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Diversification and species limits in scale-backed antbirds (*Willisornis*: Thamnophilidae), an Amazonian endemic lineage

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The genus *Willisornis* is endemic to the Amazon Basin, inhabiting upland terra firme forest, with two species and seven subspecies currently recognized. Despite numerous systematic studies, a taxonomically-dense sampled phylogeny for *Willisornis* is still lacking, which, combined with evidence of paraphyly and gene flow between its recognized species, underscores the uncertainty concerning species limits and evolutionary history of the genus. Here we present phylogenies and population genetic analyses, including all currently recognized *Willisornis* taxa, relating them to patterns of plumage variation, and reconstructing the spatiotemporal context of diversification in the genus. Our analyses have uncovered 13 independent genetic lineages in the genus, and the monophyly of all currently named taxa, which also showed robust plumage diagnoses. However, deeply coalesced genetic lineages were also found within most *Willisornis* taxa, for which no consistent variation in plumage was found. The diversification of the genus *Willisornis* is related to hydrographic and climate change cycles across Amazonia since the Plio-Pleistocene, with most genetic lineages originating in the past one million years. Based on our findings, we recommend the recognition of a total of six species in *Willisornis* (one of which polytypic) based on the congruency between deeply coalesced lineages and consistent plumage diagnoses.

ADDITIONAL KEYWORDS: Aves – biogeography – birds – cryptic diversity – drainage evolution – landscape changes – phylogeography – Pleistocene – systematics – taxonomy.

INTRODUCTION

The Amazon Basin is home to a great richness of species, yet the historical factors that contributed to this concentrated biodiversity are still little known. The quest for this knowledge has made the Amazon region the target of a large number of phylogenetic and phylogeographic studies aimed at understanding how this unique biodiversity evolved and accumulated through time (Ribas *et al.*, 2012; Smith *et al.*, 2014; Antonelli *et al.*, 2018; Ribas & Aleixo, 2019; Silva *et al.*, 2019).

Over the years, it has been verified that the major Amazonian rivers form barriers and separate species that, in most cases, are closely related (Bates, 1863). As such, the riverine barrier hypothesis is the oldest and one of the main ones trying to explain the great

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diversification in the Amazon region. It postulates that the appearance of main rivers separated populations on opposite banks, acting as vicariant barriers enhancing genetic divergences (Gascon *et al.* 2000; Haffer, 2001). Indeed, the progressive study of the Amazonian biota has confirmed that many species and subspecies are restricted to certain interfluvial areas, supporting a decisive role for major rivers as barriers separating closely related, yet genetically differentiated, taxa on their opposite banks (Aleixo, 2004; Bates *et al.*, 2004; Ribas *et al.*, 2012).

However, Haffer (1997) observed that many bird taxa restricted to some interfluves still maintained contact in specific regions of the headwaters of major Amazonian rivers, ceasing to be effective barriers for certain groups. Based on these findings, several contact zones within Amazonian interfluvia were proposed by Haffer (1997) based on the distribution of morphologically distinct avian lineages. Subsequently, more recent studies have demonstrated the existence of gene flow in the headwaters of large Amazonian rivers for pairs of genetically divergent taxa (Fernandes *et al.*, 2013; Thom & Aleixo, 2015; Weir *et al.*, 2015; Pulido-Santacruz *et al.*, 2018), corroborating that the narrowing of rivers in the headwaters can reduce their barrier effects.

The genus *Willisornis* Agne & Pacheco, 2007 (Aves: Thamnophilidae) is endemic to the Amazon Basin and relatively common in upland terra firmae forest, with several biogeographic studies in the region using it as a model group (Bates, 2000; Bates *et al.*, 2004; Fernandes *et al.*, 2014). The taxa belonging to *Willisornis* were initially grouped in a single polytypic species: *Willisornis poecilinotus* (Cabanis, 1847), including seven subspecies: *W. p. duidae* (Chapman, 1923), *W. p. griseiventris* (Von Pelzeln, 1869), *W. p. gutturalis* (Todd, 1927), *W. p. lepidonota* (Sclater & Salvin, 1880), *W. p. nigrigula* Snethlage, 1914, *W. p. poecilinotus* and *W. p. vidua* (Hellmayr, 1905). These subspecies are diagnosed mainly based on differences in female plumage characters, while males are either not distinguishable or barely distinguishable amongst subspecies (Hellmayr, 1929; Peters, 1951). The well-marked plumage differences, mainly in females, caused this type of variation to be classified as heterogynism (Hellmayr, 1929). Subsequently, Isler & Whitney (2011) carried out analyses involving vocal characters and compared their results with plumage variation. They found vocal differences among all subspecies, with *W. p. vidua* and *W. p. nigrigula* being the subspecies with the greatest degree of vocal differentiation. Voices of these two subspecies were similar to each other, but differed significantly from those of all remaining subspecies grouped under *W. poecilinotus*. This finding prompted them to suggest the treatment of *Willisornis vidua* (Hellmayr, 1905) as an independent species, with two subspecies (*W. v. vidua* and *W. v. nigrigula*). However, Isler & Whitney (2011) recognized that they had large gaps in the geographic sampling of their study, particularly in the contact zones between some neighbouring taxa, which made it difficult to evaluate the consistency in character diagnosis between them. Thus, after that review, the genus *Willisornis* included two recognized species (Del Hoyo *et al.* 2020; Gill *et al.* 2022; Remsen *et al.* 2022): *Willisornis poecilinotus* (including five subspecies: *W. p. poecilinotus*, *W. p. griseiventris*, *W. p. lepidonota*, *W. p. gutturalis* and *W. p. duidae*) and *Willisornis vidua* (including two subspecies: *W. v. vidua* and *W. v. nigrigula*). More recently, Isler *et al.* (2014) have proposed the reassessment of the generic limits in the army-ant-following clade Thamnophilidae, obtaining for the first time a phylogenetic hypothesis for the genus *Willisornis;* although not including most taxa, it demonstrated that the evolutionary history of *W. poecilinotus* and *W. vidua* remains largely unresolved.

The need for more in-depth research involving this genus has also been reinforced by recent studies that have demonstrated the existence of hybrids between *W. p. griseiventris* and *W. v. nigrigula* in the Teles Pires River region (Weir *et al.*, 2015), and also that the frequency of hybridization between these taxa is small, localized and maintained mainly by post-zygotic barriers to gene flow (Pulido-Santacruz *et al.*, 2018). Furthermore, Silva *et al.* (2019) recovered *W. poecilinotus* as a paraphyletic species with respect to *W. vidua*, highlighting the need of a revision on the species limits in the genus.

The absence of a taxonomically-dense sampled phylogeny for *Willisornis*, combined with evidence of paraphyly and gene flow between recognized species-level taxa (Isler *et al.*, 2014; Pulido-Santacruz *et al.*, 2018; Silva *et al.*, 2019), underscores the uncertainty concerning the limits between currently recognized species and subspecies. Because the genus *Willisornis* is widely distributed and endemic to the Amazon region, it is also an excellent model for reconstructing the diversification of the local biota. To fill the gaps in our knowledge we here aim to: (1) establish the phylogenetic relationships amongst *Willisornis* taxa, relating them to known patterns of plumage variation in the genus; (2) infer a spatiotemporal scenario of lineage diversification in the genus; and (3) evaluate interspecific limits and extent of gene flow between the different evolutionary lineages recovered.

MATERIAL AND METHODS

Taxon sampling and laboratory procedures

We studied 1160 specimens of which 182 were included in the molecular and 1108 in the plumage analyses

(Supporting Information, Table S1). A total of 130 specimens were evaluated for both DNA and plumage characters (Supporting Information, Table S1). Of the sequences used, 22 were downloaded from a previous study (Silva *et al.*, 2019; see Supporting Information, Table S1). The sampling covers most of the geographic range of the complex, including all seven recognized subspecies (Gill *et al.* 2022). As outgroups, we used samples of the closely related genera *Rhegmatorhina* Ridgway, 1888 and *Gymnopithys* Bonaparte, 1857 (Brumfield *et al.*, 2007; Harvey *et al.*, 2020).

Total DNA was extracted following the standard phenol-chloroform protocol (Sambrook *et al.*, 1989) or with the Wizard Genomic DNA Purification Kit. Four genes were amplified through polymerase chain reaction (PCR), two mitochondrial: cytochrome *b* (*Cytb*) and NADH dehydrogenase subunit 2 (*ND2*); and two nuclear: β-fibrinogen intron 5 (*BF5*) and muscle-specific receptor tyrosine kinase intron 4 (*MUSK*); primers listed in the Supporting Information, Table S2. These genes are frequently used in phylogeographic studies involving birds due to their highly informative phylogenetic content and availability of primers (Thom & Aleixo, 2015; Araújo-Silva *et al.*, 2017; Silva *et al.*, 2019). PCR procedures were as follows: (1) an initial step for denaturation lasting 5 min at 95 °C; (2) 35 cycles of 1 min at 95 °C; (3) 1 min at the optimum annealing temperature for each set of primers used (see Supporting Information, Table S2); (4) 1 min at 72 °C; and (5) final extension at 72 °C for 10 min. The amplifications were checked with electrophoresis using 1% agarose gel and purified following the polyethylene glycol protocol (PEG8000). Sequences were obtained using the Big Dye Terminator Cycle Sequencing Standard v.3.1 Kit on an ABI 3130 sequencer (Applied Biosystems).

