographical issue per se, the abiotic processes that could explain biotic distributions differ between these two time periods in fundamental ways. Therefore, inferring when diversification took place points to which geological and/or climatic mechanisms may have been involved.

At the end of the Tertiary, the Neogene (23–2.6 Ma) was a period of dramatic geological events in the Neotropics, such as the uplift of the Andes, the formation of a large lacustrine system in what is today western Amazonia (Lake Pebas), shifts in the courses and watersheds of major rivers, and the subsequent establishment of terrestrial conditions. Authors such as Hoorn *et al.* (2010) have argued that this tectonic activity caused changes in the landscape that provided biogeographical opportunities for new species interactions, and generated new adaptive pressures that triggered speciation. According to this scenario, most physical barriers, such as mountains and rivers, were in their current positions by the end of the Pliocene (2.6 Ma), and therefore vicariant speciation events caused by those barriers must have occurred earlier, implying that most current sister species diverged prior to 2.6 Ma (but see Ribas *et al.*, 2012).

In contrast, Haffer's (1969) Pleistocene refugium hypothesis (PRH) suggests that many extant Neotropical species originated after the Neogene (< 2.6 Ma; Cohen *et al.*, 2013) as a result of environmental fluctuations driven by repeated cycles of global cooling and warming. The PRH proposes that cold spells during the Pleistocene caused the fragmentation and replacement of moist Amazonian forest by drier grass savannas, isolating populations of forest obligate taxa, allowing allopatric differentiation and ultimately driving an

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increase in species formation. As the climate warmed again, these isolated populations had the opportunity to come back into sympatry with one another, only to be subdivided again by a subsequent cool period. This has been referred to as a 'species pump' (Haffer, 1997). Palynological evidence from the Last Glacial Maximum (LGM; *c.* 22–19 ka; Yokoyama *et al.*, 2000) indicates little change in local floral composition, and has been extrapolated to support assertions that Pleistocene forest refugia never existed (Colinvaux *et al.*, 2000; Bush & de Oliveira, 2006). However, such studies have typically examined evidence from single localities and a limited time slice (to date, all pollen cores go no earlier than 62 ka), and are mute regarding conditions elsewhere in time and space. For example, a given pollen core showing a lack of savanna-type vegetation might sample from within a forest refugium, and 'average' samples from the Amazonian fan (e.g. Maslin *et al.*, 2012) are difficult to interpret because they could represent pollen from riverine gallery forests running through non-forested areas (Hooghiemstra & van der Hammen, 1998). Further, Haffer (1969) never stated the LGM to have been more important than any other major Pleistocene glacial cycle, and in subsequent works he specifically emphasized the effect of climatic cycles throughout the Pleistocene (e.g. Haffer, 1974).

Between these alternative diversification hypotheses rests a sort of null hypothesis that speciation has been occurring continually via multiple evolutionary mechanisms, and that neither period was more important in terms of species formation (Rull, 2008; see also Hooghiemstra & van der Hammen, 1998). Such speciation events could have been driven by a variety of abiotic or biotic factors that could be unique to individual taxa, and thus are harder to test using the tools of historical biogeography, which are focused upon the inference of general explanations (patterns) for shared distributions.

Historically, the issue of the Neogene–Pleistocene timing of diversification has been debated primarily on the basis of inferences about palaeoclimate and modern-day distributions of forest taxa (see Bush, 1994, and Haffer, 1997, 2008, for reviews). In the past 20 years, however, analyses of molecular sequence data have allowed some of the biological predictions posed by these hypotheses to be tested (da Silva & Patton, 1993; Smith & Patton, 1993; Brower, 1994, 1996; Hackett, 1996; Solomon *et al.*, 2008). In general, molecular phylogeographical studies of geographical variability within and among closely related species have provided researchers with molecular clock-based estimates of divergence times, a new source of empirical data beyond fossil pollen and the congruent distributions of Amazonian taxa.

A common signature of recent discussions about the temporal origins of the Amazonian diversity has been reliance on estimates of crown group ages (Moritz *et al.*, 2000; Antonelli *et al.*, 2010; Hoorn *et al.*, 2010). As correctly pointed out by Rull (2011) and Ribas *et al.* (2012), although the ages of crown groups do represent the age of origin of a given clade, they necessarily provide an overestimate of the timing of diversification of its constituent species. The Amazon does host old lineages (e.g. *Arapaima*; Lundberg *et al.*, 2010) but their ages of origin provide neither confirmatory nor contradictory evidence for addressing alternative hypotheses for the timing of diversification of extant species. Despite these shortcomings, crown group ages have been invoked as a significant part of the evidence against the PRH (Antonelli *et al.*, 2010; Hoorn *et al.*, 2010).

