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Phylogeography and diversification of an Amazonian understorey hummingbird: paraphyly and evidence for widespread cryptic speciation in the Plio-Pleistocene

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Straight-billed Hermit *Phaethornis bourcieri* inhabits the understorey of upland *terra firme* forest throughout most of the Amazon basin. Currently, two allopatric taxa regarded as subspecies are recognized: *P. b. bourcieri* and *P. b. major*. However, the validity, interspecific limits and evolutionary history of these taxa are not yet fully elucidated. We use molecular characters to propose a phylogenetic hypothesis for populations and taxa grouped under *Phaethornis bourcieri*. Our results showed that *P. bourcieri* is part of the '*Ametrornis*' clade, along with *Phaethornis philippii* and *Phaethornis koepckeae*, and that the subspecies *major* is more closely related to the latter two species than to populations grouped under nominate *bourcieri*. Our phylogenetic hypotheses recovered three main reciprocally monophyletic clades under nominate *bourcieri* separated by the lower Negro River and the Branco River or the Branco–Negro interfluve (clades B and C) and the upper Amazon (Solimões) or lower Marañon/Ucayali Rivers (clades C and D). Based on multi-locus phylogeographic and population genetics approaches, we show that *P. b. major* is best treated as a separate species, and that *P. b. bourcieri* probably includes more than one evolutionary species, whose limits remain uncertain. The diversification of the '*Ametrornis*' clade (*P. bourcieri*, *P. philippii* and *P. koepckeae*) is centred in the Amazon and appears to be closely linked to the formation of the modern Amazon drainage during the Plio-Pleistocene.

Keywords: *Ametrornis*, biogeography, *Phaethornis bourcieri*, systematics, taxonomy.

Amazonia is the largest and most diverse tropical forest in the world, containing more than 6 million square kilometres distributed across nine countries in South America (Silva *et al.* 2005). It constitutes a heterogeneous biome in terms of both its floristic (Veloso 1962, Pires 1973, Silva *et al.* 2005) and faunistic composition (Silva *et al.* 2005). Its forests and rivers are important for the regulation of climate, regional and national hydrological regimes, and terrestrial carbon supply (Fearnside 1999). In recent decades, the biodiversity of Amazonia has been the subject of a large number of studies and debates on the events from which it originated (Haffer 1992, 1997, 2001, Räsänen *et al.* 1995, Webb 1995, Bates 2001, Antonelli *et al.* 2010, Ribas *et al.* 2012).

Molecular data, when adequately integrated with biogeography and studies of phenotypic variation, have great potential for testing hypotheses related to the factors that generate species diversity in forest biomes and assessing how they have changed over time (Moritz *et al.* 2000). In recent years, such studies have made important contributions to our knowledge of the evolutionary history of several lineages of Neotropical birds (Ribas *et al.* 2012, d'Horta *et al.* 2013, Fernandes *et al.* 2014, Thom & Aleixo 2015). By including a systematic representation of the true phyletic diversity in these lineages, they have also provided more

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accurate biogeographical interpretations which would otherwise be misleading if the species studied were treated as single lineages.

Straight-billed Hermit *Phaethornis bourcieri* (Aves: Trochilidae) is a little-studied species of hummingbird that inhabits the understorey of upland *terra firme* forest, being distributed across almost all of Amazonia (del Hoyo *et al.* 1999). It therefore represents a useful study species for evaluating historical processes involved in the biotic diversification in the region. Currently, two allopatric subspecies are recognized in *P. bourcieri*, with the Amazon and the Madeira rivers as geographical barriers separating them: *P. b. bourcieri*, occurring in the Guiana, Jaú, Imeri, Napo and Inambari areas of endemism (*sensu* Silva *et al.* 2005, Borges & Silva 2012), and *P. b. major*, restricted to the Tapajós area of endemism (*sensu* Silva *et al.* 2005).

Hinkelmann and Schuchmann (1997) produced the first phylogenetic hypothesis for some taxa of the sub-family Phaethornithinae based on 96 external morphological characters, reaching the conclusion that the position of *P. bourcieri* within *Phaethornis* was uncertain. This study also recovered *Phaethornis syrmatophorus* as sister to *Phaethornis philippii* and *Phaethornis koepckeae*. Therefore, according to these authors, the shared character between *P. koepckeae*, *P. philippii* and *P. bourcieri* – the straight rather than curved bill as in all remaining *Phaethornis* – and which prompted their previous grouping in the separate genus *Ametrornis* – had to be interpreted as homoplasy (i.e. convergence). McGuire *et al.* (2007) reconstructed a phylogeny based on molecular characters for 151 hummingbird taxa, in which *P. bourcieri* was recovered as sister to *Phaethornis hispidus*; these two species formed a monophyletic group with *Phaethornis augusti*, *P. koepckeae* and *P. philippii*, hence a different result from that obtained by Hinkelmann and Schuchmann (1997). However, the identification of a sequenced specimen attributed to *P. hispidus* that was in fact *P. bourcieri* (V. Piacentini pers. com.) cast doubt on the conclusions of the phylogenetic position of *P. bourcieri* obtained by McGuire *et al.* (2007). Indeed, the most complete phylogenetic hypothesis for the Trochilidae available to date recovered *P. bourcieri* as part of a clade including *P. koepckeae* and *P. philippii* (McGuire *et al.* 2014). Therefore, each study available so far on the phylogenetic relationships of *P. bourcieri* and the entire '*Ametrornis* assemblage' (*P. bourcieri*, *P. koepckeae* and *P. philippii*) produced different results (Hinkelmann & Schuchmann 1997, McGuire *et al.* 2007, 2014). Currently, there is no phylogenetic hypothesis available that sampled both recognized taxa grouped under *P. bourcieri* (*P. bourcieri bourcieri* and *P. b. major*), and the validity, interspecific limits and evolutionary history of these taxa are unknown. This, added to the controversial phylogenetic affinities recovered so far for this species in the genus *Phaethornis* (Hinkelmann & Schuchmann 1997, McGuire *et al.* 2007, 2014), underscores the need for a study with a dense sampling regime involving many populations and all subspecies of *P. bourcieri*.

Here, we propose a phylogenetic hypothesis for both taxa and several populations grouped in *P. bourcieri* in addition to several other *Phaethornis* species to (1) test the monophyly and determine the phylogenetic position of *P. bourcieri bourcieri* and *P. b. major* in the genus *Phaethornis*; (2) evaluate interspecific limits based on multi-locus phylogeographical and population genetics approaches; and (3) reconstruct the spatial and temporal contexts of the species' diversification in Amazonia.