PHYLOGENETIC ANALYSES

Nucleotide sequences were edited with GENEIOUS v.9.0.5 (Kearse *et al.*, 2012) software and aligned in MAFFT v.7.4.0.8 (Katoh & Standley, 2013). For the reconstruction of nuclear gene haplotypes we used the PHASE v.2.1 software, accepting results of probability > 70% (Stephens *et al.*, 2001). To assess saturation levels, transitions and transversions were plotted against genetic distances for both mitochondrial markers with the aid of the Data Analysis and Molecular Biology and Evolution – DAMBE v.6.3.109 programme (Xia & Xie, 2001).

The best-fit evolutionary models and partitions were obtained with the software PartitionFinder v.1.1.1 (Lanfear *et al.*, 2012). The best partition schemes were selected using the Bayesian information criterion (BIC). Four Bayesian phylogenies were estimated using MrBayes v.3.1.2 software (Huelsenbeck & Ronquist, 2001): one based on the mitochondrial dataset only; a second based on the entire concatenated multilocus dataset, including all four sequenced genes; and third and fourth based each, respectively, on *BF5* and *MUSK*. All searches performed involving *BF5* and *MUSK* employed unphased sequences. Searches for the best trees were carried out with two independent runs and four Markov chains Monte Carlo (MCMC) along 10 000 000 generations, and sampling trees and parameters every 500 generations. The programme TRACER v.1.4 was used to evaluate whether runs reached convergence in the estimated parameters and to verify if the effective sample size (ESS) values were greater than 200, as recommended by Drummond & Rambault (2007). The trees sampled before the Markov chains converged and reached stability were discarded as burn-in (first 500 generations).

SPECIES TREE AND SPECIES DELIMITATION

A species tree was generated in the programme BEAST v.1.8.0 (Drummond *et al.*, 2012) using major reciprocally monophyletic groups recovered in the previous Bayesian analyses as species priors. The evolutionary models were the same as those used for Bayesian inference (BI). Gene trees for the two mitochondrial markers were linked and ploidies were defined separately for each marker: mitochondrial markers were set with a ploidy of 0.5 (*Cytb* and *ND2*), while the autosomal marker (*BF5*) was set to 2.0 and the sex-linked gene (*MUSK*) set to 1.5. We used the substitution rates of the mitochondrial genes: *Cytb* at 0.0105, SD = 0.0034 (Weir & Schluter, 2008) and *ND2* at 0.0123, SD = 0.45 (Smith & Klicka, 2013), estimating those of the nuclear genes. We used an uncorrelated log-normal relaxed clock and changed the priors species.popMean and species.Yule.birthRate for log-normal with Log (mean) = 4.0 and Log (SD) = 2.0, respectively. We ran the analysis for 500 000 000 generations, sampling parameters every 1000 generations. For this analysis, we removed the known hybrid individuals sampled by us, and recovered as such by previously published genomic datasets (Weir *et al.*, 2015; Pulido-Santacruz *et al.*, 2018), to avoid any noise caused by them in coalescent inferences. In addition to these, an apparent hybrid individual inferred based on conflicting affinities derived from alternative molecular and morphological characters sets, was also removed (see Results). We used TreeAnnotator v.1.8.0 software (Drummond *et al.* 2012) to obtain the best tree, discarding the first 25% as burn-in.

To test for interspecific limits among the 13 reciprocally monophyletic groups recovered in the genus *Willisornis* by the Bayesian concatenated analyses, we carried out an unguided species delimitation analysis (Yang & Rannala, 2014) using the Bayesian Phylogenetics and Phylogeography (BP&P) software 3.2 (Yang, 2015). These 13 groups were also geographically structured and, therefore, were assumed as a priori basic evolutionary units. Due to overall poor statistical support for the internal nodes of the recovered multilocus trees, we chose an unrooted tree (i.e. option 0) as our 'speciesmodelprior' (Yang, 2015). We implemented Yang's (2015) approach, performing analyses using four parameter combinations representing different population sizes and divergence times: (1) small ancestral population sizes and ancient divergence times [θ and τ gamma priors G (2, 2000) and G (1,10)]; (2) large ancestral population sizes and ancient divergence times [θ and τ gamma priors G (1, 10) and G (1,10)]; (3) small ancestral population sizes and recent divergence times [θ and τ gamma priors G (2, 2000) and G (2, 2000)]; and (4) large ancestral population sizes and recent divergence times [θ and τ gamma priors G (1, 10) and G (2, 2000)]. We chose the reversible-jump Markov chain Monte Carlo method (Yang, 2015), with variables 0 and $\varepsilon = 2$, for 500 000 generations (sampling interval of five) and a burn-in of 10%. As in the previous analysis, the known hybrids were also removed. For each scenario, we ran analyses for 1×10^5 generations by sampling every five generations and discarding the first 20 000 as burn-in.

POPULATION GENETICS ANALYSES

To map the distribution of haplotypes in populations, haplotype networks for each gene were inferred separately using HaploViewer (Salzburger *et al.*, 2011), with populations defined a priori as corresponding to each of the 13 reciprocally monophyletic lineages recovered in the genus *Willisornis* by the Bayesian concatenated analyses. Uncorrected pairwise genetic distances (*p*-distance) were calculated within and between populations, using only the mtDNA database in the program MEGA v.10.1 (Kumar *et al.*, 2016). To verify the dynamics of effective population sizes over time, we estimated extended Bayesian skyline plots (EBSP) (Heled & Drummond, 2008) as implemented in BEAST 1.8.0 (Drummond *et al.*, 2012) for the different *Willisornis* lineages, except *W. p. poecilinotus* B and *W. p. duidae* C owing to the low number of samples. For this analysis, we used the substitution rates of *Cytb* at 0.0105, SD = 0.0034 (Weir & Schluter, 2008) and *ND2* at 0.0123, SD = 0.45 (Smith & Klicka, 2013). Ploidies of the different markers were set as in the coalescent species tree analysis. The substitution rates of the nuclear genes were estimated, and we used a strict clock and uncorrelated log-normal priors.

We used software BAPS v.6.0 (Corander *et al.*, 2008) to evaluate the level of population structure across *Willisornis* lineages. We performed separate analyses for each phased nuclear marker, and another analysis joining both sequenced mitochondrial markers. Only specimens sequenced for all markers were included in BAPS analyses. First, a genetic mixture analysis was performed to identify the best K value for the entire genus *Willisornis*. Tested K values varied from 1 to 16, which represents the maximum number of expected *Willisornis* groups based on previously described phylogenetic analyses plus 3. For each K value tested, ten independent runs were carried out. Mixture analyses were performed using 100 interactions, a minimum of three individuals per population, a reference number for each population as 200 and ten iterations of reference individuals. After finding the best K value, we performed admixture analyses. We also evaluated with BAPS the genetic structure across lineages *W. p. gutturalis* and *W. p. griseiventris* A, which are in direct contact within the Juruá–Amazon interfluve (Isler & Whitney, 2011) and for which our plumage analyses indicated at least one likely hybrid specimen coming from the contact zone between these lineages (see Results). For this, the genetic mixture analysis was performed only with K values ranging from 1 to 2, which were run independently 20 times. We used 100 iterations, a minimum of five individuals per population, a reference number of 200 and ten iterations of reference individuals. After the genetic mixture analysis, an admixture analysis was also performed.

BIOGEOGRAPHIC ANALYSES

We inferred ancestral areas of diversification in *Willisornis* with the BioGeoBEARS (Matzke, 2014) package implemented in R. The ancestral areas were coded considering the three major geological provinces of the Amazon (Silva *et al.* 2019): Guiana Shield (GS), Amazonian Foreland Basins (SB) and Brazilian Shield (BS). The analysis considered the following three different models of range evolution: Dispersal-Extinction Cladogenesis (DEC), Dispersal-Vicariance Analysis (DIVALIKE) and Bayesian Inference of Historical Biogeography for discrete areas (BAYAREALIKE). We did not consider the 'J' parameter for any model due to recent criticisms and, particularly, because *Willisornis* is a continental rather than insular radiation (Ree & Sanmartín, 2018). Model selection was performed by choosing the best LogLikelihood (ln L) and AIC values obtained directly from the software.

PLUMAGE ANALYSES

A qualitative plumage analysis was carried out for the lineages recovered in the molecular analyses with the goal of assigning them to different taxa described for the genus *Willisornis* (Isler & Whitney, 2011). To this end, we analysed a total of 1108 study skins of the following taxa: *W. p. poecilinotus* (*N* = 129), *W. p. duidae* (*N* = 65), *W. p. gutturalis* (*N* = 23), *W. p. lepidonota* (*N* = 1), *W. p. griseiventris* (*N* = 264), *W. v. nigrigula* (*N* = 239) and *W. v. vidua* (*N* = 387) (see Supporting Information, Table S1; Fig. S1). Each analysed specimen was identified to sex, adult or young life-stages and subspecies, and subsequently checked for plumage variation in comparison to other analysed specimens grouping in the same genetic clade or cluster recovered by the molecular analyses. Subspecies identification was accomplished by examination of diagnostic features related to key points of differentiation in *Willisornis* (Cory & Hellmayr, 1925; Isler & Whitney, 2011; Del Hoyo *et al.*, 2020; Zimmer *et al.*, 2020), as follows: (1) dorsal and ventral colours; (2) presence/absence and colour of the back 'lace' (i.e. the joint white-tipping effect found on the wings, back and tail of *Willisornis* populations, which is always present in males, but not always in females); (3) presence/absence and colour of the terminal tail marks; (4) throat colour; and (5) colour of the sides of the head.