The aim of this paper is to refocus the discussion about the origin of current Neotropical biodiversity onto the time of the formation of species (a different question to that about estimating diversification rates; Cicero & Johnson, 2006). Accordingly, based on pairwise mitochondrial (mt) DNA sequence comparisons, spanning 35 genera of Neotropical butterflies, we provide estimates of divergence times between 131 pairs of sister species (the blue branches in the cladogram in Fig. 1) and 56 age estimates between pairs of sister species and their closest relative (the red branches in the cladogram in Fig. 1). We compare our estimates with crown group ages reported in the literature and to similar sister taxon data for birds. Few or no speciation events more recent than 2.6 Ma would support the Neogene hypothesis, while a relatively high number of speciation events in the Pleistocene would be temporally consistent with the PRH. Although restricting our comparisons to current sister species may seem to represent a biased sample with respect to all historical speciation events, we do not dispute that speciation took place in the Neogene, nor that most extant higher taxa arose before the Pleistocene. Indeed, fossil butterflies of Oligocene age (33–23 Ma) are mostly placed in extant genera (de Jong, 2007). However, the literature abounds with statements such as 'with high species diversity evident in the early Eocene, later climate change is neither sufficient nor necessary to explain Neotropical diversity' (Knapp & Mallet, 2003, p. 72) and 'the Pleistocene accounts for only a small proportion of the cladogenetic events sampled, contrary to the expectations of the Pleistocene refugia model' (Antonelli *et al.*, 2010, p. 396), and frequent assertions that the PRH has been 'abandoned' (e.g. Hoorn *et al.*, 2010). Given this emphasis on the pre-eminent role of Neogene events in Neotropical diversification, a pertinent question is whether or not speciation continued to play a diversifying role in the Pleistocene, i.e. how old are the extant species in the Neotropics? That is the question we address herein.

MATERIALS AND METHODS

We identified pairs of sister species based on the most current phylogenetic hypothesis for each genus (the sources are provided in Appendix S1 in Supporting Information). As estimates of timing of diversification from exemplar studies that include only a subset of the species in a clade will tend to overestimate the ages of speciation events (e.g. van Velzen *et al.*, 2013), studies were assessed critically to include only those containing at least 50% of the species currently recognized in each group, based on the checklist of Neotropical butterflies (Lamas, 2004) and the Tree of Life (http://www.tolweb.org/;

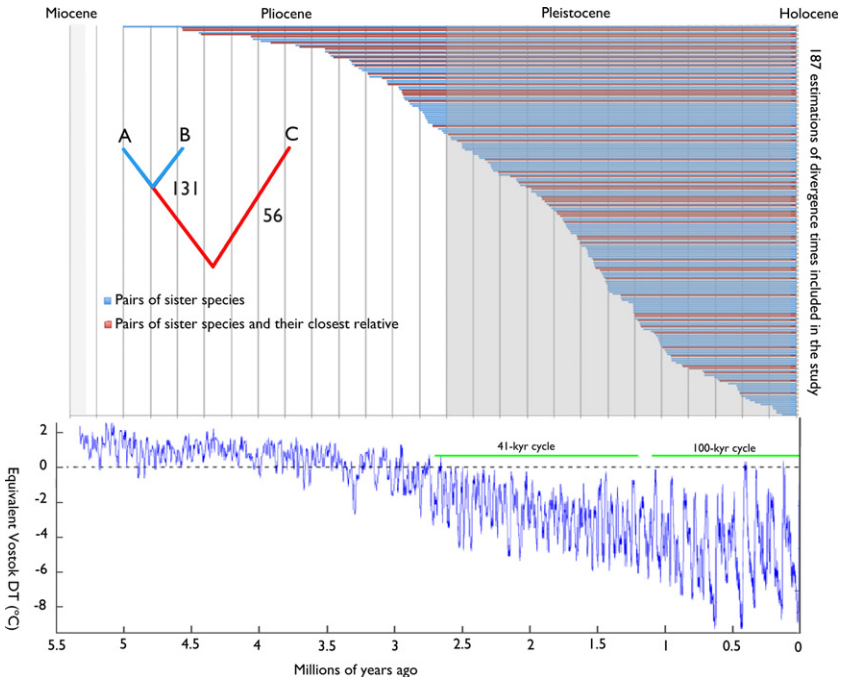

Figure 1 Composite figure showing the scheme of comparisons used in this study (and the number of each type of comparison), the results of divergence times for selected species representing 35 genera of Neotropical butterflies (sorted from the oldest to the most recent ones) and the timing of glacial–interglacial cycles over the last 5.5 Myr (modified from an original image created by Robert A. Rohde/Global Warming Art) (the geological time-scale follows Gradstein *et al.*, 2012). Divergence times between pairs of sister species are represented by blue bars; red bars represent the divergence times among sister species and their most recent common ancestor. The grey area represents the length of the Pleistocene (from 2.6 to 0.01 Ma).

last accessed 24 June 2013), to account for recent taxonomic changes. We were able to add estimations for two or three species of several genera (*Baeotus*, *Consul*, *Historis*, *Oressinoma* and *Tithorea*) based on their taxonomically necessary sister relationships, even though they have not been subject to phylogenetic analysis.