METHODS

Taxonomic sampling

We sequenced 40 *P. bourcieri* muscle tissue samples from across a significant part of the species' range, including 27 samples of *P. b. bourcieri* and 13 samples of *P. b. major* (Fig. 1, Supporting Information Fig. S1, Appendix S1) from Brazil and Peru. Additionally, one specimen of *P. b. bourcieri* from south-eastern Peru (LSUMZ B-11104) whose ND2 sequences were available on GenBank was also included in the analyses (Supporting Information Appendix S2). Due to the contentious phylogenetic placement of *P. bourcieri* in the genus *Phaethornis* as suggested by previous studies (Hinkelmann & Schuchmann 1997, McGuire *et al.* 2007, 2014), the following species were sampled as outgroups: *P. hispidus* (six samples), *P. koepckeae* (two samples including one from GenBank), *P. philippii* (three samples), *Phaethornis superciliosus* (three samples including two from GenBank), *P. ruber* (two samples, including one from GenBank), *Phaethornis aethopygus* (four samples), *Phaethornis syrmatophorus* (three samples including one from GenBank) and *Phaethornis rupurumii* (one sample). For the

following samples used, all data were from GenBank (only one sample unless otherwise noted): *Phaethornis malaris*, *Phaethornis striigularis*, *Phaethornis antophilus*, *Phaethornis augusti* (two samples), *Phaethornis pretrei*, *Phaethornis griseogularis*, *Phaethornis griseoventer*, *Phaethornis atrimentalis*, *Phaethornis longirostris*, *Phaethornis mexicanus*, *Phaethornis subochraceus*, *Phaethornis eurynome*, *Phaethornis yaruqui*, *Phaethornis guy* and *Glaucis hirsutus* (Appendices S1 and S2).

Laboratory procedures

Tissues were sequenced for the entire mitochondrial cytochrome b (cytb) and NADH dehydrogenase 2 (ND2) genes, and for intron 5 of the nuclear gene β-fibrinogen (BF5). Total DNA was extracted using standard procedures with the phenol-chloroform technique (Sambrook *et al.* 1989). Genes were amplified through PCR. The total volume of the reactions was 25 μL, containing: buffer 10× (2.5 μL), 50 ng (1 μL) of genomic DNA, 10 mM (1 μL) dNTPs, 50 mM (1.5 μL) $MgCl_2$, 1 U (0.2 μL) *Taq* DNA polymerase and 200 ng/μL (0.5 μL) of each of the primers (Table S1). The amplification profile for the different genes consisted of one initial step of 5 min at 95 °C followed by 35 cycles for 1 min at 95 °C; 1 min for association temperature of the primers (Table S1); and 1 min at 72 °C for extension; finally, a step of 5 min at 72 °C. The amplified samples were confirmed by electrophoresis throughout in 1% agarose gel and purified with polyethylene glycol (PEG-8000). The amplification products were sequenced automatically using the Big Dye Terminator Cycle Sequencing Standard Version 3.1 kit in the Applied Biosystems ABI 3130 sequencer according to the manufacturer's specifications.

Phylogenetic analyses and molecular dating

Nucleotide sequences were manually edited and aligned using the application BioEdit 7.0.5 (Hall 1999). To reconstruct the haplotypes of the nuclear gene BF5, the program PHASE 2.1 was used, accepting the results of probability >70% (Stephens *et al.* 2001).

Average uncorrected genetic divergences (*p*-distances) were calculated within and between main clades associated with *P. bourcieri* recovered by the phylogenies obtained in this study (see below). Graphic plotting of transitions and transversions was done as a function of the genetic distances for each marker using DAMBE (Xia & Xie 2001) to evaluate saturation in the number of mutations between taxa.

Phylogenies were estimated using maximum likelihood (ML), with support of branches estimated by 1000 bootstrap replicates, and Bayesian inference (BI), using the programs RAxML-7.0.3 (Stamatakis 2006) and MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001), respectively.

To determine the model that best explained the evolution of the genes sequenced, the program jModeltest 0.1.1 was used for BI and the model used in ML was GTRGAMMA. BI was undertaken with the entire dataset linked but partitioned by gene using the model chosen for each gene. BI analyses were conducted using two independent runs of 5 000 000 generations each (three hot chains and one cold per run), sampling parameters every 500 generations. The software TRACER 1.4 (Rambaut & Drummond 2007) was used to determine when the analyses reached convergence. The first 500 000 trees were discarded as burn-in.

To determine divergence times, a multilocus analysis was undertaken in BEAST v 1.6, using the option *BEAST. All individuals for whom at least one mitochondrial and nuclear genes were sequenced were used, with the substitution rate of the cytb gene fixed at 2.1% per million years (Weir & Schluter 2008), and estimating the rate of the other genes (ND2 and BF5). The priors 'Species Tree: Yule Process' and 'Relaxed clock: uncorrelated Lognormal' were chosen and run for 200 000 000 generations, with parameters sampled every 10 000 generations.

Bayesian species delimitation analysis

A Bayesian species delimitation analysis was conducted using the multilocus dataset in BPP v.2.0 (Rannala & Yang 2003, Yang & Rannala 2010). The model assumes no admixture following speciation, which is an assumption motivated by the biological species concept, and takes into account gene tree uncertainty and lineage sorting. BPP estimates divergence time (τ) and population size parameters ($\theta = 4\,Ne\mu$), where Ne is the effective population size and μ is the mutation rate per site per generation. We ran the rjMCMC analyses for 500 000 generations (sampling parameters every fifth generation) with a burn-in period of 10 000,