RESULTS

PHYLOGENETIC RELATIONSHIPS

We obtained a total of 3096 base pairs (bp) for 182 *Willisornis* specimens of the following genes: *Cytb* (926 bp), *ND2* (999 bp), *BF5* (574 bp) and *MUSK* (597 bp). The best models of molecular evolution fitting each of these datasets were: HKY+G (*Cytb* and *ND2*) and GTR+I+G (*BF5* and *MUSK*). No base saturation was observed for the mitochondrial genes. All sequences obtained were deposited in the GenBank (Supporting Information, Table S1).

We generated two trees using BI, respectively based on the mtDNA and multilocus concatenated datasets (Fig. 1), which presented some divergent relationships among the lineages recovered. The main difference referred to the basal-most phylogenetic relationships. The mtDNA tree recovered the paraphyly of *W. poecilinotus* with respect to *W. vidua*, while the multilocus tree supported the reciprocal monophyly between these two species currently recognized for the genus *Willisornis*. Another difference pertained to the number of reciprocally monophyletic groups in *W. vidua*, which varied from three (concatenated multilocus phylogeny) to four (mtDNA phylogeny), with populations of the Xingu and Belém areas of endemism grouping in separate clades in the mtDNA phylogeny, rather than in the same clade, as recovered by the concatenated multilocus phylogeny (Fig. 1).

In both phylogenies, the lineages recovered for *W. poecilinotus* (distributed in northern, western and central Amazonia) and *W. vidua* (distributed in south-eastern Amazonia) replace each other across the lower Madeira and upper Tapajós rivers, but are in direct contact with each other in the southern part of the Madeira–Tapajós and Tapajós–Xingu interfluves (Fig. 1). Most of the recognized subspecies within each *W. poecilinotus* and *W. vidua* were recovered as reciprocally monophyletic with high statistical support, with some of them subdivided into geographically structured subclades (Fig. 1). One exception was *W. v. nigrigula*, which was recovered as paraphyletic, with the subclade found in the Tapajós–Xingu interfluve grouping as sister to *W. v. vidua* rather than the *W. v. nigrigula* subclade found west of the Tapajós River (Fig. 1). The nDNA trees (Supporting Information, Fig. S2) were less resolved and lacked strong statistical support for most nodes, which together render them less informative than those including the mtDNA sequences.

The coalescent species tree (Fig. 2) recovered two main clades in *Willisornis*, resembling the BI tree based on mtDNA data, where *W. p. duidae* A/B/C, *W. p. lepidonota* A/B and *W. p. griseiventris* A/B form the sister-group of *W. vidua*, thus recovering the paraphyly of *W. poecilinotus* again. The major difference between the species tree and both BI phylogenies referred to samples of subspecies *W. v. nigrigula*, which were recovered as monophyletic by the coalescent analysis rather than paraphyletic as in the concatenated trees (Figs 1, 2).

According to the species tree, the first division within the *Willisornis* complex occurred in the Pliocene at *c.* 3.63 Mya (2.76–4.48; Fig. 2). The origin of the *W. p. poecilinotus* A/B and *W. p. gutturalis* lineages date from the Plio-Pleistocene, about 3 Mya (Fig. 2). However, it was during the Pleistocene (~ 2 Mya) that most of the diversification in the genus occurred, such as the separation of the clade containing all lineages of *W. vidua* found in the south-eastern part of the Amazon from the other lineages of *W. poecilinotus* found in western Amazonia (Fig. 2). The most recent divergence recovered (~ 200 000 years) was between the clades *W. v. nigrigula* A and *W. v. nigrigula* B currently separated the lower Tapajós River (Fig. 2). The recovered species tree contained several nodes with low posterior probability values, particularly those pertaining to the phylogenetic position of *W. p. gutturalis* and the basal-most relationships in the *W. p. duidae*, *W. p. lepidonota* and *W. p. griseiventris* clade (Fig. 2). It is important to emphasize that our data are based on coding genes and that the possibility of target selection would violate assumptions based on neutrality, which could result in more recent ages for the splitting nodes.

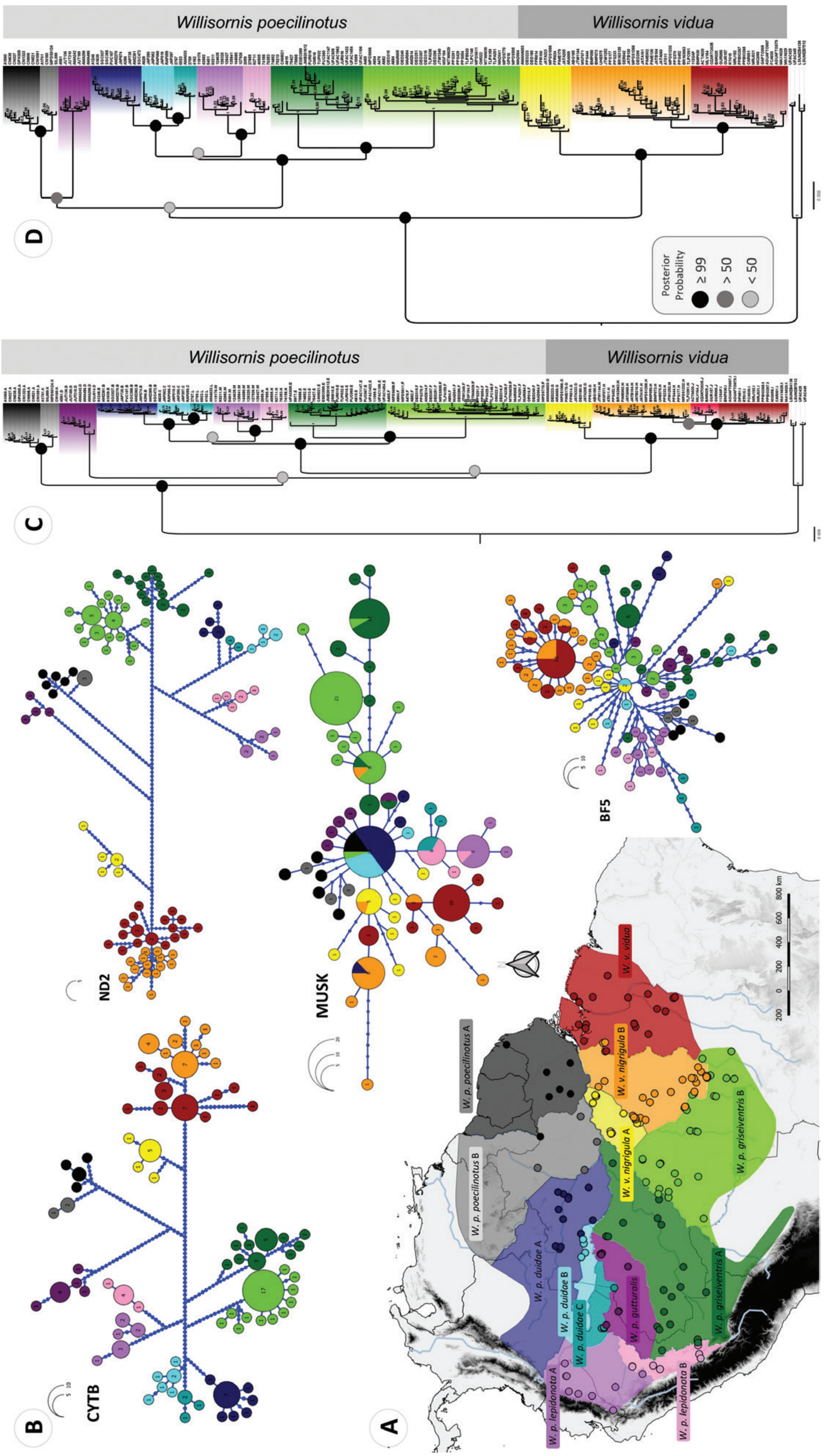

Figure 1. A, map showing the location of sequenced specimens and the distribution of recovered lineages (colour coded). B, haplotype networks for the four genetic markers (*Cytb*, *ND2*, *MUSK* and *BF5*) sequenced. Phylogenetic hypotheses obtained through concatenated Bayesian inferences (BI) based on the mitochondrial (C) and multilocus (D) datasets.