Mitochondrial cytochrome *c* oxidase subunit I (*COI*), and in some cases *COI* and cytochrome *c* oxidase subunit II (*COII*), sequences were obtained from Genbank (http://www.ncbi.nlm.nih.gov/genbank/); because protein-coding regions usually produce gap-free alignments, 'alignment' of the sequences was trivial. Sequences were compiled in Mesquite (Maddison & Maddison, 2011). When sequences from multiple individuals of a species pair were available for comparison, we used the maximum pairwise divergence, to ensure that our Neogene–Pleistocene test conservatively favoured older estimations (an inverse of the minimum used for barcode gap estimation advocated by Meier *et al.*, 2008). We used PAUP* 4.0b10 (Swofford, 2000) to calculate uncorrected pairwise distances, and from them we estimated the time of divergence between pairs of sister taxa, and among pairs of sister taxa and their closest relative, under Brower's (1994) 1.1% lineage^{-1} Myr^{-1} divergence rate estimate. Uncorrected pairwise distances were used instead of the Kimura two-parameter model because they provide better empirical distance estimates for closely related taxa (Srivathsan & Meier, 2012). Brower's (1994) 1.1% lineage^{-1} Myr^{-1} estimate was preferred over Gaunt & Miles' (2002) estimate of 0.022% lineage^{-1} Myr^{-1} for two reasons: first, the latter substitution rate was inferred from ordinal-level divergence estimates obtained from *COI* second-codon positions only, which are not informative at low levels of divergence; and second, our preliminary calculations of divergence times among sister species using Gaunt & Miles' (2002) clock in some cases doubled the crown group ages reported in the literature that had been estimated with a Bayesian clock (Wahlberg *et al.*, 2009). Finally a 'remarkably' similar mean mtDNA divergence rate estimation (2.39% Myr^{-1}) was obtained by Papadopoulou *et al.* (2010) from calibrated divergences of tenebrionid beetles, even after incorporating rate heterogeneity and using a uncorrelated lognormal relaxed clock, corroborating Brower's (1994) original rate.

RESULTS

Uncorrected pairwise distances and inferred ages of divergence for individual comparisons are presented in Appendix S1. Estimated divergence times among pairs of sister species (the blue bars in Fig. 1) averaged 1.8 Ma and ranged from 0.1 to 5.0 Ma, placing 75.5% of the speciation events within the Quaternary (the shaded area in Fig. 1). Furthermore, the split between a pair of sister species and its closest relative (the red bars in Fig. 1) fell within the Pleistocene in 64% of cases. This is in contrast to the crown group ages reported in the literature for these same groups of butterflies, which range from 5.5 to 27 Ma (see references in Appendix S1).

DISCUSSION

A high proportion of speciation events in the Pleistocene

Our findings from the butterflies are consistent with the temporal prediction formulated by the PRH, and contradict the idea that extant Neotropical biodiversity was largely generated prior to the Pleistocene (Antonelli *et al.*, 2010; Hoorn *et al.*, 2010). According to our combined results, 72% of Neotropical butterfly sister taxon speciation events occurred within the

Pleistocene (< 2.6 Ma). Furthermore, as some of the groups included here exhibit a substantial amount of shared intraspecific polymorphism (e.g. mimetic *Heliconius* and ithomiine butterflies), our results imply that their dramatic phenotypic geographical variability also evolved during the Quaternary.

Although divergences between a pair of sister species and its closest relative must be older than the divergence between that pair of sister taxa, Fig. 1 shows that, when compared across taxa, some of these divergences are more recent than the age of divergence of 'sisters' in other taxa (notice that the top part of Fig. 1 is not exclusively occupied by red bars), and that many of the divergences between a pair of sister species and its closest relative are also rather young; this should further allay the concern that sister species divergences are by definition recent events (Fig. 1). The estimated divergence times we obtained for pairs of sister species are distributed throughout the Pleistocene, with some of them extending back to the Pliocene, and therefore our data do not distinguish between a hypothesis of continuous idiosyncratic diversification with no common mechanism and a hypothesis of an escalated rate of divergence because of Pleistocene refugia. However, our data do contradict the idea that the diversity in the Amazon is predominantly of pre-Pleistocene origin. As noted by Hoorn *et al.* (2010), much work remains to be done to corroborate empirically the patterns and processes that may provide common explanations for Pleistocene speciation events.

Estimating error

Brower (1994) discussed potential sources of error in his mtDNA clock estimate at some length, and offered caveats on its employment that are still pertinent today. We have not included error bars on our point estimates of divergence times for several reasons. First of all, our data are raw measurements (rather than statistical inferences). There is no obvious way to estimate sampling error associated with individual observations of this sort. We could report a mean age of divergence of sister species with a standard deviation (1.78 Ma $\pm$ 0.97 Myr) for all the observations, implying that the 'average butterfly speciation event between extant sister taxa occurred in the Pleistocene', but that is less informative than reporting the individual observations.

A second commonly invoked type of error is underestimation of sequence divergence as a result of multiple hits, usually 'corrected' by some sort of substitution model. However, the absolute amount of sequence divergence is so low between most of the compared species that there is little chance that multiple hits have occurred at individual sites. As noted in the Materials and Methods, we intentionally rejected such corrections, based on the arguments in Srivathsan & Meier (2012).

More generally, we feel that emphasis on 'accommodating error' in model-based approaches to placing dates on phylogenetic hypotheses has the counterintuitively opposite effect of lulling researchers into a sense that they have fully represented the statistical variability in the data by, for example, the act of putting 95% Bayesian believability intervals on a BEAST tree. Such numerical precision may be completely inaccurate because of a variety of potential problems, including erroneous taxonomic placement of fossils on trees, poorly estimated ages of calibration points, incomplete taxon sampling, non-clock-like evolution of the sequences being compared, overparameterized or otherwise incorrect models and priors, and a myriad of other potential sources of error that are rarely taken into account. If the model is wrong, estimates based on it are also wrong, and any associated 'error' is effectively meaningless.

