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A palaeobiogeographic model for biotic diversification within Amazonia over the past three million years

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Many hypotheses have been proposed to explain high species diversity in Amazonia, but few generalizations have emerged. In part, this has arisen from the scarcity of rigorous tests for mechanisms promoting speciation, and from major uncertainties about palaeogeographic events and their spatial and temporal associations with diversification. Here, we investigate the environmental history of Amazonia using a phylogenetic and biogeographic analysis of trumpeters (Aves: *Psophia*), which are represented by species in each of the vertebrate areas of endemism. Their relationships reveal an unforeseen 'complete' time-slice of Amazonian diversification over the past 3.0 Myr. We employ this temporally calibrated phylogeny to test competing palaeogeographic hypotheses. Our results are consistent with the establishment of the current Amazonian drainage system at approximately 3.0–2.0 Ma and predict the temporal pattern of major river formation over Plio-Pleistocene times. We propose a palaeobiogeographic model for the last 3.0 Myr of Amazonian history that has implications for understanding patterns of endemism, the temporal history of Amazonian diversification and mechanisms promoting speciation. The history of *Psophia*, in combination with new geological evidence, provides the strongest direct evidence supporting a role for river dynamics in Amazonian diversification, and the absence of such a role for glacial climate cycles and refugia.

Keywords: Amazonia; species diversity; diversification; speciation; palaeobiogeography

1. INTRODUCTION

Amazonia is one of Earth's premier hotspots for terrestrial vertebrate endemism and species diversity [1]. Explaining this high diversity and understanding how it was assembled over time have concerned evolutionary biologists for over a century [2]. Although allopatric speciation is widely acknowledged as the primary mechanism underlying Amazonian species richness and endemism, debates about the temporal and spatial history of diversification, as well as the drivers and rate controls of these patterns, remain unresolved. Indeed, few empirically supported generalizations have emerged.

Among the numerous proposed causal mechanisms for the rate control of speciation and the origin of areas of endemism within Amazonia, two are predominant. One is the refuge hypothesis [3], which identifies orbital forcing of climate, and consequent cyclical expansion and contraction of rainforest, as the promoter of isolation and differentiation. A second explanation proposes that tectonically mediated river dynamics [4,5] have been primarily responsible for the isolation of forest biotas. Despite these two general and often-cited hypotheses [6], it is broadly understood that, while multiple causation has been operable [7], rigorous testing of these and other alternatives has been lacking [6,7]. It has been widely assumed [8] that speciation driven by isolation in forest refuges implies a relatively young history for Amazonian diversity, whereas rivers *qua* isolating barriers suggest a relatively older biotic history because of the belief that major features of the Amazonian drainage substantially predate Quaternary climatic fluctuations [3].

These predictions have not been evaluated with respect to a large-scale model of Amazonian diversification owing to substantial uncertainty over the palaeogeographic and environmental history of the region from the Miocene to the present [9–13]. Two large-scale palaeogeographic models for this time period have been proposed. Both accept evidence that much of western Amazonia was covered by a large, mostly freshwater, long-lived lake system, Lake Pebas, which formed by the Early Miocene and grew to a million square kilometres in the Middle Miocene

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(by approx. 16.0 Ma) as Andean uplift delivered more water to the developing Solimões Basin [9,14,15]. The highly dynamic lake system was probably bounded on the east by the Purus Arch, located approximately 300 km west of Manaus [12,13,15]. Under the first hypothesis, this deltaic, fluvial and lacrustine environment would have precluded extensive, long-term *terra firme* habitats until the Late Miocene, when the Purus Arch was breached and a transcontinental Amazon drainage created around 11.8–10.0 Ma [8,12]. This was followed [12] by a sixfold increase in sedimentation on the Amazon fan between 6.8 and 2.4 Ma, and then a fourfold increase from 2.4 Ma to the present, which was interpreted as increased sedimentation during Plio-Pleistocene glacial cycles.

An alternative palaeogeography for western Amazonia points to a younger, Plio-Pleistocene origin for a transcontinental Amazon River drainage [10,11,13]. Based primarily on Plio-Pleistocene stratigraphy and palaeoenvironmental analysis, it has been proposed that a continuous sedimentary system between the Solimões Basin in the west and the Amazon Basin in the east did not appear until approximately 6.0–5.0 Ma [13] to approximately 2.5 Ma ([11]; see electronic supplementary material).

Each model has profound consequences in understanding the assembly of the Amazonian biota. The first implies that western Amazonian *terra firme* forests and at least part of the current drainage system were in place by the Late Miocene (approx. 11.0–9.0 Ma), whereas the second predicts that the drainage system and those forests are much younger than previously assumed, and are Plio-Pleistocene in age. The temporal and spatial history of endemic clades in Amazonia has the potential to test these models and provide additional insights and predictions in the absence of critical geological data.