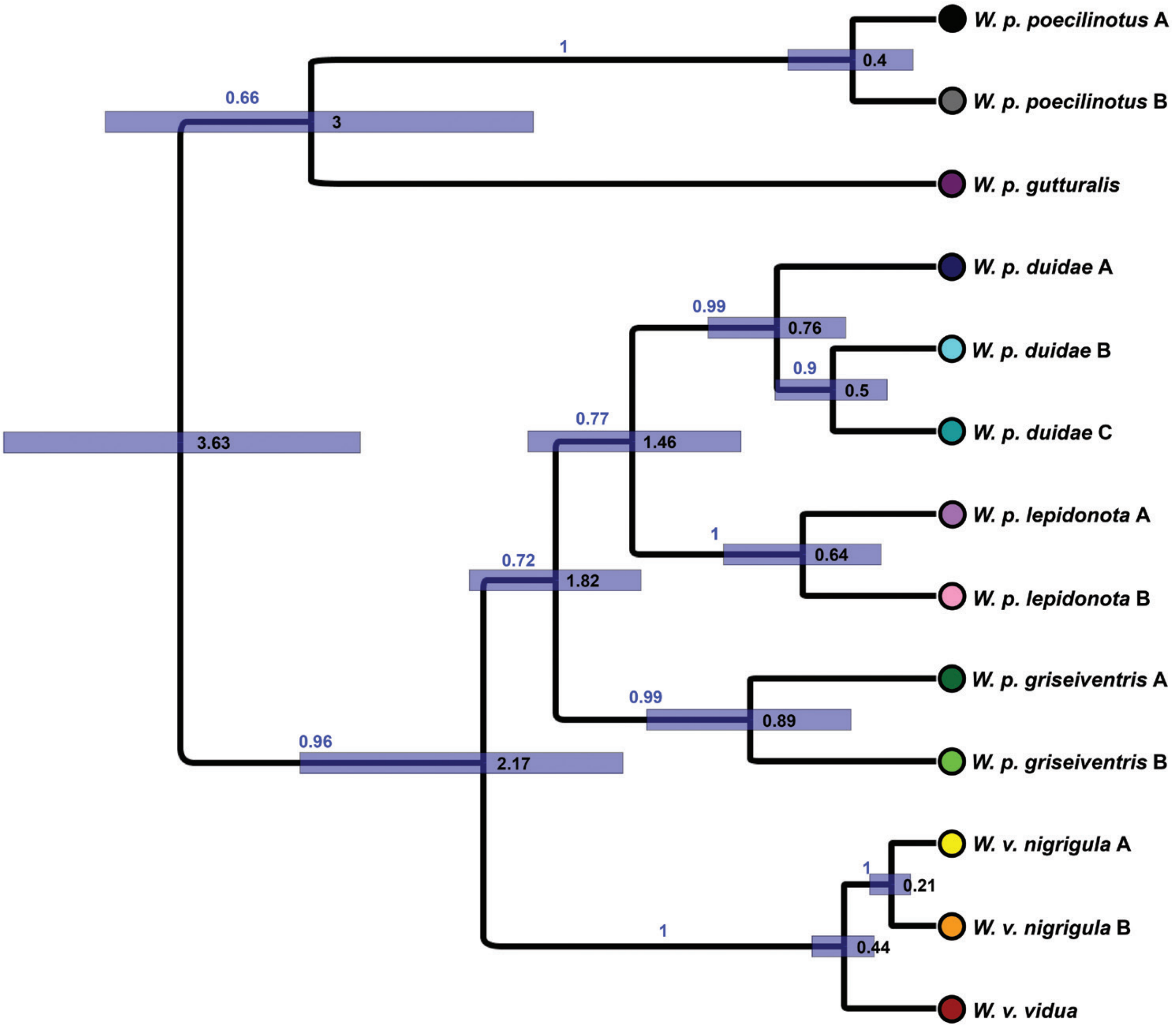

Figure 2. Multilocus coalescent species tree of *Willisornis* lineages identified in previous molecular analyses (Fig. 1). Nodes contain posterior probabilities of clades (above line) and associated confidence intervals (95% HPD) for splitting times (blue bars). Numbers on the timescale below represent millions of years. The colours are the same as used for the recovered groups in Figure 1.

SPECIES DELIMITATION

Unguided species delimitation tests performed by BP&P, regardless of the demographic and divergence time model considered, supported all 13 main reciprocally monophyletic lineages recovered in *Willisornis* by the Bayesian analyses as hypothetical species (Fig. 3).

POPULATION GENETICS

Haplotype networks (Fig. 1) for mtDNA markers did not reveal shared haplotypes amongst the 13 distinct lineages identified in the phylogenetic analyses, underscoring the high level of geographic structure across *Willisornis* lineages. Pairwise mitochondrial uncorrected *p*-distances between clades of *Willisornis* ranged from 0.74% (*W. p. nigrigula* B × *W. p. vidua* A) to 7.04% (*W. p. griseiventris* B × *W. p. gutturalis*), with divergences within clades ranging from 0.05% to 0.56% (Supporting Information, Table S3).

In contrast, haplotype networks for the nuclear markers showed a much shallower degree of differentiation, with some alleles shared amongst lineages, but overall coarse phylogeographic structure still present. In both nuclear markers sequenced, alleles grouped together into three main clusters representing mostly lineages from northern, south-western and south-eastern Amazonia (Fig. 1).

A

Lineage	BP&P – Posterior Probability			
	Scenario 1 Θ = G(2,2000) τ = G(1,10)	Scenario 2 Θ = G(1,10) τ = G(1,10)	Scenario 3 Θ = G(2,2000) τ = G(2,2000)	Scenario 4 Θ = G(1,10) τ = G(2,2000)
W. p. poecilinotus A	1.00000	0.98210	1.00000	0.99380
W. p. poecilinotus B	1.00000	0.98210	1.00000	0.99380
W. p. duidae A	1.00000	1.00000	1.00000	1.00000
W. p. duidae B	1.00000	1.00000	1.00000	1.00000
W. p. duidae C	1.00000	1.00000	1.00000	1.00000
W. p. lepidonota A	1.00000	1.00000	1.00000	1.00000
W. p. lepidonota B	1.00000	1.00000	1.00000	1.00000
W. p. gutturalis	1.00000	1.00000	1.00000	1.00000
W. p. griseiventris A	1.00000	1.00000	1.00000	1.00000
W. p. griseiventris B	1.00000	1.00000	1.00000	1.00000
W. v. nigrigula A	1.00000	1.00000	1.00000	1.00000
W. v. nigrigula B	1.00000	1.00000	1.00000	1.00000
W. v. vidua	1.00000	1.00000	1.00000	1.00000

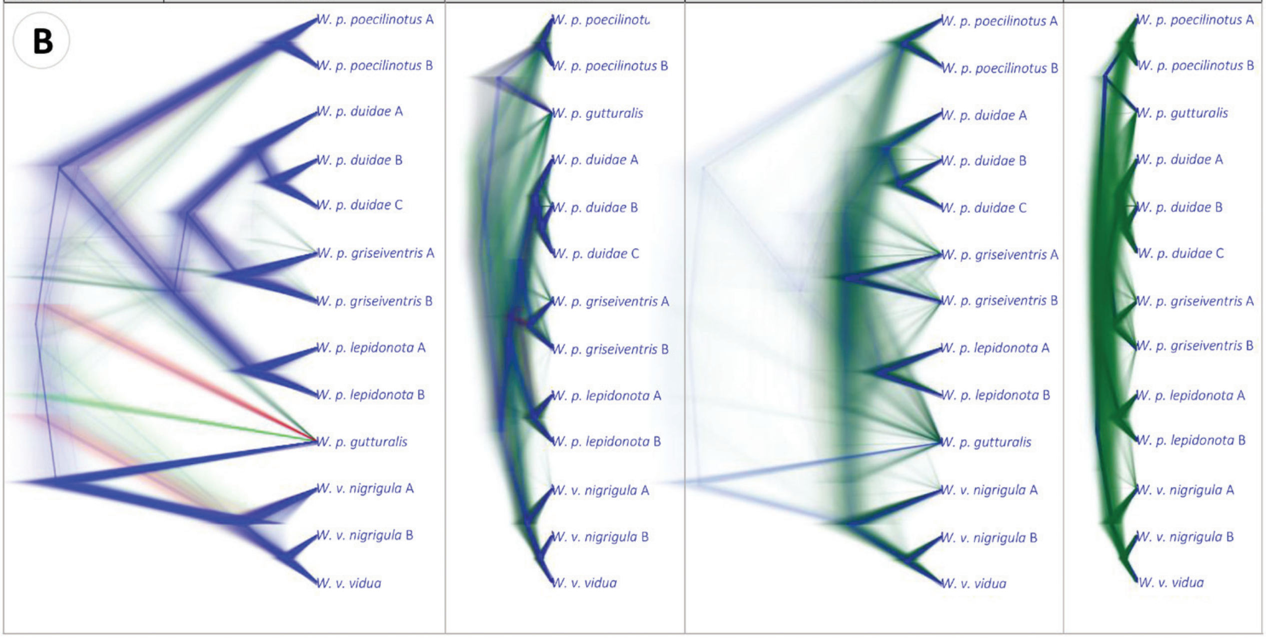

Figure 3. A, BP&P posterior probabilities for different prior settings corresponding to the degree of significant coalescence between reciprocally monophyletic *Willisornis* lineages. Θ corresponds to the population size parameter and τ to the divergence time priors at the root of the species tree. ε, α and m are different values of fine-tune parameters. B, overlapping coalescent trees for each scenario generated by BP&P.

Our multilocus historical demography analyses recovered patterns consistent with population expansion for *W. v. vidua*, *W. v. nigrigula* B, *W. p. poecilinotus* A, *W. p. gutturalis*, *W. p. duidae* A and *W. p. griseiventris* A/B at the end of the Pleistocene and beginning of the Holocene (Supporting Information, Fig. S3). However, for the other lineages (*W. p. duidae* B/C, *W. p. lepidonota* A/B and *W. v. nigrigula* A), our results support a pattern of overall demographic stability (Supporting Information, Fig. S3).