Appropriateness of crown groups for estimating ages of diversification

The general conclusion that biotic diversification in the Neotropics is old, and that the majority of extant species diverged during the Neogene, is largely based on estimates of crown group ages (Moritz *et al.*, 2000; Antonelli *et al.*, 2010; Hoorn *et al.*, 2010), a practice that is prevalent particularly for vertebrates. By definition, a crown group includes all of the descendants of the most recent common ancestor of some clade of extant species (Jefferies, 1979). Because of this, the age of any crown group containing more than two species must be older than the ages of divergence of most of the species contained in the group. Clearly the ancestors of extant Amazonian species or clades must have been present before the diversification of their groups; however, the phenomenon to be explained is not, for example, 'when did the butterfly genus *Adelpha* evolve?' (11 Ma; Mullen *et al.*, 2011) but rather 'how old are extant *Adelpha* species?' (the 13 speciation events we compared average 2 Ma). Another example of this scenario would be the tree genus *Inga,* whose crown age is reported to be *c.* 10 Ma but 30% of the speciation among sister species occurred within the last 2 Myr (Richardson *et al.*, 2001). The same is true for the birds of the genus *Psophia* (Ribas *et al.*, 2012) and would be true for any other taxon in which recent, rapid diversification has occurred.

Furthermore, because any clade of extant taxa could be selected as a crown group of interest, ages of different lineages cannot be expected to provide comparable evidence across taxa. The circumscription of a crown group, and therefore delimitation of its age, is arbitrary. To illustrate this principle, Fig. 2 shows that, given a phylogenetic tree, any subjectively identified clade (large or small) could be chosen as the 'crown group' of interest. Clades of different sizes, perhaps representing genera, tribes or subfamilies, could be arbitrarily compared and reported. The ages of these groups are not comparable unless they are sister taxa (in which case they are, by definition, the same age). The crown group 'life' arose some 3.8 billion years ago, but that does not mean that the 'diversity' of Neotropical species is 3.8 billion years old. In contrast, the time of divergence of sister species represents speciation events between equivalent evolutionary units that are comparable among taxa. Divergence of sister species is not constrained to represent exclusively recent events,

because lineages could have split at any point in time (note that, in Fig. 2, the splits of two species pairs fall before the Pleistocene, two other during and one after). Therefore, we consider that comparison of the ages of pairs of sister species provides a direct and objective assessment of whether or not most diversification among closely related taxa took place in the Neogene or in the Pleistocene.

The role of extinction

There has been some discussion regarding the potential effect of incomplete sampling or extinction on molecular clock estimations (e.g. Milne, 2009). To begin, let us state that any inference made based on the study of extant taxa could be affected by extinction events, but scientific research is constrained to the empirical realm, and invocation of unobserved phenomena is, at least, unparsimonious. Regarding our metric, extinction of one of the members of a pair of sister taxa would lead to an 'incorrect' estimate of the age of the speciation event (B in Fig. 2). However, unobserved extinction events can only make divergences between extant sister species appear to be older than they actually were, which would tend to produce observations contradicting our hypothesis. In contrast, extinction has no effect at all on crown group ages because crown groups are, by definition, dependent on the age of common ancestors of extant taxa. Whether extinction happened, and where in a tree it took place, is impossible to know in the absence of fossils, and therefore, as noted, unless we are following a preconceived model of evolution, concern that extinctions may be blurring our interpretation of reality seems to us to be a metaphysical endeavour.

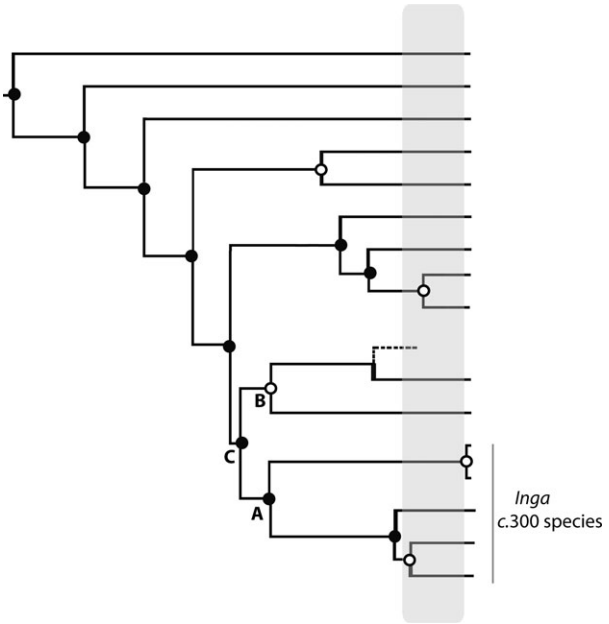

Figure 2 Differences between the questions assessed by crown groups and sister taxa, and the effects of extinction on age estimates based on each metric. A hypothetical phylogenetic tree highlights all the potential crown groups (black nodes) and pairs of sister species (white nodes). The dotted line represents an extinct lineage. The grey shadow represents the Pleistocene. Because the age of a crown group is by definition the age of the most recent common ancestor of a group of extant species, it only expresses how long a given clade has existed, and not when the diversification of the group took place. Consider clade A (e.g. the genus *Inga*), the crown age of which places its origin long before most of the diversification of the group; in this case the reported crown age ignores the fact that most speciation occurred recently. Extinction of one of the members of a pair of sister species means that, in practice, an older age is being inferred for the time of speciation (clade B). The estimated crown age of clade C, however, should not be affected by the extinction of one of its derived members.