The monotypic trumpeters (*Psophia*; Psophiidae) have distant relationships to cranes (Gruidae) and limpkins (Aramidae) [16], and include species in each of the major areas of endemism within Amazonia (see electronic supplementary material). Trumpeters have long been of interest to those trying to understand the history of diversification within this region and have been a key exemplar supporting the refuge hypothesis [17]. Here, we use phylogenetic and biogeographic analyses, along with independent estimates of the *Psophia* time-tree, to reveal an unexpectedly 'complete' picture of diversification within Amazonia that makes predictions about its geological and environmental history. These results are also used to evaluate alternative drivers of allopatric speciation, including tectonically mediated landscape change and orbitally driven climatic cycles, and provide new evidence for the relative importance of these historical processes in shaping the assembly of Amazonia's extant biota.

2. MATERIAL AND METHODS

(a) *Sampling, distribution and morphological variation*

We obtained DNA from fresh tissue ($n = 47$) and museum specimens ($n = 15$) for a total of 62 individuals (electronic supplementary material, table S1), including representatives of all species and subspecies that have been described to date. *Psophia* is one of the few avian genera endemic to Amazonia, with species in all major areas of endemism [1,17–19]. To better understand their distribution, we accessed and georeferenced the available locality information (electronic supplementary material, figure S1 and appendix S1). Morphological variation (plumage patterns) was assessed through examination of skin specimens belonging to all *Psophia* taxa housed at the AMNH and MPEG collections (see electronic supplementary material).

(b) *Data collection*

Standard methods were used to extract, isolate, amplify and sequence mitochondrial and nuclear genes (electronic supplementary material). We obtained sequences of the mitochondrial genes cytochrome b (cyt b, 993 bp) and NADH dehydrogenase 2 (ND2, 1041 bp) for all 62 *Psophia* individuals and outgroups. Sequences of the intron 7 of the nuclear gene beta fibrinogen (BFib7, 950 bp) were collected for 48 *Psophia* individuals (representing all taxa except *Psophia obscura*, for which there was no fresh tissue available). Sequences of the nuclear gene *RAG2* were obtained for 24 individuals representing 22 species from five different avian orders, plus a representative of the northern clade of *Psophia* (*Psophia crepitans*) and one representative of the southern clade (*Psophia viridis*; electronic supplementary material, table S2). All primers used are given in electronic supplementary material, table S3.

(c) *Phylogenetic analysis*

Following previous studies [16], we included the limpkin (Aramidae: *Aramus guarauna*), a crane (Gruidae: *Grus canadensis*), a rail (Rallidae: *Rallus limicola*) and the sunbittern (Heliornithidae: *Heliornis fulica*) in a preliminary analysis, with a stork (Ciconiidae: *Ciconia ciconia*) as a more distant outgroup. This analysis confirmed *Aramus* and *Grus* as being most closely related to *Psophia*, and these were used as outgroups. Phylogenetic analyses were performed using maximum likelihood (ML) and maximum parsimony (MP) in PAUP* [20], and Bayesian inference (BI) in MrBayes v. 3.1 [21]. Evolutionary models for ML were selected by Modeltest v. 3.7 [22], under the Akaike information criterion, and for BI analyses by MrModeltest [23]. Bayes factors were employed to determine the optimal number of partitions to be used by the Bayesian analysis [24], and convergence for all parameters was assessed by evaluating stationarity of the Markov chain using Tracer v. 1.5 [25] and by sampling of tree topologies using AWTY [26] (see electronic supplementary material). Because of the fact that outgroups are distantly related to the ingroup, we performed an additional phylogenetic analysis in BEAST without outgroups to confirm rooting. The relaxed molecular clock analysis implemented in BEAST simultaneously estimates topology and branch lengths, and thus allows rooting in the absence of outgroups [27].

(d) *Molecular dating*

Two independent estimates were used to date the diversification history of *Psophia*. First, a bracket for substitution rates for cyt b, from 1.6 (0.008 substitutions per site per lineage per Myr [28]) to 2.1 per cent (0.01 substitutions per site per lineage per Myr [29]) was used to calibrate the *Psophia* tree using the strict-clock option in BEAST [27]. A likelihood ratio test did not reject a molecular clock within *Psophia*.

The second approach was designed to date the basal node of *Psophia*; thus, a dataset was constructed using the slowly evolving nuclear protein-coding gene *RAG2*. The taxon sampling included two species of *Psophia*, representing the northern and the southern clade, plus representatives of

other avian lineages (electronic supplementary material, table S2) [16]. An ML tree with branch lengths was analysed by penalized likelihood (PL) with the truncated Newton (TN) algorithm implemented in r8s [30]. The *RAG2* matrix was also analysed by a Bayesian approach in BEAST using a relaxed clock model (uncorrelated lognormal) and applying a Yule process as the tree prior. Three analyses of 20 million generations each were run, and the outputs were combined to estimate divergence dates and confidence intervals. Convergence was evaluated as mentioned above for Bayesian analyses.