Our BAPS analyses, based on all 117 specimens sequenced for the mtDNA markers, recovered an optimal value of K = 8, consistent for the most part with currently recognized species/subspecies limits in *Willisornis* (Fig. 4). Important exceptions to this rule were as follows: (1) north-eastern Peruvian populations traditionally classified as *W. p. lepidonota* clustered with other populations attributed to *W. p. duidae*; (2) populations currently classified as *W. p. griseiventris* clustered in two independent groups, separated by the Madeira River; and (3) the easternmost population of *W. v. nigrigula* (*W. v. nigrigula* B) clustered with populations attributed to *W. v. vidua*, rather than with the remaining populations of *W. v. nigrigula* distributed west of the Tapajós River (*W. v. nigrigula* A; Fig. 4). BAPS analyses carried out for the nuclear markers revealed smaller numbers of optimum clusters ranging from K = 3 (*BF5*) to K = 6 (*MUSK*; Fig. 4). Indices of

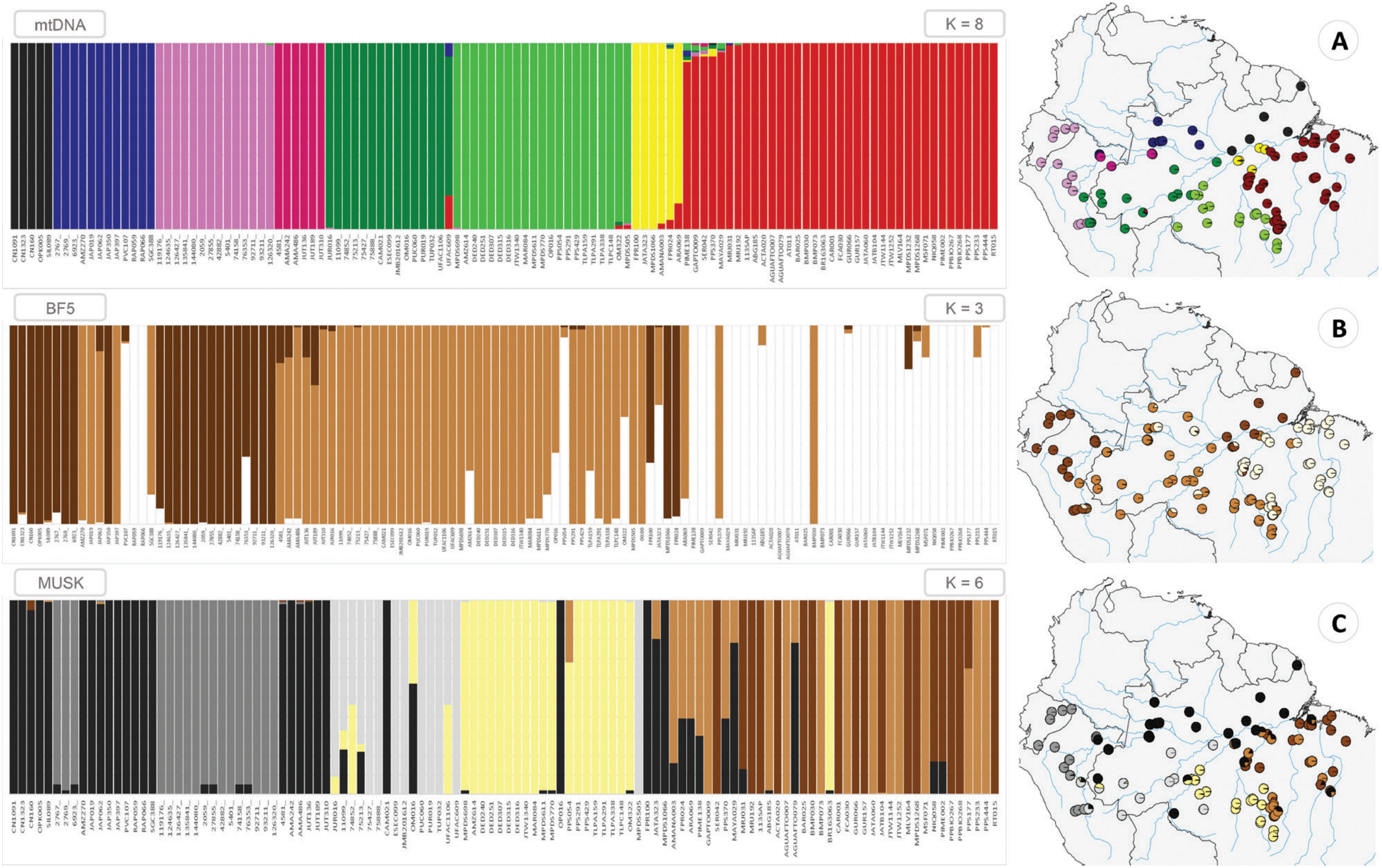

Figure 4. Graphs of BAPS clusters and geographic distribution of individuals analysed when considering A, mitochondrial (K = 8); B, *BF5* (K = 3); C, *MUSK* (K = 6) datasets. Samples were plotted on the map in the pie chart format representing the percentage of admixture recovered by the bar graphs.

admixture were much higher for both nuclear markers than for the mtDNA genes (Fig. 4).

This same trend was also observed between *W. p. gutturalis* and *W. p. griseiventris* A specimens, with several individuals presenting comparatively high levels of genetic admixture for the nDNA genes according to the BAPS analysis performed (Fig. 5). One particularly relevant specimen is CAM021 (MPEG 74415), an adult female from the left bank of the upper Juruá River, which was classified within the *W. p. griseiventris* A cluster when the mtDNA data was considered, but in *W. p. gutturalis* (with no admixture proportions) when the nuclear genes were considered (Fig. 5). Plumage attributes of this particular specimen are also consistent with introgression between *W. p. gutturalis* and *W. p. griseiventris* (Supporting Information, Fig. S4).

BIOGEOGRAPHICAL RECONSTRUCTION

The best-fit ancestral area reconstruction model recovered by BioGeoBEARS was DIVALIKE. The western Amazonian foreland basin was recovered as the most likely ancestral area for the origin of the *Willisornis* diversification (Fig. 6). Alternative area relationships with smaller but still relevant probabilities were also recovered, whereby the *Willisornis* ancestor was distributed in the Guiana and Brazilian shields or in the Amazonian foreland basins and the Brazilian Shield areas (Fig. 6). Here, we will describe in detail and discuss only the scenario with the highest probability.

From the *Willisornis* ancestral range in the western Amazonian foreland basin, two major inferred dispersal events took place: (1) towards the Guiana Shield in the north (leading to the split between *W. p. gutturalis* and *W. p. poecilinotus*); and (2) to the Brazilian Shield in the south (leading to the diversification of the remaining *Willisornis* lineages) (Fig. 6). Despite the inference of those two dispersal events eastwards, the western foreland basins remained a centre of cladogenesis for *Willisornis*, with the further split of *W. p. griseiventris*, *W. p. duidae* and *W. p. lepidonota* (Fig. 6). Diversification within both the Brazilian and Guiana shields was more recent, and in the case of the former geological province, it involved two independent lineages (*W. vidua* and *W. p. griseiventris*), which also differed in their extent of differentiation in this area (Fig. 6).

COMPARATIVE PLUMAGE AND GENETIC VARIATION

Adult males in all *Willisornis* lineages have a general blackish or grey colour, with parts of the back, wings and tail usually tipped white. In contrast, females in all groups tend to have a reddish brown colour overall, with a great diversity of shades, and with white-tipping on the back, wings and tail, present in some lineages but absent in others (Del Hoyo *et al.*, 2020; Zimmer *et al.*, 2020). Below, we provide a summary of the plumage diagnoses found for each genetic lineages recovered by our analyses, linking them to available taxon names following current taxonomy (Del Hoyo *et al.*, 2020; Gill *et al.*, 2022; Zimmer *et al.*, 2020).

WILLISORNIS POECILINOTUS POECILINOTUS (CABANIS, 1847)

Taxonomy

As discussed below, genetic lineages possessing the plumage attributes described below can be unequivocally assigned to the nominate *W. poecilinotus* (type locality: 'British Guiana'; Peters, 1951), distributed in north-eastern Amazonian Brazil, the Guianas and southern Venezuela (Gill *et al.*, 2022).

Plumage attributes

Males with overall grey colour and a slightly paler ventral region. Presence of 'lace' (see Plumage analyses above). Black tail with white markings and white tips. Females with rufous upperparts overall and underparts mostly grey. 'Lace' on the dorsal region similar to that in males, but in black and rufous (instead of white) colours. The tail is black with terminal and intermediate markings also rufous.

Genetic differentiation

Specimens exhibiting the plumage characteristics above belong to a distinct genetic lineage completely sorted for their mitochondrial DNA, but less so for the nuclear genes, and which is distributed on the eastern part of the Guiana Shield, from Amapá in Brazil and the Guianas westward to the lower east bank of the Rio Negro (Fig. 1). We found significant genetic structure within this group, roughly across the Trombetas River near the Amazonas/Pará border, which prompted us to distinguish them as *W. p. poecilinotus* A (east of the Trombetas) and *W. p. poecilinotus* B (west of the Trombetas) (Fig. 1). Our genetic sampling does not allow for a resolution of the relationships in the contact zones between *W. p. poecilinotus* A and *W. p. poecilinotus* B, nor between *W. p. poecilinotus* as a whole and the neighbouring *Willisornis* lineages.

Plumage variation

We did not identify noticeable variations in plumage that were consistent with the genetic structure found for this group.