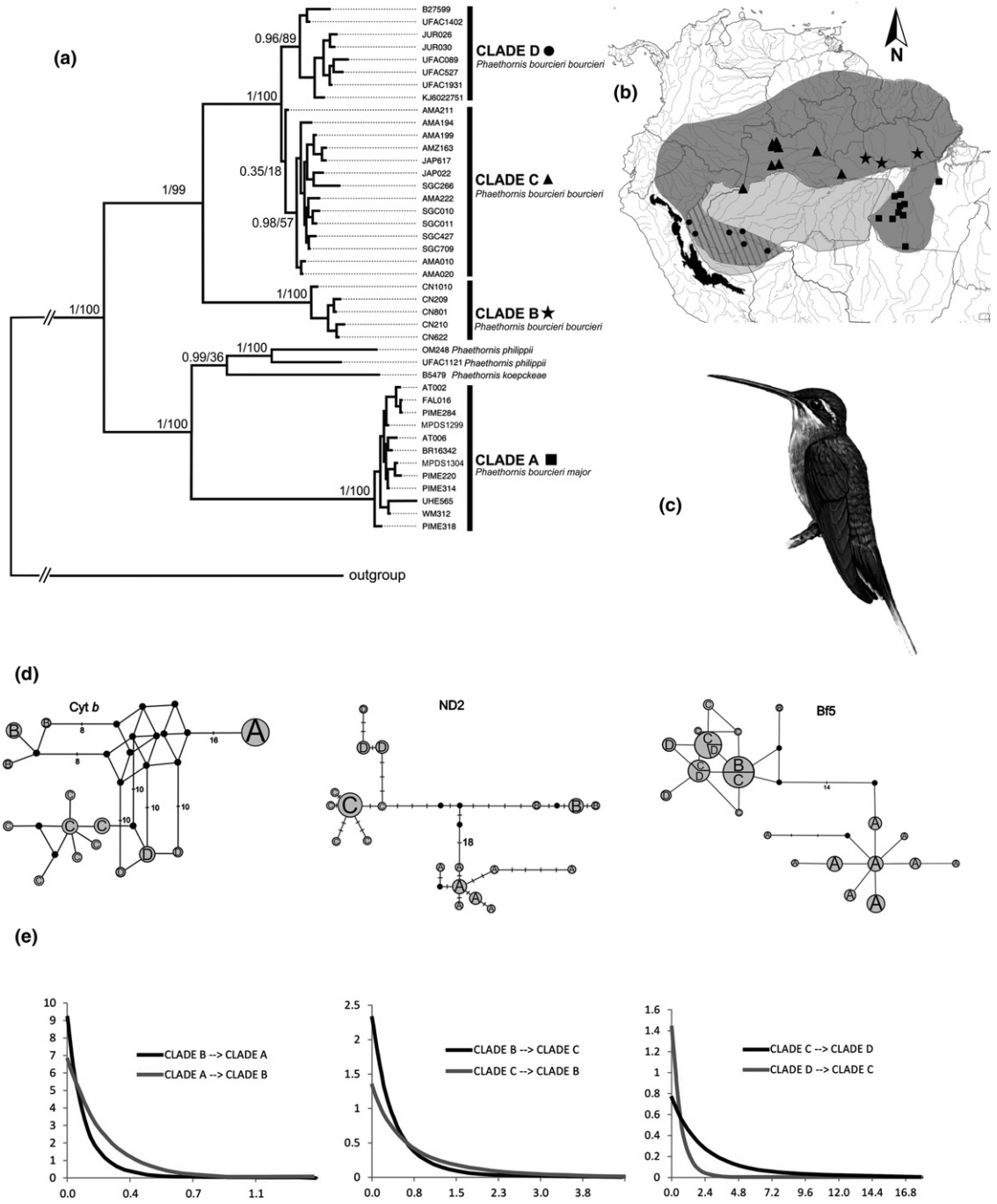

Figure 1. Results of molecular analyses. (a) Multilocus phylogenetic hypothesis obtained through BI and ML. For species included in these analyses see Appendices S1 and S2. Numbers at nodes denote respectively BI posterior probabilities and ML bootstrap values. Low support values are depicted as pale numbers. (b) Distribution map showing the known ranges of *P. bourcieri* (in dark grey), *P. philippii* (in light grey) and *P. koepckeae* (in black) with symbols denoting *P. bourcieri* samples sequenced in this study (for the key to symbols see below). Ranges were based primarily on del Hoyo *et al.* (1999) and Hinkelmann (1989), with modifications. (c) *Phaethornis bourcieri bourcieri* (modified from *Handbook of the Birds of the World* available at: http://www.hbw.com/). (d) *P. bourcieri* haplotype networks estimated for each sequenced gene. Letters denote clades as shown in Fig. 1a. (e) IMA2 graphs showing the absence of gene flow between parapatric clades of *P. bourcieri*. Symbols represent clade membership as follows: squares – Clade A; stars – Clade B; triangles – Clade C; circles – Clade D.

and using the species tree topology as the starting tree. As BPP has been shown to be sensitive to the choice of prior distributions of theta and divergence times, we implemented the approach of Leaché and Fujita (2010) and performed analyses using three combinations of priors: (1) large ancestral population sizes and deep divergences (θ G (1,10) and τ G(1, 10)); (2) small ancestral population sizes and shallow divergences among species (θ G(2, 2000) and τ G(2, 2000)); and (3) large ancestral populations sizes and shallow divergences (θ G(1, 10) and τ G(2, 2000)). Each analysis was run at least twice to confirm consistency between runs. We considered speciation probability values ≥0.95 under all three prior scenarios as strong support for a speciation event.

Biogeographical reconstructions

To perform biogeographical area reconstructions we used the R package BioGeoBEARS (Matzke 2013; http://cran.r-project.org/web/packages/BioGeoBEARS/index.html). BioGeoBEARS models probabilistic biogeographical scenarios onto a user-defined phylogeny. We performed six different area reconstruction analyses, testing models including the dispersal-extinction cladogenesis (DEC), a likelihood version of the dispersal-vicariance analysis ('DIVALIKE'), and a version of the Bayesian inference of historical biogeography for discrete areas (BAYAREALIKE), as well as '+J' versions of these three models, which include founder-event speciation, an important process left out of most inference methods (Matzke 2013). In these analyses, we used a time-calibrated ultrametric tree, and defined as biogeographical units seven major Amazonian areas of endemism inhabited by *P. bourcieri* lineages: Gui = Guiana; Ina = Inambari; Ime = Imeri; Jau = Jaú; Nap = Napo; Ron = Rondônia; and Tap = Tapajós (*sensu* Silva *et al.* 2005, Borges & Silva 2012).

Population genetics analyses

To examine the geographical distribution and relationships of the recovered haplotypes we constructed median-joining networks (Bandelt *et al.* 1999) using NETWORK 4.5.1.0 (www.fluxus-engineering.com). As the tree-building reconstruction methods described above assume that no gene-flow occurred between lineages, we used the isolation–migration model (Nielsen & Wakeley 2001, Hey & Nielsen 2004) implemented in IMA2 (Hey 2010) to estimate levels of gene flow between clades of *P. bourcieri*. IMA2 estimates were obtained only for reciprocally monophyletic parapatric pairs of *P. bourcieri* populations potentially in contact across the banks of major Amazonian rivers. IMA2 analyses employed the HKY model (Hasegawa *et al.* 1985) for all markers; an inheritance scale of 0.25 for the mtDNA and 1 for nDNA; a substitution rate for the cytb gene of 2.1% sequence divergence per million years per generation (estimating those of ND2 and BF5) and a 1-year generation time. Several runs were performed to establish the best priors for effective population sizes, time of divergences and migration parameters. Three final runs were performed using 10^5 generations as burn-in, 10^5 trees sampled during 10^6 generations and 20 chains. Finally, to test whether a model of isolation without gene flow fitted the data better than a model with gene flow, we used Nielsen and Wakeley's (2001) approach and also the likelihood-ratio tests of different models implemented in the IMA2 L mode.

To analyse population size dynamics through time for both loci combined, we used extended Bayesian skyline plots (EBSPs, Heled & Drummond 2010) implemented in BEAST. EBSP analyses were performed for each population used in the IMA2 analysis. The best-fit substitution model for each marker, substitution rates, priors and the MCMC run strategy were the same described above for the phylogenetic analyses.