Three minimum age calibrations were used (electronic supplementary material, table S4): crown anseriforms at 66 Ma, origin of *Fregata* lineage at 53 Ma and stem sphenisciforms at 60 Ma. Additionally, the basal-most node of the Neoaves was constrained between 100 and 85 Ma based on previous molecular dating analyses that used nuclear data [31], and on biogeographic evidence that several groups of Neoaves (e.g. parrots, passerines) were already diversifying when New Zealand became isolated from other landmasses, around 85 Ma [32,33]. From the *RAG2* dating analysis, we calculated divergence dates and confidence intervals (PL and Bayesian methods) for the northern versus southern split in *Psophia* (*P. crepitans* and *P. viridis*), using these results to calibrate the species-tree derived from the combined mtDNA data (cyt b and ND2). To calculate the errors under PL, 1000 trees were randomly sampled from the Bayesian stationary distribution, independently dated, and the results summarized to obtain median values and 95 per cent credibility intervals of node ages.

(e) *Population variation and structure*

Haplotype networks were generated for mtDNA lineages and for BFib7 using TCS v. 1.18 [34]. Allelic phase for BFib7 was determined using a Bayesian algorithm implemented in PHASE [35].

A main prediction of the refuge model is contraction and expansion of forest and non-forest ecosystems with consequent changes in population sizes; we therefore used several approaches to test for past demographic fluctuations. Estimates of nucleotide diversity and statistical tests for detecting past population growth [36] were performed in DnaSP v. 4.10.2 [37]. Independent assessments of demographic expansion were obtained for taxa with more than 10 individuals by analysing pairwise nucleotide differences (mismatch distribution) implemented in Arlequin [38] and through extended Bayesian skyline plots (EBSP) [39] estimated in BEAST (see electronic supplementary material).

3. RESULTS

(a) *Species and species limits*

Traditionally, three 'biological' species of *Psophia* have been widely recognized [40]. Our genetic and museum-based analyses, however, confirm that two of these are not monophyletic and the third includes several distinct taxa. Our data instead identify eight diagnosably distinct phylogenetic species [41], each endemic in a major area of endemism (figure 1; electronic supplementary material, figure S1 and table S1).

(b) *Phylogeny and biogeography*

Phylogenetic analysis of 2181 bp of mtDNA using all methods of phylogenetic reconstruction recovered a single well-supported topology (figure 1; electronic supplementary material, figure S2), with only node 5 having relatively poor support values. The data identify two main clades (figure 1). North of the Amazon River, *Psophia napensis* (Napo area of endemism) is the sister species of *P. crepitans* (Guiana and Imeri areas) and *Psophia ochroptera* (Rio Negro area). A second clade of five species is distributed south of the Amazon, with *Psophia leucoptera* (Inambari area) being the sister group to all others, *P. viridis* (Rondônia area) the sister to the remaining three and *Psophia obscura* (Belém area) resolved as the sister of *Psophia dextralis* (Tapajós area) and *Psophia interjecta* (Xingu area).

Phylogenetic analyses of BFib7 recovered the northern and southern clades found in the mtDNA analysis with high bootstrap support, but with no internal resolution (electronic supplementary material, figure S3). A 4 bp deletion was common to taxa in the northern clade. Within the latter, all individuals of *P. ochroptera* shared the same haplotype. The only individual of *P. napensis* was a heterozygote and shared one of its two haplotypes with *P. crepitans*. More importantly, the haplotype network including all 48 individuals (96 alleles) for which BFib7 sequences were obtained (25 distinct haplotypes) revealed that in both clades, the ancestral haplotypes (central in the network) and the greatest haplotype diversity are found in eastern Amazonia, with derived haplotypes (tips of the network) and lower diversity being present in the western populations (electronic supplementary material, figure S4), suggesting that the most recent common ancestor of *Psophia* was originally in eastern Amazonia.

(c) *Temporal pattern of diversification*

Time estimates using different data and methods are consistent in dating the basal split (figure 1, node 1) of *Psophia* as being of Late Pliocene age (table 1; electronic supplementary material, figures S5 and S6). Four divergences within *Psophia* took place in western Amazonia approximately 2.0–0.7 Ma in the Late Pliocene–Middle Pleistocene (figure 1, nodes 2–5), whereas the remaining two species lineages in southeastern Amazonia arose in the Middle Pleistocene (figure 1, nodes 6 and 7).

(d) *Population history within species of* Psophia

Intraspecific nucleotide diversity in mtDNA was generally low (electronic supplementary material, table S5), with the highest values being found for *P. viridis* (0.450%) and *P. napensis* (0.315%). Despite the denser sampling, both *P. crepitans* and *P. leucoptera* exhibited low values (0.079% and 0.097%). BFib7 nucleotide diversity within species varied from all individuals of *P. ochroptera* having the same haplotype to a high of 0.333 per cent within *P. napensis*.