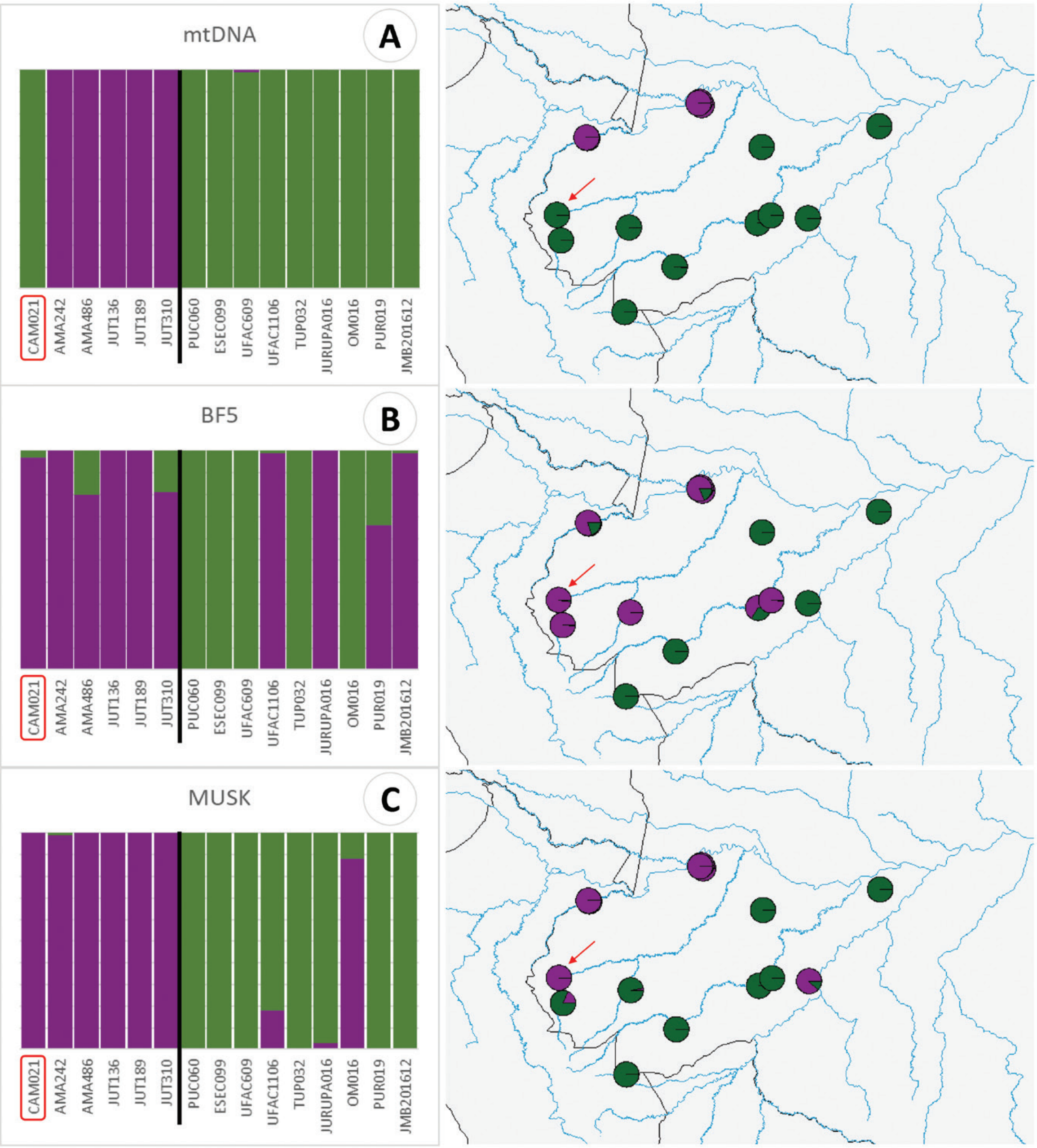

Figure 5. Clusters of BAPS for *Willisornis poecilinotus gutturalis* and *W. p. griseiventris* A in western Amazonia. Each graph corresponds to analyses made with A, mitochondrial; B, *BF5*; C, *MUSK* datasets. The coloured bars represent the proportion of genetic similarity to each population for each specimen included in the analysis while the maps on the right show the geographic distribution of the samples used. Specimen CAM021 is highlighted by a red rectangle and arrow.

WILLISORNIS POECILINOTUS DUIDAE (CHAPMAN, 1923)

Taxonomy

Genetic lineages possessing the plumage attributes discussed below can be unequivocally assigned to *W. p. duidae* (type locality: 'Mount Duida, Amazonas, Venezuela'; Peters, 1951), distributed in north-western Amazonian Brazil, southern Venezuela and eastern Colombia (Gill *et al.*, 2022).

Plumage attributes

Males similar to *W. p. poecilinotus*, but distinguished by an overall darker colour, both in the dorsal and ventral regions. Females distinguished from those in any other

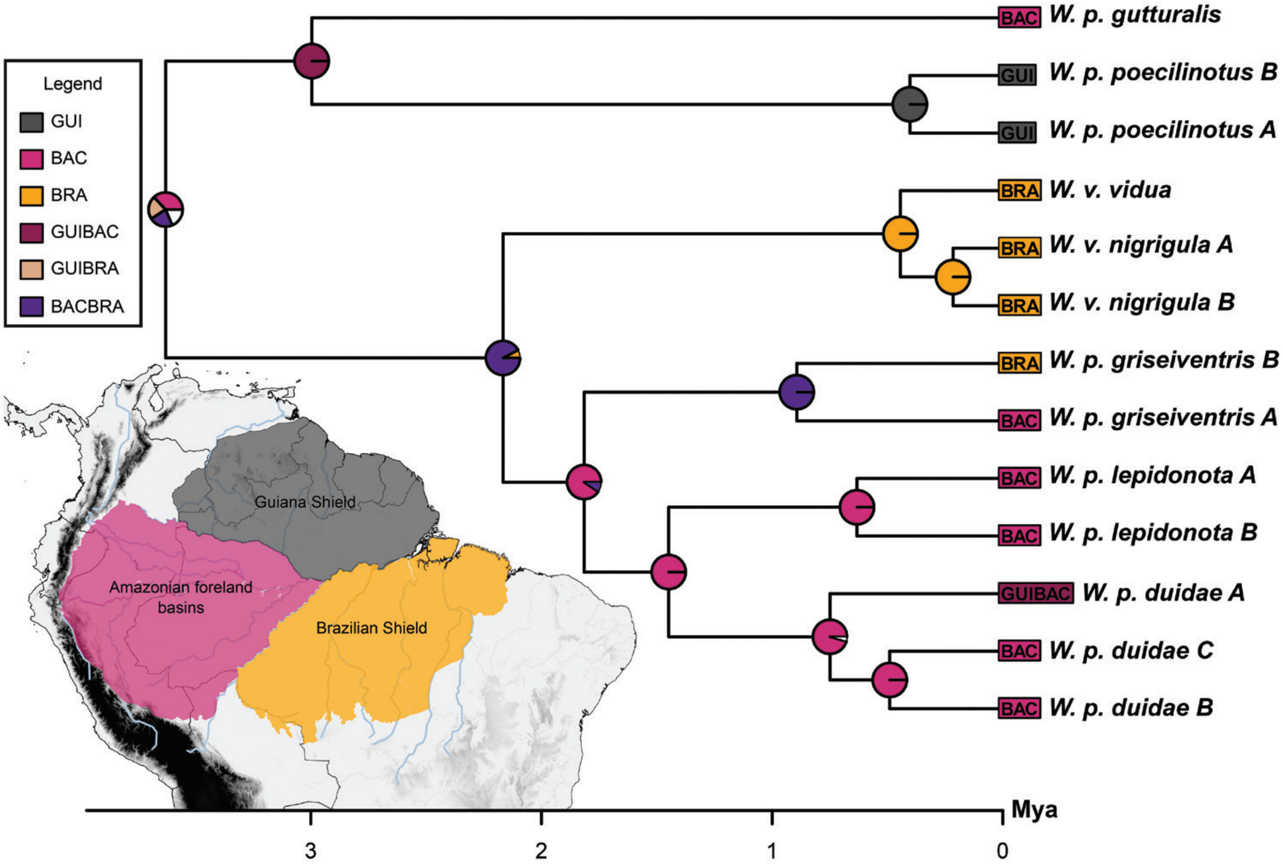

Figure 6. Ancestral range estimates for the genus *Willisornis* obtained with BioGeoBEARS. The model selected was DIVALIKE (see text for details). Node pie charts represent the likelihoods of ancestral area states. Colours represent major Amazonian geological provinces selected as areas for the analysis, with those differing from the ones in the map corresponding to combinations of areas: GUI, Guiana Shield; BAC, Amazonian foreland basins; BRA, Brazilian Shield; GUIBAC, Guiana Shield + Amazonian foreland basins; GUIBRA, Guiana Shield + Brazilian Shield; and BACBRA, Amazonian foreland basins + Brazilian Shield. Numbers on the timescale below represent millions of years.

Willisornis taxon by an intense rufous colour below, with a slightly paler quality in the upperparts. 'Lace' similar to those in males (i.e. in black-white colour). Tail rufous as well, with terminal and intermediate white dots.

Genetic differentiation

Specimens with the plumage characteristics above belong to three distinct and closely related genetic lineages that are completely sorted for their mtDNA, but less so for the nuclear genes and which are distributed from both sides of the Middle and Upper Rio Negro, all the way to the north bank of the Amazonas/Solimões (Fig. 1). The three lineages within this group were named *W. p. duidae* A, *W. p. duidae* B, *W. p. duidae* C and appear to replace each other across the Japurá/Caquetá and Içá rivers (Fig. 1). Interestingly, four specimens sequenced from northern Peru obtained from the north bank of the Amazon and Napo rivers (and previously attributed to *W. p. lepidonota*; Isler & Whitney, 2011) also clustered in this lineage (see Discussion below).

Plumage variation

Females of *W. p. duidae* C (which extends from northernmost Brazil to northern Peru on the north bank of the Amazonas and Napo rivers) have a lighter brown colour, less intense than in the other females of *W. p. duidae* A and B, and approaching those in *W. p. gutturalis*. Males belonging to *W. p. duidae* C are similar to those in *W. p. duidae* A and *W. p. duidae* B.