Appropriateness of 'community divergence' for estimating ages of diversification

While multiple studies with butterflies have examined divergence between closely related species to assess the utility of DNA barcoding (e.g. Brower, 2006; Elias *et al.*, 2007; Wiemers & Fiedler, 2007; Meier *et al.*, 2008), very few (e.g. Brower, 1996) have explicitly attempted to test the validity of the PRH. One of these is that of Whinnett *et al.* (2005). Based on GTR+I+Γ distances, these authors estimated divergence times among members of a community of 31 ithomiine butterfly species present at a suture zone near Tarapoto, Peru. Their test was based on Coyne & Orr's (2004) criterion that co-distributed taxa should exhibit concordant divergence times if a simultaneous vicariance event was responsible for their initial isolation. Whinnett *et al.* (2005) reported striking variation in divergence times among pairs of compared taxa, and suggested that these results strongly reject the PRH. They argued instead that the rich Amazonian biota originated from ongoing diversification caused primarily by idiosyncratic parapatric evolution driven by natural selection. But Coyne & Orr's (2004) method also stipulates that comparisons should be made between sister species, and only two pairs of taxa in the Whinnett *et al.* (2005) dataset represent known sister species. The rest are arbitrary pairs of congeners. Thus, as with the crown group ages already discussed, most of the variation Whinnett *et al.* (2005) found in divergence times is the result of inappropriate comparisons, and therefore the metric they used to assess predictions from the PRH represents an invalid test.

The LGM is not (all of) the Pleistocene

Although most Pleistocene-age palynological cores provide data that go back only as far as *c.* 60,000 years (Hooghiemstra & van der Hammen, 1998; Behling *et al.*, 2010; Maslin *et al.*, 2012), there were at least six other glacial cycles prior to the LGM (Ehlers & Gibbard, 2007; Ribas *et al.*, 2012) that could have affected the distributional patterns and population dynamics of Amazonian groups. If Pleistocene climate fluctuations did play a role in vicariant splitting of sister taxa or otherwise structuring populations (e.g. geographical races), then it seems reasonable to suspect that older cycles might have had a more dramatic effect on community com-

position, and that subsequent cycles would be expected to have a diminishing impact, as the taxa that persist have already survived previous events. Therefore, it is not surprising to find that the LGM has had no observed effect on the speciation of forest taxa (e.g. Ribas *et al.*, 2012). In fact, such a pattern has also been demonstrated in other groups of birds using evidence similar to our butterfly data, albeit with much smaller sample sizes (Klicka & Zink, 1997; Johnson & Cicero, 2004; Weir & Schluter, 2004; Lovette, 2005). For this reason, emphasis on the LGM as a major generator of current species diversity (e.g. Solomon *et al.*, 2008; Maldonado-Coelho *et al.*, 2013; Ramirez-Barahona & Eguiarte, 2013), and using data pertinent only to the LGM to extrapolate patterns and processes during the entire Pleistocene epoch, seems fundamentally unjustified. Although we are convinced that events during the Pleistocene played an important role in the production of extant Neotropical diversity, all things being equal we would expect the LGM to have had the smallest effect on the Amazonian biota of any of the Pleistocene cool periods.

General patterns

Although attempts have been made to test the various diversification hypotheses (Patton & da Silva, 1998; Lougheed *et al.*, 1999; Maldonado-Coelho *et al.*, 2013), those based on vicariance scenarios have received considerably more attention than ecological ones. Vicariance events in the Neogene, such as the Andean uplift, the formation of the Amazon river (the riverine barrier hypothesis; Wallace, 1852, 1876), the rise of sea level and marine incursions (the marine incursion hypothesis; Nores, 1999) and Quaternary fragmentation of the forest caused by climatic oscillations (PRH), are large-scale, extrinsic physical phenomena that would have affected a variety of taxa simultaneously and therefore have a higher explanatory power (Nelson & Platnick, 1981) than ecological models based on selection or other idiosyncratic eventualities affecting individual taxa. Within the realm of historical sciences (Cleland, 2002), a match between the timing of extrinsic events and the timing of species formation offers important circumstantial evidence supporting vicariant diversification hypotheses. Palynological evidence of the fragmentation of the forest by dry savanna (cf. Hooghiemstra & van der Hammen, 1998), supporting the potential existence of refugia, and DNA-based data that species formed during the Pleistocene, such as we present here, provide key spatial and temporal evidence, respectively, that support or are at least consistent with the PRH. Our study shows that a considerable amount of diversification in Neotropical butterflies occurred within the last 2.6 Myr, and is in agreement with the estimated divergences of sister species for other taxa, including plants (Richardson *et al.*, 2001), birds (Ribas *et al.*, 2012; Lutz *et al.*, 2013; Sousa-Neves *et al.*, 2013) and monkeys (Chiou *et al.*, 2011). In the absence of a more parsimonious interpretation of this result, the PRH cannot yet be ruled out as an explanation of Amazonian species diversity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Uncorrected pairwise distances, divergence times and sources of sister pairs of Neotropical butterfly species.

BIOSKETCHES

Ivonne J. Garzón-Orduña is a postdoctoral research associate at Middle Tennessee State University; she is interested in the phylogenetic systematics and patterns of diversification of Neotropical butterflies.

Jennifer E. Benetti-Longhini is a fledgling systematist whose research interests lie in the systematics and biogeography of Neotropical butterflies.