Mitochondrial haplotype networks for *P. crepitans* in the Guianan area of endemism and *P. leucoptera* in Inambari are composed of one most common haplotype with several other haplotypes directly connected to it by one or two mutational steps (electronic supplementary material, figure S7). Also, for these two taxa, most test statistics were significant, rejecting the null hypothesis of demographic stability (electronic supplementary material, table S5). The results for *P. crepitans* and *P. leucoptera* are indicative of recent population expansion. A different pattern is seen in *P. viridis* mtDNA: not only is the nucleotide diversity higher within this taxon, but the test statistics are all

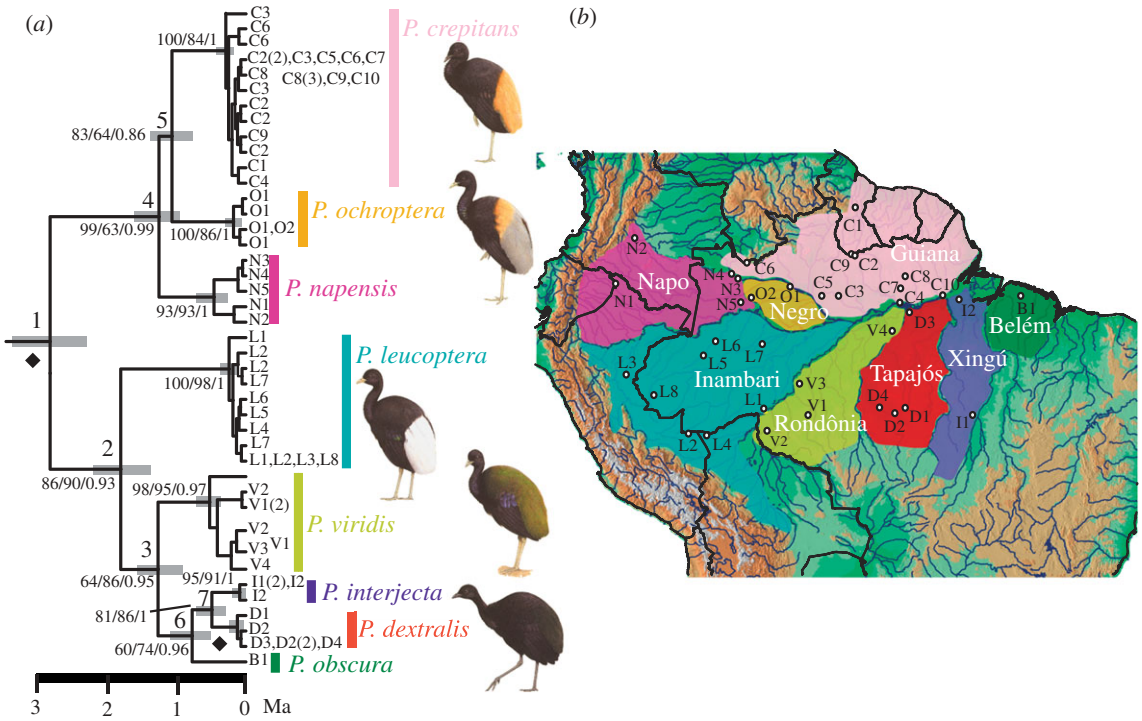

Figure 1. (*a*) Chronogram derived from a Bayesian analysis of cyt b and ND2 sequences (2181 bp) with a calibration derived from an analysis of the *RAG2* nuclear gene (table 1). Support values correspond to MP bootstrap/ML bootstrap/posterior probability. Filled diamonds indicate maximum support values (100/100/1.0) in all analyses. Bars correspond to confidence intervals. (*b*) Map showing current distribution and collection localities of samples included in the molecular analysis.

Table 1. Dates and confidence intervals (CI) derived from four temporal analyses of the mtDNA dataset for *Psophia* using Bayesian inference (BI) and penalized likelihood (PL) methods. The calibrations used include an upper–lower bracket of the published rates for sequence evolution of cyt b and the dates from a dating analysis of a *RAG2* dataset. The first two Bayesian inference analyses include only cyt b data with fixed evolutionary rates. In the second set of analyses, rates were estimated using *RAG2* and fossil calibrations, and both cyt b and ND2 sequences were analysed by PL and BI, respectively. Node numbers correspond to those indicated on figure 1.

method calibration	BI rate: 2.0%	BI rate: 1.6%	PL *RAG2* analysis	BI *RAG2* analysis
evol. rate[a] mt data	0.0100 cyt b	0.0080 cyt b	0.0092 cyt b + ND2	0.0067 cyt b + ND2
node 1	2.07 (1.56–2.63)	2.59 (1.95–3.31)	2.01[b]	2.74[c]
node 2	1.62 (1.14–2.11)	2.02 (1.42–2.64)	1.05 (0.82–1.32)	1.79 (1.40–2.25)
node 3	0.91 (0.58–1.24)	1.14 (0.74–1.56)	0.76 (0.35–1.15)	1.25 (0.95–1.60)
node 4	0.87 (0.58–1.19)	1.08 (0.71–1.45)	1.00 (0.70–1.41)	1.21 (0.91–1.56)
node 5	0.73 (0.47–1.03)	0.91 (0.58–1.28)	0.82 (0.52–1.23)	1.04 (0.77–1.39)
node 6	0.52 (0.25–0.80)	0.65 (0.32–1.01)	0.51 (0.30–0.87)	0.76 (0.49–1.06)
node 7	0.37 (0.16–0.58)	0.47 (0.20–0.73)	0.31 (0.15–0.63)	0.48 (0.27–0.70)