WILLISORNIS POECILINOTUS LEPIDONOTA (SCLATER & SALVIN, 1880)

Taxonomy

Genetic lineages with plumage characteristics described below are apparently related to *W. p. lepidonota* (type locality: Sarayacu, Pastaza, Ecuador; Peters, 1951; Isler & Whitney, 2011), distributed in southern Colombia, eastern Ecuador and eastern Peru (Isler & Whitney, 2011; Gill *et al.*, 2022).

Plumage attributes

Due to logistical constrains (see Discussion), we analysed the plumage of only one female specimen within the known range of *W. p. lepidonota*. This particular specimen presents an intense rufous colour above, but with slightly paler underparts, resembling females of *W. p. gutturalis*. In comparison with females of *W. p. duidae*, the rufous colour of the examined specimen is noticeably less intense. The 'lace' is present and is similar to those in males (i.e. in black-white colour; as in Zimmer *et al.*, 2020). Tail rufous, with terminal and intermediate white dots.

Genetic differentiation

The female plumage characteristics described above belong to a specimen (MPEG 54179) obtained from a locality (Peru, Amazonas, Rio Comaina) within the range of *W. p. lepidonota* lineage A (Fig. 1). Along with *W. p. lepidonota* B (which apparently replaces *W. p. lepidonota* A across the Huallaga River), these lineages are completely sorted for their mtDNA, but less so for the nuclear genes (Fig. 1). Together, they occupy the foothills of the Andes from eastern Ecuador to central Peru eastward to the Ucayali and possibly the Napo rivers, respectively, in southern and northern Amazonia (Supporting Information, Table S1; Fig. 1).

Plumage variation

Because only one female specimen was examined, no plumage variation within and between *W. p. lepidonota* A and *W. p. lepidonota* B could be assessed. As discussed above, four Peruvian specimens sequenced by us from the north bank of the Amazon and Napo rivers (and thought previously to belong *W. p. lepidonota*; Isler & Whitney, 2011) grouped, in fact, with *W. p. duidae* C, suggesting either introgression or lack of consistent plumage diagnoses between these taxa.

WILLISORNIS POECILINOTUS GUTTURALIS (TODD, 1927)

Taxonomy

Genetic lineages with plumage characteristics described below can be unequivocally assigned to *W. p. gutturalis* (type locality: São Paulo de Olivença, on the south bank of the upper Amazon; Peters, 1951), distributed in south-western Amazonian Brazil and north-eastern Peru (Gill *et al.*, 2022).

Plumage attributes

Males are similar to those of *W. p. duidae* lineages, but readily distinguished by a conspicuous a narrow black throat patch. Females are pale rufous above and below, paler underneath. 'Lace' similar as in males, in black–white colour. Tail pale rufous, with white spots in the middle part and on the terminal edges.

Genetic differentiation

Specimens with the plumage characteristics above belong to a highly distinct genetic lineages that is completely sorted for their mitochondrial DNA, but less so for the nuclear genes and which is distributed in a restricted area between the Juruá and Amazon/Solimões rivers (Fig. 1).

Plumage variation

We have not identified conspicuous variations in plumage.

WILLISORNIS POECILINOTUS GRISEIVENTRIS (VON PELZELN, 1869)

Taxonomy

Genetic lineages sharing plumage characteristics discussed below are unequivocally assigned to *W. p. griseiventris* (type locality: Borba, on the right/eastern bank of the Madeira River; Peters, 1951), distributed in south-eastern Peru, northern Bolivia and south-western Amazonian Brazil, eastwards to the upper Xingu River (Pulido-Santacruz *et al.*, 2018; Gill *et al.*, 2022).

Plumage attributes

Males similar to those in *W. p. poecilinotus*. Females pale plain rufous throughout the dorsal region, extending to the forehead and the sides of the head. 'Dark-light lace' on the back and wings absent. Throat and ventral region grey. Upper and under tail parts concolorous with the body, but with narrow distinct white terminal edges.

Genetic differentiation

Specimens with the plumage characteristics above belong to a widespread genetic lineage with two major geographic sister groups replacing each other across the Madeira River and referred herein as *W. p. griseiventris* A and *W. p. griseiventris* B. These two lineages are completely sorted for their mitochondrial DNA, but less so for the nuclear genes, occurring throughout most of south/central Amazonia, from the upper Juruá River eastward to the headwaters of the Xingu River (Fig. 1).

Plumage variation

We identified a geographic gradient in male plumage colour in *W. p. griseiventris* A and B running from west to east, with the darkest individuals found in the west (considering the overall body colour) and the palest in the east. Despite this clear trend, major shifts in the overall plumage colour intensity were not correlated with interclade boundaries and the presence of the Madeira River itself.

WILLISORNIS VIDUA NIGRIGULA (SNETHLAGE, 1914)

Taxonomy

Genetic lineages possessing plumage characteristics described below can be assigned to *W. v. nigrigula* (type locality: Boim, on the left/western bank of the Tapajós River; Peters, 1951), distributed in the lower Madeira–Tapajós interfluve and between the Tapajós and Xingu rivers in the Brazilian states of Amazonas, Mato Grosso and Pará (Isler & Whitney, 2011). Plumage attributes: males similar to *W. p. gutturalis*, with both taxa sharing a conspicuous black throat patch not found in males of any other *Willisornis* taxa. However, *W. v. nigrigula* males have ventral and dorsal parts paler grey. Females, on the other hand, are similar to those in *W. p. griseiventris*, with pale rufous colouring throughout the dorsal region and a missing 'dark-light lace' from the back and wings. However, in *W. v. nigrigula* females, the dorsal colour does not extend to the sides of the head, being limited to the crown, forehead and supercilium. The ventral grey colour extends to the sides of the head, with the throat presenting a pale grey colour.

Genetic differentiation

Specimens sharing the plumage characteristics above belong to two distinct genetic lineages that are completely sorted for their mitochondrial DNA, but less so for the nuclear genes, and which are distributed from the lower east bank of the Madeira to the left bank of the Xingu River (Fig. 1). These lineages replace each other across the Tapajós River and are referred herein as *W. v. nigrigula* A and *W. v. nigrigula* B (Fig. 1).

Plumage variation

Specimens of *W. v. nigrigula* differ with respect to the size of the gular patch. In *W. v. nigrigula* A (distributed in the northern part of the Tapajós–Madeira interfluve), the black throat patch tends to be slightly smaller and narrower than in *W. v. nigrigula* B (from the Tapajós–Xingu interfluve). However, some individuals in both groups deviate from this general pattern and, therefore, this phenotypic variation is not exactly congruent with the lineages recovered in molecular analyses.

WILLISORNIS VIDUA VIDUA (HELLMAYR, 1905)

Taxonomy

Genetic lineages with plumage characteristics discussed below can be assigned to *W. v. vidua* (type locality: Igarapé-Açu, state of Pará, in the Belém area of endemism; Peters, 1951).

Plumage attributes

Males similar to *W. v. nigrigula* overall, but without a black throat patch. Ventral region grey, with lighter throat. Females are also similar to those in *W. v. nigrigula*, but with the white interscapular spot absent or inconspicuous.

Genetic differentiation

Specimens with the plumage characteristics above belong to a genetic group with some geographic structuring recovered for the mitochondrial genes across the Tocantins River and which are referred herein as *W. v. vidua* A and *W. v. vidua* B. These groups are completely sorted for their mitochondrial DNA, but less so for the nuclear genes, occupying together the area comprised between the east bank of the Xingu eastward to Maranhão in easternmost Brazilian Amazonia (Fig. 1).

Plumage variation

We did not detect any conspicuous geographic variation in plumage that could be related to the comparatively shallow level of genetic structure recovered within this group.

DISCUSSION

PHYLOGEOGRAPHIC STRUCTURE, CONTACT ZONES AND PLUMAGE VARIATION

The diversity in the genus *Willisornis* is greater than previously thought (Hellmayr, 1929; Isler & Whitney, 2011; Isler *et al.*, 2014). Our analyses corroborate the existence of 13 independent genetic groups in the genus, only seven of which are currently formally recognized as named taxa. In general, the plumage of *Willisornis* taxa has darker tones in wetter western Amazonia, with a tendency towards lighter colours in drier and more seasonal eastern Amazonia, mirroring

Gloger's rule and other similar geographic patterns of plumage variation detected previously for Amazonian birds (Ferreira *et al.*, 2016; Santana *et al.*, 2021). All currently recognized *Willisornis* taxa have robust plumage diagnoses (see Results), but for the subspecies genetic subdivisions documented herein (A/B/C clades), no consistent variation in plumage was found.