RESULTS

Data characteristics

The combined dataset consisted of a total of 2608 base pairs (bp) sequenced for 80 *Phaethornis* specimens with 1001 bp of cytb, 1036 bp of ND2 and 571 bp of BF5. Only ND2 sequences were available for all 80 individuals analysed, whereas complete cytb and BF5 sequences were available only for the ingroup (*P. b. bourcieri* and *P. b. major*), *P. hispidus*, *P. aethopygus*, *P. superciliosus*, *P. philippii*, *P. koepckeae*, *P. syrmatophorus*, *P. ruber* and *P. rupurumii* (Appendices S1 and S2). Saturation was not detected among populations of *P. bourcieri* for any of the sequenced genes. Among the markers used, the one having the greatest number of phylogenetically informative sites among all *Phaethornis* taxa analysed was ND2 (291), followed by cytb (173) and BF5 (33). The best

models of sequence evolution (BIC) found for the different genes sequenced were: HKY+I for cytb, TIM1+I+G for ND2 and HKY+G for BF5.

Phylogeny and molecular dating

We estimated one inclusive BI phylogenetic hypothesis based on ND2 sequences of all *Phaethornis* specimens sampled to be used as a guide tree to test the monophyly and species boundaries in *P. bourcieri* based on multilocus coalescent methods. The ND2 BI gene tree obtained showed with strong statistical support that *P. bourcieri* is in fact paraphyletic with respect to the closely related *P. koepckeae* and *P. philippii*, which together comprise a monophyletic group, hence supporting the monophyly of the '*Ametrornis* assemblage' (Fig. S1). Due to the fact that we did not sample all species in the genus *Phaethornis*, it was not possible to determine which species or group of species is closest to the '*Ametrornis* assemblage'. Nevertheless, among some of the *Phaethornis* taxa for which all genes were sequenced, *P. hispidus* was closest to the '*Ametrornis* assemblage'; therefore, it was used as outgroup in the following analyses.

Both ML and BI multilocus phylogenies recovered essentially the same topology of the ND2 gene tree whereby the paraphyletic *P. bourcieri* included four clades (clades A–D) whose ranges were apparently bound by large Amazonian rivers (Fig. 1a,b). Clade A included the population that inhabits the Tapajós-Xingú interfluve in southeastern Amazonia belonging to *P. b. major* (type locality Caxiricatuba in the Tapajós area of endemism; Hinkelmann 1989). Phylogenetic analyses demonstrated with strong support that clade A is more closely related to *P. koepckeae* and *P. philippii* than it is to the remaining *P. b. bourcieri* clades (B, C and D), which in turn comprised a monophyletic group with good statistical support. The well-supported clade B included individuals inhabiting the north bank of the Amazon River, from either the Branco-Negro interfluve or Branco River to the Guianas and Amapá in Brazil (Guiana area of endemism). Clade C included specimens inhabiting the north bank of the Amazon River, from the western bank of the Branco River or west of the Branco-Negro interfluve to at least the northern bank of the upper Amazon (Solimões) River. Finally, clade D contained individuals from the west bank of the mid-Ucayali (Marañon – Ucayali interfluve; LSUMZ 27599) to the Madeira River in the Inambari area of endemism (Fig. 1b). The precise boundary between clades C and D could not be established due to our lack of samples from both banks of the lower Marañon, Ucayali and Napo Rivers. At any rate, the reciprocal monophyly between clades C and D is poorly supported due to the presence of one specimen from the north bank of the upper Amazon (Tabatinga, Brazil; AMA 211) whose relationships with either all remaining specimens from the north bank (clade C) or those from the south bank (clade D) are weakly supported according to the multilocus and ND2 gene tree estimates, respectively (Figs 1a & S1).

The levels of pairwise uncorrected genetic divergences (p-distances) within and between clades A, B, C and D of *P. bourcieri* (Fig. 1) varied from 0.02% to 1% and 1% to 6%, respectively (Table 1). The pairwise genetic distance between specimens attributed to *P. b. major* (clade A) and those of other clades of *P. bourcieri* (6%) was equal to or greater than the distance separating closely related taxa that have always been treated as distinct biological species in the genus *Phaethornis*, such as *P. philippii*, *P. koepckeae* and *P. bourcieri* (Table 1). In the case of populations attributed to *P. b. bourcieri* (clades B, C and D), the highest pairwise divergence between them was 3%, comparable to that between populations of the monotypic *P. philippii*, but much greater than that obtained within populations of the respective clades (0.02–0.03) or between different populations of *P. hispidus* (0.02; Table 1).

Table 1. Pairwise uncorrected genetic divergences (p-distances) among different clades of straight billed *Phaethornis* species (*P. bourcieri*, *P. philippii* and *P. koepckeae*). A – *P. bourcieri* clade A; B – *P. bourcieri* clade B; C – *P. bourcieri* clade C; D – *P. bourcieri* clade D; *P. p* – *Phaethornis philippii*; *P. k* – *Phaethornis koepckeae*; *P. h* – *Phaethornis hispidus*; D.p – p-distance within each lineage.

	A	B	C	D	*P. p*	*P. k*	D.p
A							1%
B	6%						0.03%
C	6%	3%					0.02%
D	6%	3%	1%				0.03%
P. p	5%	6%	6%	6%			3%
P. k	6%	5%	5%	6%	5%		–
P. h	10%	10%	10%	10%	10%	11%	0.02%

The species tree recovered a slightly different topology from that generated by ML and BI based on the concatenated genes, despite confirming the paraphyly of *P. bourcieri* with respect to *P. philippii* and *P. koepckeae* and the formation of clades A, B, C and D (Fig. 2). Nevertheless, it recovered clade A as sister to *P. koepckeae* rather than a *P. philippii*–*P. koepckeae* clade, albeit with very low statistical support (Fig. 2).

Based on the estimated chronogram, the oldest divergence occurred between 4.4 and 3.1 million years ago, separating populations attributed to *P. b. bourcieri* (clades B, C and D) from the clade comprising *P. b. major* (clade A), *P. philippii* and *P. koepckeae*. The second divergence occurred between 3.5 and 1.7 million years ago, separating *P. philippii* and the clade formed by *P. b. major* and *P. koepckeae*. The divergence separating *P. b. major* and *P. koepckeae* occurred between 2.9 and 1.25 million years ago.

Between 2.7 and 1.1 million years ago, the separation between clade B from the Guiana area of endemism and the ancestors of clades C and D of *P. b. bourcieri* took place. The population that inhabits the Inambari area of endemism (clade D) separated from clade C (Napo, Imeri and Jaú areas of endemism) more recently, between 0.6 and 0.2 million years ago (Fig. 2).