[a]Substitutions per site per lineage per million years.
[b]Estimate obtained for the basal node within *Psophia* in the PL *RAG2* analysis (CI 0.67–3.02).
[c]Estimate obtained for the basal node within *Psophia* in the BI *RAG2* analysis (CI 0.26–7.99).

non-significant (electronic supplementary material, table S5), and the haplotype network shows clear signs of structure (electronic supplementary material, figure S7). Mismatch distribution analyses of mtDNA and EBSP based on both mtDNA and BFib7 data also show evidence for population expansion in both *P. crepitans* and *P. leucoptera* (electronic supplementary material, figure S8 and table S6). Based on mismatch distribution analysis, the values estimated for τ and mutation rates for mtDNA derived from molecular clock analysis, the time since the demographic expansion ranged from about 7000 to 10 000 years ago for *P. crepitans*, and from 8000 to 12 000 years ago for *P. leucoptera*. EBSP analyses indicate expansion between 40 000 years and the present, with a great reduction in the confidence intervals for population size over the last 20 000 years (electronic supplementary material, figure S8).

There is little geographical structuring of populations within most areas of endemism, and in several instances specimens from very distant localities share identical haplotypes (e.g. at L1, L2 and L3; figure 1). Importantly, in some cases specimens from the headwaters of large rivers delimiting areas of endemism have the same haplotypes as those found close to their mouths (e.g. at I1 and I2, and at D2

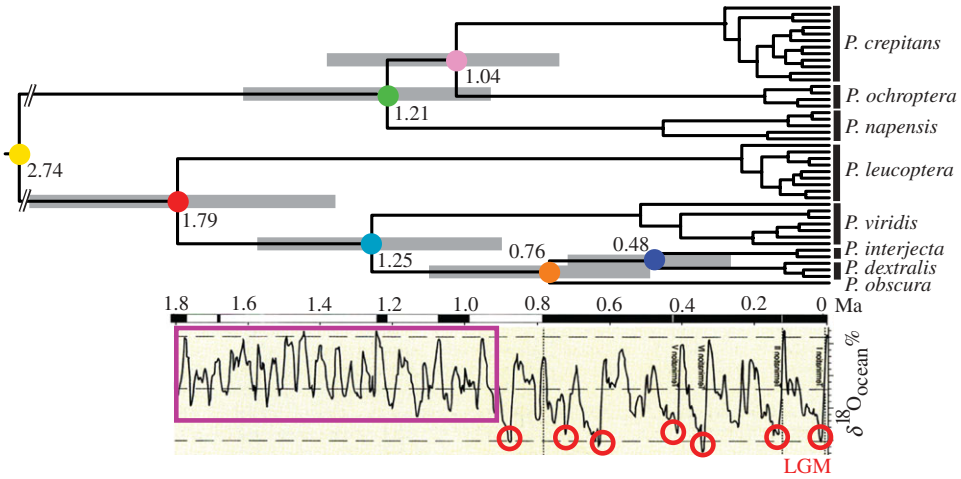

Figure 2. The mean age of speciation events (coloured dots) for lineages of *Psophia* calculated from genetic dating analysis plotted against the Quaternary climate curve derived from $\delta^{18}O$ (modified from 70) provides a phylogenetic test of the refuge hypothesis. The estimated age of the youngest species-pair, *P. interjecta* and *P. dextralis* (dark blue dot), indicates that the two species existed in their respective rainforest areas of endemism through four major glacial cycles (red circles), including the LGM. The next youngest speciation event, giving rise to *P. obscura* and the ancestor of *P. dextralis* + *P. interjecta* (orange dot), was prior to two additional cold cycles. Prior to approximately 1.0 Ma, in the '41 kyr world', climate was warmer and sensitivity to orbital forcing was lower (purple box; see text), hence orbital cycling would have been more unlikely to have created refuges at this time. The species history of *Psophia* is a falsifying instance of the refuge hypothesis inasmuch as they persisted throughout Amazonian wet forest areas of endemism during multiple glacial cycles.

and D3; figure 1). There is no evidence in the mtDNA data for the sampled specimens of inter-taxon hybridization at the headwater regions of the different areas of endemism. Thus, the pattern seen in *Psophia* points to a lack of isolation-by-distance of populations within areas of endemism, but strong isolation between adjacent areas.

4. DISCUSSION

(a) *Testing the refuge hypothesis*

The last glacial maximum (LGM), with its estimated 5–6°C drop in temperature, had a major climatic and environmental influence on Amazonia [42] and has been predicted to represent favourable conditions for the creation of refuges [43]. Our data suggest that speciation in *Psophia* was unaffected by LGM environmental change. All species were established prior to the LGM (table 1); more significantly, all existed prior to the first three major glacial maxima (figure 2). Thus, if these cycles had an influence on the distribution of rainforests within Amazonia, that history of perturbation was neither sufficient to disrupt the geographical patterning of genetic diversity in *Psophia* nor, presumably, to extirpate populations in any interfluvium. We further note that prior to approximately 1.0 Ma, the climate response (sensitivity) to orbital forcing was less than in the Pleistocene [44], and at the same time global climate was warmer, thus making it more difficult to sustain the refuge hypothesis (figure 2; electronic supplementary material).