Andrew V. Z. Brower has been studying the systematics, biogeography and speciation of Neotropical butterflies for 25 years.

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SUPPORTING INFORMATION

Timing the diversification of the Amazonian biota: butterfly divergences are consistent with Pleistocene refugia

Ivonne J. Garzón-Orduña, Jennifer E. Benetti-Longhini and Andrew V. Z. Brower

Appendix S1 Uncorrected pairwise (*p*) distances, divergence times and sources of sister pairs of Neotropical butterfly species.

The first column provides an identification number for each of the comparisons, the second column records the genus and the species compared, the third and fourth columns provide uncorrected *p* distances among pairs of sister species, and between pairs of sister species and their most recent ancestor, respectively. Estimations of divergence times are shown in the fifth column. Crown group ages, if reported in the literature, are provided in the sixth column, and the last column presents the reference used as the source of the phylogeny. The first row for each taxon features, in addition to the genus, the averaged estimated age of the speciation events included, followed by the crown group age. Nomenclature follows Lamas (2004).

				Estimated age (Ma)		
	Species pair compared	*p* distance among sister taxa	*p* distance between three taxa*	Brower (1994) 1.1% Myr^{-1}	Crown group age reported in literature (Ma)	Source of phylogeny
	Nymphalidae: Danaine					
	FORBESTRA				*c*. 6.40	Elias *et al.* (2007)
1	*olivencia–proceris*	0.002		0.09		
2	1–*equicola*		0.051	2.32		
	HYALIRIS				*c*. 6.00	Arias-Mejia (2012)
3	*antea–oulita*	0.013		0.59		
	HYPOSCADA			**1.57**	Not available	de Silva *et al.* (2010)
4	*zarepha–anchiala*	0.034		1.55		
5	*schausi–virginiana*	0.034		1.55		
6	5–*kena*		0.036	1.63		
	HYPOTHYRIS			**1.18**	*c*. 7.00	Arias-Mejia (2012)
7	*ninonia–mansuetus*	0.027		1.23		
8	7–*mamercus*		0.025	1.14		
	ITHOMIA			**1.11**	3.00–8.00	Mallarino *et al.* (2005)
9	*lagusa–xenos*	0.038		1.73		

10	*iphianassa–salapia*	0.004		0.18		
11	10–*cleora*		0.033	1.50		
12	*celemia–heraldica*	0.031		1.41		
13	*eleonora–ellara*	0.003		0.14		
14	13–*praeithomia*		0.018	0.82		
15	*amarilla–arduinna*	0.031		1.41		
16	15–*jucunda*		0.038	1.73		
	NAPEOGENES			**1.80**	*c*. 13.00	Elias *et al*. (2009)
17	*glycera–N*. species 1	0.040		1.82		
18	17–*pharo*		0.042	1.91		
19	*larina–aethra*	0.050		2.27		
20	*duessa–stella*	0.037		1.68		
21	*apulia–gracilis*	0.008		0.36		
22	21–*inachia*		0.017	0.77		
23	*lycora–harbona*	0.046		2.09		
24	*sodalis–benigna*	0.021		0.95		
25	24–*sulphureophila*		0.064	2.91		
26	*cranto–flossina*	0.070		3.18		
	OLERIA			**2.05**	Not available	de Silva *et al*. (2010)
27	*aquata–sexmaculata*	0.062		2.82		
28	*tigilla–assimilis*	0.050		2.27		
29	*enania–quintina*	0.056		2.55		
30	*ilerdina–onega*	0.060		2.73		
31	*Oleria* sp. nov 17–511 *estella*	0.022		1.00		
32	31–*gunilla*		0.039	1.77		
33	*rubescens–paula*	0.025		1.14		
34	33–*zelica*		0.033	1.50		
35	*boyeri–deronda*	0.063		2.86		

36	35–*derondina*		0.063	2.86		
37	*radina–baizana*	0.023		1.05		
38	*santineza–fumata*	0.034		1.55		
39	*athalina–fasciata*	0.065		2.95		
40	*attalia–cyrene*	0.052		2.36		
41	40–*bioculata*		0.068	3.09		
42	*tremona–makrena*	0.031		1.41		
43	*makrena–padilla*	0.022		1.00		
	TITHOREA[†]				Not available	A. Zubek[‡], unpublished data
44	*harmonia–tarricina*		0.098	4.45		
	Nymphalidae:Satyrinae					
	BLEPOLENIS			**0.68**	15.00[§]	Penz *et al.* (2011)
45	*catherinae–batea*	0.010		0.45		
46	45– *bassus*		0.020	0.91		
	DASYOPHTHALMA			**3.52**		Penz (2009)
47	*rusina–geraensis*	0.055		2.50		
48	47–*creusa*		0.100	4.55		
	FORSTERINARIA **SUBCLADE**			**1.88**	*c.* 10.00	Matos-Maravi *et al*. (2013)
49	*antje–pseudinornata*	0.026		1.18		
50	*neonympha–inornata*	0.041		1.86		
51	*coipa–boliviana*	0.027		1.23		
52	*pilosa–pichita*	0.046		2.09		
53	52–*guaniloi*		0.057	2.59		
54	*punctata–rotunda*	0.043		1.95		
55	*Guaianaza pronophilina–F. necys*	0.050		2.27		