Species delimitation and biogeographical reconstruction

We tested species limits based on alternative topologies obtained with the concatenated multilocus BI and ML trees (with *P. philippii* and *P. koepckeae* as sister taxa) and the coalescent multilocus species tree (which recovered clade A as sister to *P. koepckeae*) using BPP. We obtained similar results using the three prior combinations related to ancestral population sizes and divergence times for both topologies used as guide trees, which showed consistently strong support for an advanced degree of divergence and hence speciation separating clades A, B, C and D of *P. bourcieri* (Fig. 2). As BPP showed all speciation scenarios to be equally likely, we chose to use the concatenated multilocus BI and ML topology – with *P. philippii* and *P. koepckeae* as sister taxa – for the ancestral area reconstructions for several reasons. First, *P. philippii* and *P. koepckeae* are morphologically very similar to each other (del Hoyo *et al.* 1999), and therefore plumage characters favour a sister relationship between them to the exclusion of clade A (*P. b. major*). Secondly, *P. philippii* and *P. koepckeae* ranges are continuous in southwestern Amazonia, whereas those of *P. koepckeae* and *P. b. major* are allopatric and separated by thousands of kilometres, implying a much more complex range

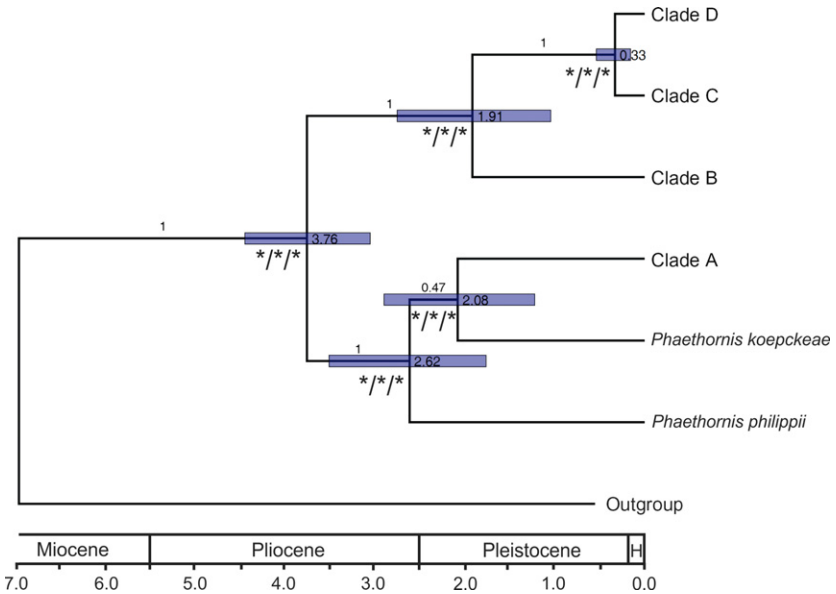

Figure 2. Chronogram obtained with a multilocus species tree analysis. Numbers on top of branches represent posterior probability values. Numbers on nodes represent the median divergence time in million years whereas bars denote the confidence interval of the estimated divergence times. Asterisks below branches represent maximum posterior probability values (1.0) obtained for the three different coalescence scenarios tested on the species tree topology with BPP. Numbers on the time scale below represent millions of years.

of inheritance scenarios. As already explained above, the tree used in ancestral area reconstructions was rooted in *P. hispidus*, one of the species recovered as closer to the '*Ametrornis* assemblage' for which sequences of all sampled genes were available. We chose this strategy because, given true outgroup uncertainty, adding more species to the root could potentially increase bias of the estimates. Our results showed that the best ancestral area reconstruction model fitting the *P. bourcieri* species tree was 'DIVALIKE + J' (log-likelihood ratio test = –13.53) (Table S2, Fig. 3).

The ancestor of the '*Ametrornis* assemblage' was probably distributed in western Amazonia (Imeri, Jaú, Napo, Inambari and Rondônia areas of endemism; Fig. 3). A first split occurred between the ancestors of *P. philippii-koepckeae-bourcieri major* (clade A) and *P. bourcieri bourcieri* (clades B, C and D), apparently by vicariance across the Amazon River (Fig. 3). A second diversification event probably occurred through dispersal across the Tapajós River and separated the ancestor of *P. philippii-koepckeae* (probably occurring in the Rondônia and Inambari areas of endemism) from *P. b. major* (clade A; Fig. 3). Then, *P. philippii* and *P. koepckeae* split after dispersal to the Andean foothills from an ancestor probably distributed in southwestern Amazonia (Rondônia and Inambari areas of endemism). Finally, two subsequent diversification events involved *P. b. bourcieri* lineages (clades B, C and D), whose ancestor occupied northwestern Amazonia (Imeri, Jaú and Napo areas of endemism; Fig. 3). Both these events were estimated as caused by dispersal followed by vicariance; a first event probably took place across the Branco River or the Branco–Negro interfluve,