Our data suggest, however, that population demography of individual species may have been variably influenced by glacial conditions and recovery from them, as documented recently for frog assemblages in the Brazilian Atlantic forest [45]. In *P. leucoptera* in the Inambari area of endemism and *P. crepitans* in the Guianan area there is evidence of population expansion after the LGM (also see electronic supplementary material). But in other species, most notably *P. napensis* in the Napo and *P. viridis* in Rondônia, there is strong genetic structuring in mtDNA, suggesting the presence of widespread forests in those areas during the LGM. Importantly, the coalescence of alleles sampled from the current populations is much more recent than the origin of the different species, indicating that any demographic events detected by these data occurred after, and were not directly related to, the speciation events.

The history of *Psophia* is therefore inconsistent with the refuge hypothesis as a generator of species diversity. The environmental effects of glacial cycles, and the LGM in particular, were apparently localized rather than regional across Amazonia; thus we conclude that the driver of speciation in *Psophia* was not climatic cycles. Our approach provides a rigorous methodology to test further the efficacy of the refuge hypothesis (see electronic supplementary material).

(b) *Testing alternative palaeogeographic histories of Amazonia*

Our results (figure 1) provide support for diversification occurring between 3 and 0.5 Ma, and for patterns of speciation being strongly associated with river locations, suggesting that drainage evolution, and not refugia, was the main driver of diversification.

These results also support a palaeogeographic model of Amazonian history in which a transcontinental Amazon River and its western drainage system were established in Plio-Pleistocene times [10,11,13], and not in the Early–Late Miocene [8,9,12,14]. This conclusion is strengthened by the inference that, as the clade arose, species of *Psophia* have been tied ecologically to *terra firme* forest, and behaviourally to a lifestyle as terrestrial foragers. Critical also is the observation that species' distributions are precisely limited by the large river systems within Amazonia, and that multiple lines of evidence point to these rivers as the isolating barriers for sister species.

An alternative model of speciation—sequential long-distance dispersal or 'island hopping' across pre-existing