	HARJESIA			**1.82**	*c*. 7.00	Matos-Maravi *et al*. (2013)
56	*obscura*–spp DNA99044	0.013		0.59		
57	56– *blanda*		0.067	3.05		
	LYMANOPODA			**1.92**	*c*. 27.00	Casner & Pyrcz *et al*. (2010)
58	*acraeida–venosa*	0.050		2.27		
59	*caeruleata–caucana*	0.038		1.73		
60	*melia–tolima*	0.062		2.82		
61	*caracara–hazelana*	0.071		3.23		
62	*ionius–pieridina*	0.034		1.55		
63	*excisa–nivea*	0.042		1.91		
64	*labda–nadia*	0.059		2.68		
65	*araneola–hockingi*	0.027		1.23		
66	*dietzi–lecromi*	0.033		1.50		
67	*confusa–obsoleta*	0.031		1.41		
68	*albomaculata–apulia*	0.031		1.41		
69	68–*affineola*		0.029	1.32		
	MORPHO			**2.51**	*c*. 20.00	Penz *et al*. (2012)
70	*amathonte–menelaus*	0.064		2.91		
71	*achilles–helenor*	0.029		1.32		
72	71–*granadensis*		0.072	3.27		
73	*deidamia–epistrophus*	0.067		3.05		
74	*cisseis–hecuba*	0.063		2.86		
75	*hercules–theseus*	0.058		2.64		
76	*rhetenor–cypris*	0.053		2.41		
77	*marcus–eugenia*	0.036		1.64		

	ORESSINOMA				Not available	Kodandaramaiah *et al.* (2010)
78	*sorata–typhla*	0.065		2.95		
	PARATAYGETIS					Matos-Maravi *et al*. (2013)
79	*lineata–albinotata*	0.050		2.27		
	***PSEUDODEBIS* SUBCLADE**			**2.70**	*c.* 8.00	Matos-Maravi *et al*. (2013)
80	*Taygomorpha celia–T. puritana*	0.043		1.95		
81	80–*Pseudodebis marpessa*		0.076	3.45		
	TAYGETIS			**1.78**	*c.* 7.50	Matos-Maravi *et al*. (2013)
82	*mermeria–larua*	0.060		2.73		
83	82–*Taygetis* sp. PM01-14		0.055	2.50		
84	*tripunctata*–nr *virgilia*	0.061		2.77		
85	*rufomarginata–virgilia*	0.045		2.05		
86	85–*acuta*		0.044	2.00		
87	*echo–Taygetis* sp. PM01-06	0.027		1.23		
88	*thamyra–sosis*	0.031		1.41		
89	88–*Taygetis* sp. PM04-04		0.026	1.18		
90	*uncinata–Taygetis* sp. UNO261	0.020		0.91		
91	90–*laches*		0.022	1.00		
	***TAYGETINA* SUBCLADE**			**3.30**	*c.* 9.00	Matos-Maravi *et al*. (2013)
92	*banghaasi–Harjesia oreba*	0.072		3.27		
93	*Taygetis kerea–coeruleotaygetis peribaea*	0.073		3.32		
94	93–*Taygetis weymeri*		0.073	3.32		

	Nymphalidae: Charaxinae					
	ARCHEOPREPONA+ NOREPPA			**2.89**	Not available	Ortiz-Acevedo & Willmott (2013)
95	*demophon–camilla*	0.054		2.45		
96	95–*phaedra*		0.081	3.68		
97	*licomedes–chromus*	0.064		2.91		
98	*meander–amphimachus*	0.022		1.00		
99	98–*chalciope*		0.097	4.41		
	CONSUL				Not available	Inferred from taxonomy
100	*fabius–panariste*	0.010		0.45		
101	100–*electra*		0.049	2.23		
	PREPONA–AGRIAS			**1.39**	Not available	Ortiz-Acevedo & Willmott (2013)
102	*amydon–hewitsonius*	0.021		0.95		
103	*praeneste–deiphile neoterpe*	0.027		1.23		
104	*pylene–deiphile ibarra*	0.055		2.50		
105	*A. claudina–A. narcissus*	0.019		0.86		
	Nymphalidae: Nymphalinae					
	BAEOTUS			**3.55**		Inferred from taxonomy
106	*aelius–beotus*	0.067		3.05		
107	106–*deucalion*		0.089	4.05		
	ERESIA			**1.80**	*c.* 15.00	Wahlberg & Freitas (2007)
108	*eunice–nauplius*	0.055		2.50		
109	*carme–polina*	0.032		1.45		
110	109–*emerantia*		0.043	1.95		

111	*perna–clio*	0.034		1.55		
112	111–*letitia*		0.039	1.77		
113	*casiphia–stricta*	0.038		1.73		
114	113–*datis*		0.036	1.64		
	GNATHOTRICHE				*c*. 16.00	Wahlberg & Freitas (2007)
115	*exclamationis–mundina*	0.011		5.00		
	HYPANARTIA			**2.20**	Not available	Willmot *et al.* (2001)
116	*lethe–godmanii*	0.033		1.50		
117	116–*bella*		0.064	2.91		
	HISTORIS					Inferred from taxonomy
118	*odius–acheronta*	0.088		4.00		
	JANATELLA			**1.43**	*c*. 10.00	Wahlberg & Freitas (2007)
119	*leucodesma–hera*	0.023		1.05		
120	119–*fellula*		0.040	1.82		
	JUNONIA			**1.48**	Not available	Pfeiler *et al*. (2012)
121	*vestina–genoveva*	0.024		1.09		
122	121–*evarete*/*coenia*		0.041	1.86		
	Nymphalidae: Biblidinae					
	HAMADRYAS			**1.67**	*c*. 23.00 (95% HPD 20–27.5)	Garzón Orduña *et al.* (2013)
123	*epinome–iphthime*	0.057		2.59		
124	*belladonna–amphinome*	0.032		1.45		
125	124–*arinome*		0.026	1.18		
126	*laodamia–velutina*[¶]		0.032	1.45		