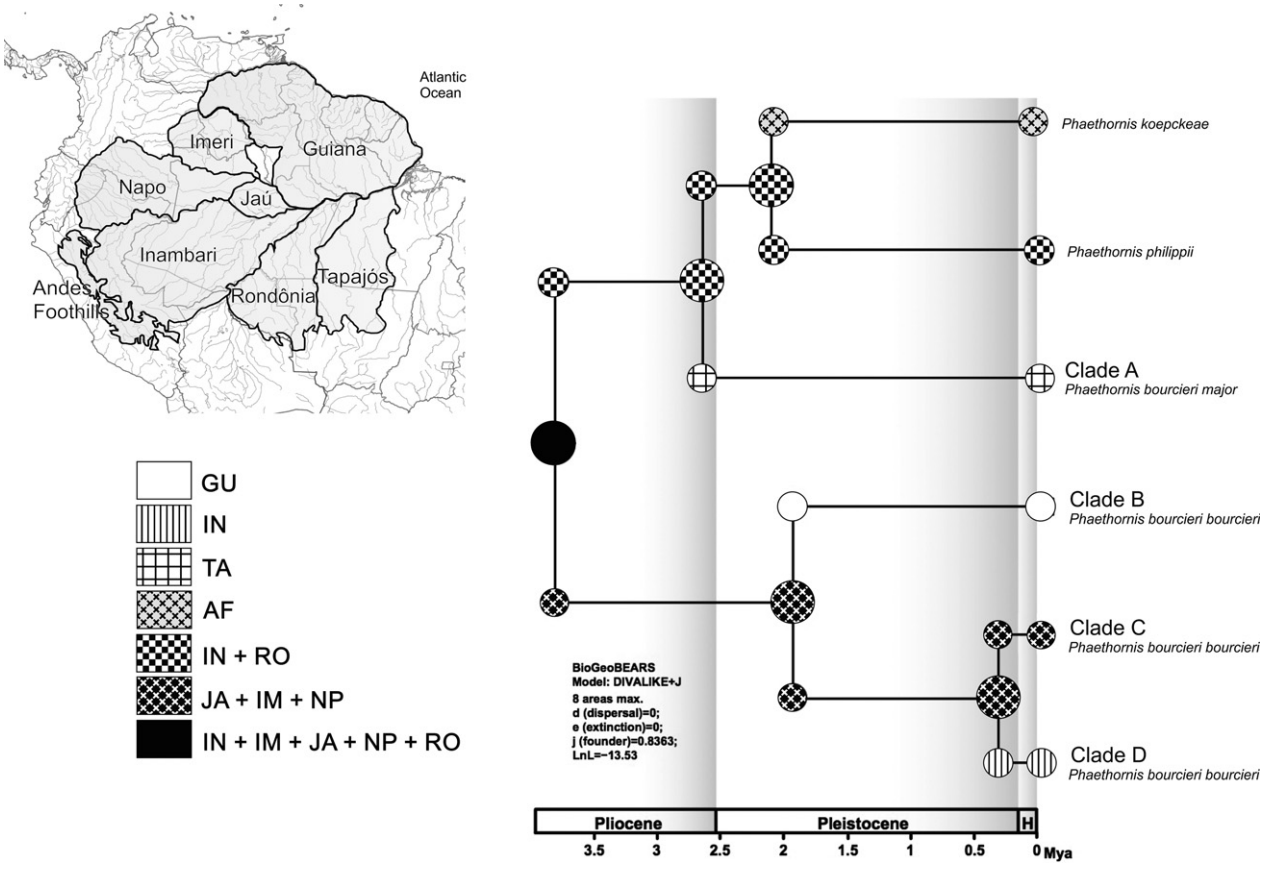

Figure 3. Biogeographical history of the Straight-billed Hermit assemblage (*P. bourcieri*, *P. koepckeae* and *P. philippii*) favoured by BioGeoBEARS under the DIVALIKE+J model (see Table S2). Top left: areas of endemism occupied by the different lineages (adapted from Silva *et al.* 2005 and Borges & Silva 2012); bottom left: different patterns represent recognized ancestral areas, as follows: AF = Andes Foothills; GU = Guiana; IN = Inambari; IM = Imeri; JA = Jaú; NP = Napo; RO = Rondônia; TA = Tapajós; and combined areas. Numbers on the time scale below represent millions of years. See Fig. S4 for detailed ancestral area probabilities associated with each node.

Table 2. Migration rates estimated with IMA2 between *Phaethornis bourcieri* clades in contact across Amazonian riverine barriers. 2N1M1→2, migration rate into population 1 from population 2 per generation; 2N2M2→1, migration rate into population 2 from population 1 per generation. 95% HPD confidence intervals are shown in parentheses. In all comparisons, scenarios with migration rates different from zero were rejected by likelihood ratio tests.

	2N1M1→2	*P*	2N2M2→1	*P*
CLADE A × CLADE B (Amazon River)	0.003 (0.0–0.433)	6.882	0.004 (0.0–0.393)	9.578
CLADE B × CLADE C (Lower Negro/Branco rivers)	0.007 (0.0–1.762)	2.372	0.007 (0.0–3.711)	1.347
CLADE C × CLADE D (Solimões – Upper Amazon River)	0.007 (0.0–5.510)	0.77	0.015 (0.0–2.114)	1.455

which led to the separation of the Guiana area of endemism lineage (clade B), followed by a second event across the upper Amazon (Solimões) River, and which resulted in the separation of the Inambari area of endemism lineage (clade D; Fig. 3).

Population genetics and historical demography

Haplotype networks obtained for the mitochondrial genes were consistent with the estimated phylogenies whereby *P. bourcieri* clades A, B, C and D formed four separate structured groups with no haplotype-sharing among them (Fig. 1d; see also Figs S2 and S3.). In contrast, the haplotype network obtained for the nuclear marker recovered essentially two major groups, one including only clade A (*P. b. major*) alleles and a second with clades B, C and D alleles, including one allele shared between clades B and C, and two alleles shared between clades C and D (*P. b. bourcieri*) (Fig. 1d; see also Figs S2 and S3).

The posterior distributions of parameters in all IMA2 runs had a clear peak and the right tails converged on zero (Fig. 1e). Gene flow analyses contrasting only *P. bourcieri* clades potentially in contact across riverine barriers detected migration rates not statistically different from zero (Table 2; Fig. 1e), indicating strong genetic isolation.

Historical demography analyses (EBSPs) implemented in BEAST recovered similar patterns of recent demographic expansions in all *P. bourcieri* clades during the Holocene (clades A and D) and Late Pleistocene (clade C; Fig. 4), except for clade B from the Guiana area of endemism.

DISCUSSION

Taxonomic implications and cryptic diversification

Molecular data revealed that straight billed species of the genus *Phaethornis* (*P. bourcieri*, *P. philippii* and *P. koepckeae*; formerly grouped in the separate genus *Ametrornis*) form a well-supported clade according to different phylogeny estimates (Figs 1a, 2 & S1). This result was also recovered by the most complete phylogenetic hypothesis for the genus *Phaethornis* available to date (McGuire *et al.* 2014). However, a complete phylogeny for the genus *Phaethornis* is still lacking and a major taxonomic overhaul of this genus will be only possible when such a phylogeny becomes available.

Within the *Ametrornis* clade, all phylogenies obtained recovered a paraphyletic *P. bourcieri* as currently defined (Figs 1a & S1). The paraphyly of *P. b. major* (clade A) with respect to *P. b. bourcieri* (clades B, C and D) is matched by the morphological differentiation between these taxa (Hinkelmann 1989, Piacentini 2011). Clade A birds (*P. b. major*) have much lighter grey bellies than those in clades B, C and D, in addition to bolder gular and malar stripes and reddish rather than yellowish mandibles (Piacentini 2011, L.E. Araújo-Silva and A. Aleixo pers. obs.). Therefore, a heavily marked throat and red mandible are shared by *P. b. major* and *P. philippii* plus *P. koepckeae*, mirroring phylogenetic relationships recovered for these taxa. When interpreted together with the results from our Bayesian species delimitation analysis (Fig. 2) and the estimates of gene flow (Table 2), the combined dataset consistently supports a separate species level rank for *P. b. major* (hereafter *Phaethornis major* Hinkelmann 1989), whose sister group is a clade containing *P. philippii* and *P. koepckeae*.

Similarly, the genetic data support the recognition of three additional species within *P. b. bourcieri*, both according to the Bayesian species delimitation analysis and the obtained estimates of migration rates. These results together support scenarios of significant coalescence and little or no gene flow among clades B, C and D (Fig. 2, Table 2), consistent with separate evolutionary species-level status for each of them.