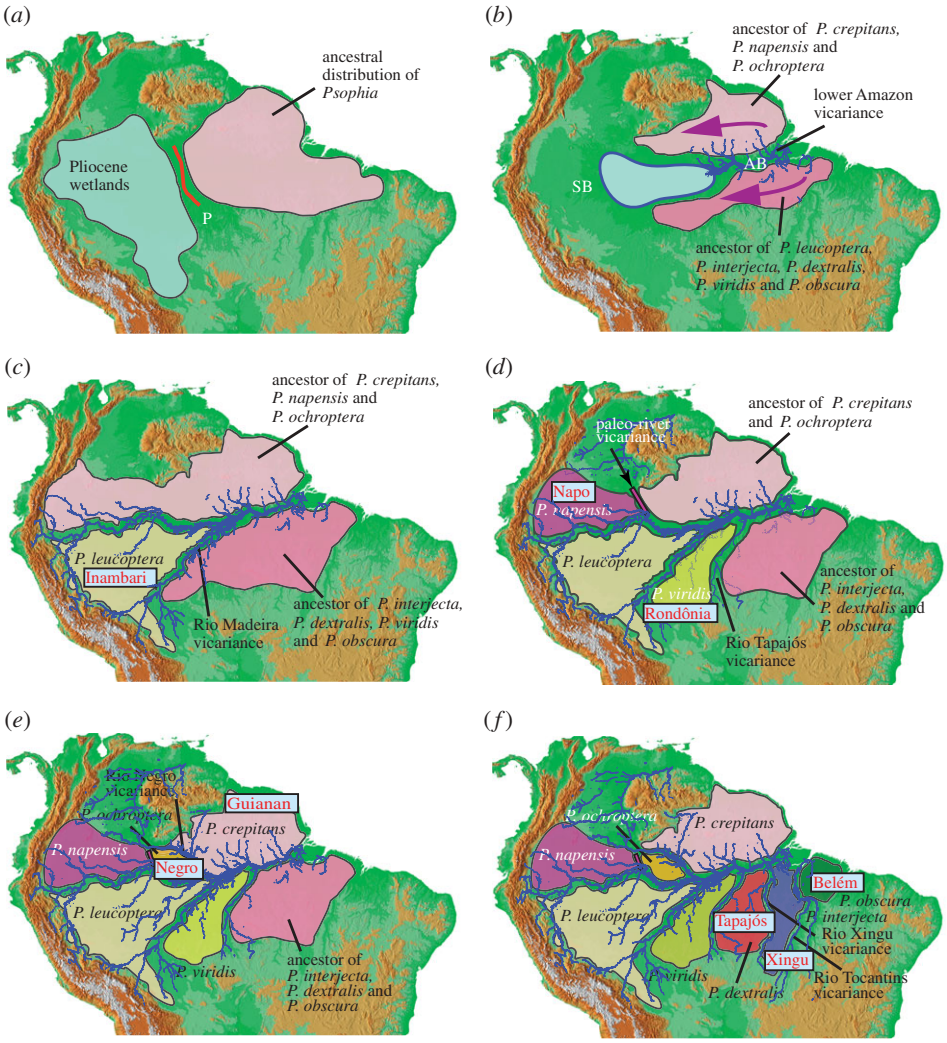

Figure 3. A palaeobiogeographic model for terrestrial environments of Amazonia for the last 3.0 Myr based on the evolutionary history of the trumpeters (*Psophia*) and geological data (see main text). Historical distributions are estimated from specimen locations (electronic supplementary material, figure S1). Rivers and their tributaries are depicted as in the present, but their palaeopositions may have differed. Indicated ages of river drainages are interpretable as ages of isolating events, not necessarily of river entrenchment. (*a*) Approximately 3.0–2.7 Ma: western lowland Amazonia is a large interconnected wetland/lake/river system; its boundaries are not known with precision. (*b*) Approximately 2.7–2.0 Ma: the wetland system drained significantly and the lower Amazon River, which isolated northern and southern populations of *Psophia*, was established. As *terra firme* forests progressively developed, these populations expanded westward. (*c*) Approximately 2.0–1.0 Ma: the Rio Madeira drainage was established, thus promoting speciation of *P. leucoptera* and the formation of the Inambari area of endemism. (*d*) Approximately 1.3–0.8 Ma: the Rio Tapajós drainage system developed, resulting in the differentiation of *P. viridis* and the establishment of the Rondônia area of endemism. At the same time, a barrier (red bar) is postulated to have isolated the Napo area of endemism within which *P. napensis* differentiated (electronic supplementary material). (*e*) Approximately 1.0–0.7 Ma: an isolating barrier associated with the lower Rio Negro formed, giving rise to *P. ochroptera* (Negro area) and *P. crepitans* (Guianan area). (*f*) Approximately 0.8–0.3 Ma: two drainage systems on the Brazilian Shield, the Rio Tocantins and the Rio Xingu, were established as isolating barriers (table 1, nodes 6 and 7), thus creating three areas of endemism and their endemic species. AB, Amazon Basin; SB, Solimões Basin; P, location of Purus Arch.

rivers— is not supported, as we do not see any evidence of mtDNA gene non-monophyly involving individuals of taxa in different interfluvia, even with samples collected close to river boundaries between taxa, indicating lack of current and recent dispersal across rivers. On the other hand, data show evidence of high levels of gene flow within interfluvia. These facts point to large rivers as strong and long-lasting barriers for these birds.

(c) *A palaeobiogeographic model for Amazonia*

Our data and interpretations provide a framework for a palaeobiogeographic model that integrates the phylogenetic and biogeographic history of *Psophia* with current knowledge about the palaeogeographic history of Amazonia over the past 3.0 Myr (figure 3). Under a vicariance model of speciation, the *Psophia* time-tree is predictive of the temporal history of the Amazonian drainage system, but we interpret these ages as isolating events rather than as specific geological arguments for river entrenchment at their current location.

Geological evidence [11,13] points to the lowlands of western and central Amazonia being covered with extensive fluvial and lacustrine conditions (figure 3*a*) in the Late Pliocene, in which *terra firme* forest would have been found primarily on the uplands of the Andean

forelands, the Brazilian and the Guianan shields, and the Amazon sedimentary basin. Our data indicate a separation of *Psophia* into two lineages north and south of the present-day Amazon River between 2.7 and 2.0 Ma (figure 3*b* and table 1, node 1). Both genetic (electronic supplementary material, figure S4) and palaeogeographic [11,13] evidence support the ancestral distribution of *Psophia* being in eastern Amazonia, followed by subsequent westward expansion. This is consistent with a Pliocene age for the establishment of the transcontinental Amazon River based on recent geological interpretations [11,13].

Between 2.0 and 1.0 Ma a vicariance event associated with the current location of the Madeira River isolated *P. leucoptera* in the Inambari area of endemism (figure 3*c* and table 1, node 2). This was followed by another isolating event on the south bank of the Amazon at approximately 1.3–0.8 Ma, associated with the current location of the Tapajós River, which gave rise to *P. viridis* in the Rondônia area of endemism (figure 3*d* and table 1, node 3).

Just prior to approximately 1.0 Ma, the drainage system of northwestern Amazonia began to organize. We propose that a vicariance event north of the Solimões River isolated *P. napensis* in the Napo area of endemism (figure 3*d* and table 1, node 4). There is currently no easily identifiable barrier between *P. napensis* and species to the east, although the lower Rio Japurá separates *P. napensis* and *P. ochroptera* in the south near the Solimões River. Moreover, the mid-Rio Negro basin is occupied by extensive areas of open vegetation growing over white-sand soil, which may have formed over ancient palaeochannels and today may function as a barrier for forest species [46].