	PERISAMA			**0.70**	Not available	A. Zubek‡, unpublished data
127	*albipennis–bomplandii*	0.007		0.32		
128	127–*moronina*		0.023	1.05		
129	*ambatensis–phenix*	0.006		0.27		
130	*latimargo–calamis*	0.011		0.50		
131	*klugii–dorbignyi*	0.008		0.36		
132	*alicia–paralicia*	0.022		1.00		
133	*goeringi–tryphena*	0.010		0.45		
134	133–*affinis*		0.015	0.68		
135	*guerini–humboldti*	0.003		0.14		
136	135–*comnena*		0.009	0.41		
137	*vitringa–cabirnia*	0.004		0.18		
138	137–*yeba*		0.013	0.59		
139	*philinus–tristrigosa*	0.020		0.91		
140	139–*canoma*		0.035	1.59		
141	*hazama–ouma*	0.026		1.18		
142	*Orophila cecidas–O.diotima*	0.033		1.50		
	Nymphalidae: Heliconiinae					
	EUEIDES			**2.41**	*c.* 18.4**	Beltrán *et al.* (2007)
143	*lybia–tales*	0.077		3.50		
144	143–*aliphera*		0.076	3.45		
145	*lineata–isabella*††	0.061		2.77		
146	*vibilia–lampeto*	0.025		1.14		
147	146–*pavana*		0.026	1.18		
	HELICONIUS			**2.29**	*c.* 18.4**	Beltrán *et al*. (2007)
148	*charitonia–peruvianus*	0.037		1.68		

149	*sara–leucadia*	0.059		2.68		
150	eleuchia–congener	0.053		2.41		
151	150–*sapho*		0.077	3.50		
152	*clysonimus–telesiphe*	0.058		2.64		
153	152–*hortense*		0.064	2.91		
154	*hierax–xanthocles*	0.073		3.32		
155	154–*doris*[‡‡]		0.086	3.91		
156	*burneyi–wallacei*	0.035		1.59		
157	156–*egeria*		0.059	2.68		
158	*ethilla–nattereri*	0.035		1.59		
159	*hecale–atthis*	0.009		0.41		
160	*elevatus–pardalinus*	0.015		0.68		
161	*numata–ismenius*	0.037		1.68		
162	*melpomene–cydno*[§§]	0.049		2.23		
163	*demeter–eratosignis*	0.061		2.77		
	Nymphalidae: Limenitidinae					
	ADELPHA			**1.97**	*c.* 11.00	Mullen *et al.* (2011)
164	*justina–olynthia*	0.052		2.36		
165	*jordani–naxia*	0.009		0.41		
166	165–*malea aethalia*		0.100	4.55		
167	*erotia–lycorias*	0.031		1.41		
168	167–*phylaca*		0.042	1.91		
169	*mesentina–thesprotia*	0.021		0.95		
170	*irmin–leucophthalma*	0.033		1.50		
171	170–*cocala*		0.070	3.18		
172	*rothschildi–sichaeus*	0.038		1.73		
173	*iphicleola–iphiclus*	0.015		0.68		
174	173–*thessalia*		0.039	1.77		
175	*epione–ethelda*	0.030		1.36		

176	*cytherea–salmoneous*	0.082		3.73		
	Papilionidae: Troidina					
	PARIDES–EURYADES			**2.20**	*c*. 27.00 (95% HPD 23–32.4)	Condamine *et al.* (2012)
177	*ascanius–buchinus*	0.040		1.82		
178	*proneus–agavus*	0.089		4.05		
179	*aeneas–tros*	0.070		3.18		
180	*panthonus–burchellanus*	0.020		0.91		
181	180–*lysander*		0.031	1.41		
182	*eurimedes–zacynthus*	0.026		1.18		
183	182–*neophilus*		0.047	2.14		
184	*childrenae–sesostris*	0.076		3.45		
185	*erithalion–vertumnus*	0.028		1.27		
186	185–*anchises*		0.045	2.05		
187	*E. corethrus–E. duponchelii*	0.061		2.77		

*In a comparison ((A,B),C) the largest distance between A–C and B–C is reported.
†Not sister taxa; there is a new species described from Colombia more closely related to *T. harmonia* from which there is not COI available yet (Willmott & Lamas, 2004).
‡Zoological Museum of the Institute of Zoology, Jagiellonian University, Kraków, Poland.
§Species diversification reported to be around 2.5 Ma.
¶The sister pair is probably *laodamia–arete* but there is not COI for *arete*.
**Time of divergence of *Heliconius* and *Eueides*.
††There might be two other species in between these two taxa, according to Beltran *et al.* (2007).
‡‡The largest distance is between *doris* and *hierax*, but *hierax* is a long branch.
§§Largest divergence between races of *melpomene* and *cydno*.