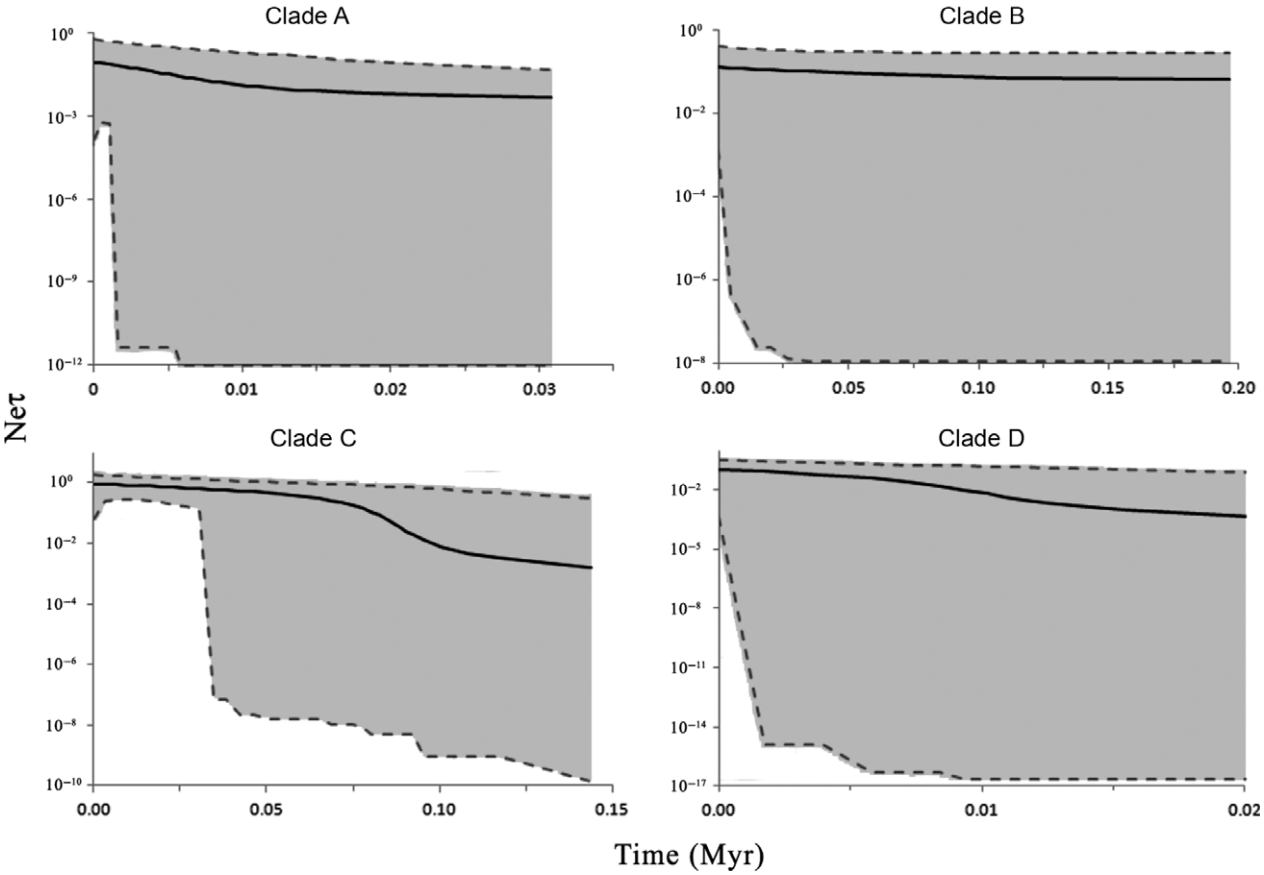

Figure 4. Demographic histories of clades A–D of *Phaethornis bourcieri* inferred through extended Bayesian skyline plots based on mtDNA (cytb and ND2), and BF5 sequences. Black solid lines represent median values and dashed lines correspond to 95% confidence intervals.

However, our data show some ambiguity concerning the position of one *P. b. bourcieri* specimen (AMA 211), which groups alternatively with clades C or D depending on the phylogenetic inference and loci considered (see above; Figs 1a & S1). Therefore, despite the strong support offered by BPP for the recognition of clades C and D as separate evolutionary species, our small sampling across the headwaters of the Amazon River cannot rule out paraphyly between these clades, compromising their recognition as separate evolutionary species. Furthermore, despite the high degree of genetic and even some vocal differentiation among clades B, C and D, no diagnostic morphological features appear to distinguish them (Piacentini 2011). Based on a large series of 441 *P. b. bourcieri* specimens from throughout the ranges of clades B, C and D, Piacentini (2011) detected some variation in plumage, which was nevertheless not structured geographically. This finding contrasted with an earlier study, which concluded that specimens attributed to clade B birds were distinct in plumage from those in clades C and D (Zimmer 1950), as also suggested by vocal characters (Piacentini 2011). From a nomenclature standpoint, the type locality of nominate *bourcieri* (whose type specimen is lost) is known only generically as Brazil, and therefore could refer to any of the clades B, C and D. In contrast, the names *whitelyi* (type locality Mount Roraima in Guyana) and *abnormis* (type locality Marabitanas on the western bank of the Negro River, Brazil) refer unequivocally to clades B and C, respectively, whereas no available name appears to be unequivocally associated with clade D. Given the smaller genetic distances and apparent lack of straightforward morphological diagnoses among clades B, C and D, coupled with the nomenclatural issues associated with the taxon

name *bourcieri*, and the apparent lack of an available name for clade D, we recommend not to formally recognize any of these clades separately as a named species-level taxon until a more thorough multi-character taxonomic review becomes available.

Regardless of the taxonomic and nomenclatural issues surrounding clades B, C and D, the contrasting levels of plumage and genetic differentiation documented for them suggest that they constitute separate cryptic evolutionary species of Pleistocene origin (Fig. 2). The recognition of clades B, C and D as divergent groups under a wide range of ancestral population sizes and times of diversification is probably best explained by the complete sorting of the mitochondrial genes among them, which contrasts with the incomplete sorting verified for the only nuclear marker sequenced (BF5). However, this mito-nuclear discordance is best explained as retained ancestral polymorphism rather than gene flow, as demonstrated by the IMA2 results, which recovered essentially no gene flow among clades B, C and D (Table 2, Fig. 2).

Instances of similar patterns of cryptic diversification have been documented for many suboscine passerine avian lineages also inhabiting the understorey of Amazonian forests and may be related to their poor dispersal abilities and lack of selective pressures for changing phenotypes (Whitney *et al.* 2013, Fernandes *et al.* 2014, Thom & Aleixo 2015). That these same patterns have also been identified in other unrelated non-passerine avian lineages such as *Psophia* (Ribas *et al.* 2012), which are also typical understorey upland *terra firme* dwellers, argues for a common response to major barriers by understorey *terra firme* avian lineages irrespective of their phylogenetic affinities.

Biogeography

Amazonia has been the focus of a large number of studies and debates on the events that have contributed to its vast biological richness (Haffer 1992, 1997, 2001, Räsänen *et al.* 1995, Webb 1995, Bates 2001, Antonelli *et al.* 2010, Ribas *et al.* 2012, Thom & Aleixo 2015). However, there is still no synthetic theory explaining the process of diversification of the rich Amazonian biota as, depending on the individual taxon and its ecological and evolutionary characteristics, patterns of divergence could have been influenced by different events that did not necessarily affect other lineages (Aleixo & Rossetti 2007, Smith *et al.* 2014). As is true for other avian taxa (Ribas *et al.* 2012, Fernandes *et al.* 2014, Thom & Aleixo 2015), Amazonian rivers consistently delimit reciprocally monophyletic populations of *P. bourcieri* lineages (Fig. 1a). Therefore, diversification in *P. bourcieri* seems to have been greatly influenced by dispersal and vicariance events related to the formation of Amazonian rivers, which have apparently imposed serious restrictions on recent migration events between populations located on opposite banks of the upper Amazon (Solimões), Amazon and Tapajós rivers.