At about approximately 1.0–0.7 Ma, the lower Negro River was established as a barrier, resulting in the creation of the Negro area of endemism [19], as well as the separation of *P. ochroptera* and *P. crepitans* (figure 3*e* and table 1, node 5). The palaeogeographic mechanism for this vicariance event apparently involved the establishment of the current course of the lower Negro River in response to a series of neotectonic faulting events [10,47,48] (see electronic supplementary material). Supporting this molecular dating result, Soares *et al*. [47] found evidence for an age of about 0.4 Ma for the initiation of sedimentation covering the Miocene Novo Remanso formation along the lower Negro River.

Finally, two major river barriers were established on the Brazilian Shield during the last 1.0 Myr. The first was associated with the Tocantins River at approximately 0.8–0.5 Ma (figure 3*f* and table 1, node 6), which isolated the Belém area of endemism and its endemic species, *P. obscura*. This age estimate is consistent with a north-northwesterly trending Tocantins palaeodrainage of Plio-Pleistocene age that shifted sharply northeast in the Middle/Late Pleistocene to form the modern-day river [49]. This event was followed at 0.5–0.3 Ma by another involving the Rio Xingu, which created the Tapajós area of endemism housing *P. dextralis* and the Xingu area of endemism with *P. interjecta* (figure 3*f* and table 1, node 7).

(d) *Generality of the model*

Our palaeobiogeographic model describes the causal spatio-temporal linkages between palaeoenvironmental evolution and speciation within *Psophia* during the Plio-Pleistocene. Major rivers are the primary distributional boundaries for a significant proportion of Amazonian vertebrates, but the importance of river dynamics in promoting allopatric speciation has not gained wide acceptance. It has long been thought that major features of the Amazonian drainage were entrenched well before current species arose and that, unlike forest refugia, they therefore could not be drivers of speciation [3]. Moreover, some meandering rivers such as the Juruá and Purus appear not to have acted as primary barriers for speciation in some *terra firme* vertebrate taxa [50–52]. Nevertheless, many Amazonian terrestrial vertebrate groups of the *terra firme* forest exhibit taxonomic disjunctions across the same rivers that separate reciprocally monophyletic lineages in *Psophia*, and these interfluvial areas harbour high endemicity [17,18,53]. Recent molecular phylogenies have also revealed multiple examples of disjuncts associated with major rivers [54–58].

It is difficult to compare the pattern seen in *Psophia* with other taxa in the same areas of endemism because few rigorous time-trees have been determined for these groups, and most studies only report sequence divergences among taxa. In *Psophia*, the Solimões-Amazonas, Negro and Madeira rivers are associated with older divergences, whereas the rivers of the Brazilian shield (Tapajós, Xingú and Tocantins) are related to younger diversification events. Compared with *Psophia*, slightly larger genetic divergences across the Amazon River are seen in *Capito* barbets (5% uncorrected divergence [59]) and *Glyphorhynchus* woodcreepers (4.9–6.6% uncorrected divergence [54]), whereas smaller distances separate species of *Xiphorhynchus* woodcreepers (1.6–1.9% corrected [51]) and *Pteroglossus* araçaris (several divergences less than 1.5 Myr [58]). None of these argue for Miocene vicariance across the Amazon River. It has recently been argued that most clades of vertebrates diversified within Amazonia prior to the Pleistocene [8,52], an argument based on a geological model that postulates a mid-Miocene establishment of the terrestrial environment in western Amazonia, as well as Miocene and Pliocene ages for 'crown groups' of vertebrates using molecular dating. Our study does not address crown group ages, but rather ages of species origins. Comparable age estimates for species in other groups are uncommon in the literature, as it is necessary to have a resolved taxonomy with species limits that adequately represent diversity, complete species sampling and well-dated trees (see electronic supplementary material). Thus, we agree with Rull [60] that the use of genera, or any other group of species, as surrogates for the age of Amazonian taxa [8] significantly overestimates the timing of speciation within those groups.

Large-scale mountain building, small-scale neotectonic change at regional and local landscape levels, and Milankovich forcing of climate all can influence river dynamics. Extreme climatic events have created cold or dry conditions, which have led to significant reductions in Amazonian river size and discharge in the past [61]. These have the potential to eliminate or dampen the effects of riverine vicariance barriers, especially in headwaters, which would promote cosmopolitanism of biotas, followed by barriers then reappearing during subsequent wetter periods. Thus, drainage evolution is dynamic and probably important for understanding the origin of current diversity patterns. These patterns may also be related to shifts in vegetation zones associated with climate change. Teasing apart this multiple causation will depend on other well-dated studies of speciation.

Our palaeobiogeographic model offers the potential to reframe the analysis of Amazonian diversification, first by linking the temporal history of specific speciation events to identifiable changes in the history of the Earth and by providing a more precise temporal framework, and, second, by refocusing attention on the role played by river dynamics in driving allopatry (and therefore diversification). The model is expected to have the most generality for terrestrial, understorey taxa in *terra firme* forests. Importantly, the same forces of the Earth's history that influence river evolution also create dynamic change in biome composition and distribution. Only by integrating riverine dynamics, climatic and biotic landscape change, and other manifestations of the Earth's history will we truly begin to answer how Amazonian diversity was generated.

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