In the case of *P. bourcieri* clades, the oldest divergence occurred between the end of the Pliocene and the Pleistocene, separating the *major–philippii–koepckeae* clade (distributed to the south of the Amazon River between the Xingu River and the foothills of the Andes) from clades B, C and D (predominantly distributed to the north of the Amazon River, with only one population (clade D) occurring to the south of this river in the western portion of the Inambari area of endemism). Therefore, this initial event could have been correlated with dispersal and vicariance due to the Solimões River, as the estimated confidence interval of time for this divergence (4.4–3.1 million years ago) overlaps with that estimated for the formation of the Amazon River as a barrier based on the phylogeny of several avian lineages also associated with the *terra firme* forests of the Amazon (Ribas *et al.* 2012, d'Horta *et al.* 2013). However, under the ancestral area reconstruction scenario favoured by BIOGEOBEARS (Fig. 3), clade D would have crossed the upper Amazon much later and colonized the Inambari area of endemism after the formation of the Amazon River, possibly across its headwaters. This appears to be further supported by two lines of evidence: (1) clade D, along with clade C, is derived and has diversified more recently among all lineages of *Phaethornis* with straight beaks (Fig. 1b; see below); and (2) the distribution of *P. bourcieri* in the Inambari area of endemism is concentrated in its western portion, near the upper Amazon and its tributaries (Fig. 1), where it overlaps with the distribution of *P. philippii* (Fig. 1b). This is a closely related and more widely distributed lineage inhabiting the same area of endemism, implying a secondary contact in this particular sector of the Amazon.

Between 2.9 and 1.25 million years ago (Plio-Pleistocene), a divergence occurred between clade

A (*major*) and the clade formed by *P. philippii* and *P. koepckeae*, separated by the Tapajós River. This estimate is similar to those inferred for the separation between *Xiphorhynchus spixii* and *Xiphorhynchus elegans* (Aleixo 2004) and *Thamnophilus aethiops* western and eastern lineages (2.70–1.86 million years ago; Thom & Aleixo 2015), despite the dating method of the former study having not been based on a Bayesian or coalescent timetable, therefore limiting this comparison. Therefore, lineages such as *P. bourcieri*, *X. spixii/elegans* and *T. aethiops* together provide support for a lower bound timing estimate (average dates around 2–3 million years ago) for the emergence of the Tapajós River as a major biogeographical barrier in Amazonia (Aleixo 2004, Thom & Aleixo 2015).

Between 2.75 and 1.1 million years ago (Plio-Pleistocene), clade B split from the ancestors of clades C and D, apparently as a response to the Branco River or associated landscape changes in the Negro–Branco interfluve (Naka *et al.* 2012). Our BioGeoBears results suggest that this diversification occurred through a dispersal event from northwestern Amazonian (composite Imeri, Jaú and Napo areas of endemism) into the Guiana area of endemism (Fig. 3). Both sides of the upper Negro River (São Gabriel da Cachoeira area) are occupied by a single *P. bourcieri* lineage (clade C), a pattern consistent with that documented for 31 other *terra firme* forest avian taxa whereby the upper Negro river does not appear to be an important barrier to gene flow, in sharp contrast to the Branco River and wide savannah areas in the Branco–Negro interfluve (Naka *et al.* 2012).

As already discussed above, the split between clades C and D across the upper Amazon was probably preceded by a dispersal event (Fig. 3) and became complete between 0.6 and 0.2 million years ago. Other studies recovered divergences across the upper Amazon and some of its tributaries (e.g. the Marañon) dating back between 1.8 and 4 million years ago (*Myrmeciza hemimelaena* complex; Fernandes *et al.* 2012), 0.8–2.0 million years ago (*Lepidothrix coronata*; Cheviron *et al.* 2005) and 0.29–1.4 million years ago (*T. aethiops*; Thom & Aleixo 2015). Therefore, overlap between the estimates of the last two groups and those obtained for the separation between clades C and D may indicate responses to the same event. A demographic analysis revealed that populations of *L. coronata* to the south of the upper Amazon (with distribution analogous to that of clade D) showed signs of expansion, while those to the north of the river (with distribution analogous to clade C) were in equilibrium (Cheviron *et al.* 2005). In contrast, we found evidence for relatively recent population expansion events in both clades C and D (Fig. 4). Therefore, an alternative possibility is that the separation between *P. bourcieri* clades C and D is not necessarily correlated with the river formation, but instead with the acquisition of reciprocal monophyly due to isolation by distance among populations distributed around the headwaters of the Amazon, Marañon and Ucayali rivers. As is true of other avian lineages throughout Amazonia, the pattern of diversification documented herein for the straight-billed *Phaethornis* assemblage confirms the important role of Amazonian rivers as major promoters of speciation, at the same time that it highlights the importance of dispersal as a concurrent event that can potentially explain widely disparate splitting times across Amazonian rivers and other major biogeographical barriers such as the Andes (Smith *et al.* 2014).

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SUPPORTING INFORMATION

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

Figure S1. Phylogenetic hypothesis obtained through BI based only on mitochondrial ND2 sequences obtained for all *Phaethornis* specimens sampled in this study (see Appendices S1 and S2 for sample identification and origin). Numbers on the top of branches denote BI posterior probabilities.

Figure S2. Phylogenetic hypothesis obtained through BI based only on mitochondrial ND2 and cytb sequences obtained for all *Phaethornis* specimens sampled for both genes in this study (see Appendices S1 and S2 for sample identification and origin). Numbers on the top of branches denote BI posterior probabilities.

Figure S3. Phylogenetic hypothesis obtained through BI based only on nuclear BF5 sequences obtained for all *Phaethornis* specimens sampled for this gene in this study (see Appendices S1 and S2 for sample identification and origin). Numbers on the top of branches denote BI posterior probabilities.

Figure S4. Biogeographical history of the straight-billed hermit assemblage (*P. bourcieri*, *P. koepckeae* and *P. philippii*) favoured by BioGeoBEARS under the DIVALIKE+J model (see Table S2). Top left: areas of endemism occupied by the different lineages (adapted from Silva *et al.* 2005 and Borges and Silva 2012); bottom left: different colours represent recognized ancestral areas, as follows: (AF = Andes Foothills; GS = Guiana; IN = Inambari; IM = Imeri; JA = Jaú; NP = Napo; RO = Rondônia; TA = Tapajós; and combined areas). Numbers on the time scale below represent millions of years. Pie charts denote probabilities of the different ancestral areas associated with each node.

Table S1. Primers used to amplify genes sequenced in this study.

Table S2. Models and number of parameters of ancestral area reconstructions carried out in BioGeoBEARS based on the *P. bourcieri* chronogram. Values represent dispersal (d), extinction (e), founder event (J) and Log-Likelihood (ln *L*) probability values as well as Akaike Information Criterion (AIC), ΔAIC and Akaike weight (ωi) scores.

Appendix S1. Specimens of *Phaethornis* sequenced in this study for the cytb, ND2 and BF5 genes.

Appendix S2. Sequences of ND2 of *Phaethornis* spp. from GenBank used in this study.