When the current taxonomy is considered (Gill *et al.*, 2022), the most widespread species in the genus is apparently paraphyletic (*W. poecilinotus*), while all but one subspecies-level taxon (*W. v. nigrigula*; Fig. 1) were consistently recovered as monophyletic. For most subspecies, we recovered two reciprocally monophyletic lineages with high statistical support and geographic structure. Our divergence-time estimates showed that these subdivisions, within currently accepted subspecies, date back to a period roughly between 900 000 and 200 000 years (Fig. 2). However, these genetic divergences within currently recognized subspecies were not matched by consistent differences in plumage characters. More detailed quantitative morphological analyses will help clarify whether divergent phenotypes exist. Regarding subspecies *W. v. nigrigula*, the two independent groups (A and B) were recovered as monophyletic only by the coalescent multilocus species tree analysis (Fig. 2). In contrast, both mitochondrial and multilocus concatenated Bayesian analyses consistently recovered *W. v nigrigula* as paraphyletic, with *W. v. nigrigula* B clustering as sister to *W. v. vidua* distributed east of the Xingu, rather than with *W. v. nigrigula* A (Fig. 1). However, plumage variation in *W. v. nigrigula* A and B is subtle, with no consistent differentiation between them, agreeing with the topology recovered by the multilocus species tree. The discrepancy observed between concatenated and coalescent phylogenies indicates that more complex evolutionary scenarios could be involved in the differentiation of *W. v. nigrigula* A and B, maybe with at least one episode of introgression with *W. v. vidua*. Alternatively, ancestral polymorphism and incomplete lineage sorting can also be behind topology conflicts, particularly because only three genetic loci were employed in the phylogenetic analyses. Therefore, the phylogenies presented herein should be interpreted with caution, and will serve as a reference points to future studies based on next-generation sequencing (NGS) approaches.

The clade grouping *W. p. gutturalis* specimens is the only one fully consistent with current taxonomy and with no internal phylogeographic structure (Fig. 1). The position of this taxon within *Willisornis* is still uncertain and needs further investigation. Most of our analyses support a sister-group relationship with *W. p. poecilinotus*. However, the Bayesian mtDNA tree recovered *W. p. gutturalis* as sister to a clade grouping *W. p. duidae*, *W. p. griseiventris*, *W. p. lepidonota* and the polytypic *W. vidua* (Fig. 1). This conflict between the mitochondrial and the joint mitochondrial and nuclear datasets could be due to the apparent introgression between *W. p. gutturalis* and *W. p. griseiventris*. Next-generation sequencing data encompassing a much greater number of loci could help clarify the basal relationships in the *Willisornis* tree as a whole.

As the sampling of specimens used in morphological analyses ($N = 1108$) was nearly ten times greater than the sampling employed for molecular analyses ($N = 182$), it was possible to draw a better view of the geographic limits of the different taxa currently recognized in *Willisornis*, including the definition of contact zones between some taxa. In *W. p. poecilinotus* from the Guiana Shield, for instance, our genetic sampling (incomplete in the headwaters regions) suggests that the Rio Branco acts as a barrier. However, the larger sampling employed in plumage analyses showed that, in fact, *W. p. poecilinotus* occurs on both banks of the upper Rio Branco (Supporting Information, Fig. S1; see also: Isler & Whitney, 2011). However, given the limitations of our sampling, it was not possible to determine exactly where the contact zone between *W. p. poecilinotus* and *W. p. duidae* lies between the Rio Branco and Rio Negro in north-western Amazonia (see also: Naka & Brumfield, 2018). Overall, the genetic data obtained herein and that in Naka & Brumfield (2018) agree with anecdotal evidence supporting a constancy in plumage characters diagnosing both *W. p. poecilinotus* and *W. p. duidae* species along their entire contact zone (Zimmer 1934), which extends into southern Venezuela (see: Isler & Whitney, 2011).

Our data have also confirmed a contact zone between *W. p. gutturalis* and *W. p. griseiventris* in the Juruá–Ucayali interfluve (see also: Isler & Whitney, 2011; Del-Rio *et al.* 2021), with a specimen from the left bank of the upper Juruá River (CAM021; Fig. 4; Supporting Information, Fig. S4) exhibiting a mixture of characters belonging to both *W. p. gutturalis* and *W. p. griseiventris*. This adult female has the dorsal plumage pattern similar to those of *W. p. griseiventris* females (absence of markings on the back and wings), while the ventral region is similar to that in *W. p. gutturalis* females (ventral region plain rufous). DNA sequences obtained for the same specimen also pointed to a mixed background, with those belonging to the mitochondrial genes placing the specimen firmly in *W. p. griseiventris*, whereas those of the nuclear markers assigning it to *W. p. gutturalis* (Fig. 5; Supporting Information, Fig. S4). These findings represent the best supporting evidence so far that *W. p. gutturalis* and *W. p. griseiventris* are not only in direct contact in the upper Juruá River, but also may hybridize in this area.

Our data have confirmed the existence of a direct contact zone between *W. p. griseiventris* and *W. v. nigrigula* in northern Mato Grosso State (Figs

1, 4; Supporting Information, Fig. S1), as documented in detail by Pulido-Santacruz *et al.* (2018). Our plumage analyses demonstrated that some genetically identified hybrids by Weir *et al.* (2015) also present hybrid phenotypes, with intermediate plumage characteristics, never documented before. The black gular spot, present in *W. v. nigrigula* males, is reduced and less marked in these hybrid males, which have variable amounts of grey feathers on the throat, approaching the smooth, grey throat of *W. p. griseiventris* (Supporting Information, Fig. S5). However, our dataset did not include any specimens supporting the instance of sympatry between *W. p. griseiventris* and *W. v. nigrigula* as reported by Isler & Whitney (2011) along the left (western) bank of the Middle Tapajós River near Jacareacanga, despite our sampling of several individuals in this area (Fig. 1; Supporting Information, Fig. S1; Table S1). However, our sampling on the right (eastern) bank of the Madeira River included only *W. v. nigrigula* specimens and did not extend southward to Borba, the known type locality of *W. p. griseiventris*, so we have missed at least this part of the contact zone between these taxa. Despite the relatively small number of samples and genetic markers included in our phylogenetic and population structure analyses (Figs 1, 4), the high levels of genetic and morphological differentiation recovered between these taxa within the middle-lower Madeira–Tapajós interfluve suggest that, even if present, gene flow has not been widespread to the point of promoting character homogenization between them. Such a scenario has been demonstrated for the contact zone between *W. v. nigrigula* B and the same *W. p. griseiventris* B group southward, near the Teles Pires River, where hybridization is present, although localized and with evidence for heavy selection against hybrids (Weir *et al.*, 2015; Pulido-Santacruz *et al.*, 2018). The highly dynamic nature of the Madeira and Tapajós river drainages during the Quaternary may be associated with these contact zones that do not correspond to modern major rivers, as observed in other parts of the range of the *Willisornis* complex (Hayakawa & Rossetti, 2015; Rossetti *et al.*, 2015).

In contrast with currently recognized subspecies limits, our molecular results uncovered an unexpected pattern, whereby sequences of specimens from the north bank of the Amazon and Napo rivers in north-eastern Peru clustered with *W. p. duidae* rather than *W. p. lepidonota* (Figs 1, 4), as implied by current taxonomy (Isler & Whitney, 2011). Therefore, the contact zone between *W. p. duidae* and *W. p. lepidonota* in north-western Amazonia might have complex dynamics, as also discussed previously by Zimmer (1934). Future studies should investigate this in more detail.

HISTORICAL DIVERSIFICATION IN THE AMAZON

The biogeographic history of the genus *Willisornis* reveals that its ancestor was widely distributed in the western Amazonia, with a greater probability of being found in the western foreland basins (Fig. 6). The first split in the genus dates to *c.* 3.5 Mya. (4.47–2.09), probably separating northern from southern western foreland basin populations, which could have already occupied the Guiana and Brazilian shields, respectively (Fig. 6). This time frame roughly coincides with the breaching of the Purus arch, leading to the formation of the transcontinental Amazon River (Hoorn *et al.*, 2010; Albert *et al.*, 2021). Although the dating for the origin of the modern Amazon River is still being debated (Late Miocene or Pliocene, see: Campbell *et al.*, 2006), the first split in *Willisornis* could be spatially and temporality matched with the onset of the modern transcontinental drainage, which would have isolated distinct northern and southern populations.

From that first separation, the lineages that seemed to have their distribution restricted to the north and west of the Amazon basin were again separated around 3 Mya (3.99–1.93) to the north and south of the upper Amazon/Solimões River (Fig. 6). This date coincides with the probable reorganization and modern establishment of the Solimões River (Campbell *et al.*, 2006; Nogueira *et al.*, 2013), so that *W. p. gutturalis* became restricted to the Inambari area of endemism, while *W. p. poecilinotus* A and B were isolated in the Guiana Shield. The onset of the Pleistocene coincided with the beginning of the most intensive period of diversification in *Willisornis*, being centred both in the western foreland basins and the Brazilian Shield (Fig. 6). By 2.17 Mya (3.05–1.50), a major division occurred in this clade, roughly separating the Brazilian Shield (*W. vidua*) from the western foreland basin populations (*W. p. duidae*, *W. p. griseiventris* and *W. p. lepidonota*), and possibly coinciding with the establishment of the Madeira River, analogous to the scenario proposed for the separation between *Psophia leucoptera* Spix, 1825 and *Ps. viridis* Spix, 1825 (Ribas *et al.*, 2012). Subsequently, by 1.82 Mya (2.24–1.42), a vicariant event took place within the western foreland basins separating populations south-east (*W. p. griseiventris*) and north-west of the upper Amazon/Ucayali rivers (*W. p. duidae* and *W. p. lepidonota*), coinciding spatiotemporally with the scenarios for the diversification of *Phaethornis philippii* (Bourcier, 1847)/*Ph. koepckeae* Weske & Terborgh, 1977 (Araujo-Silva *et al.*, 2017) and *Sciaphylax hemimelaena* (Sclater, 1857)/*S. castanea* (Zimmer, 1932) (Fernandes *et al.*, 2012), and possibly explained by further episodes of drainage reorganization in this sector of Amazonia (Nogueira *et al.*, 2013). Additional splitting events took place in the north-western part of the western foreland basins, which culminated with the separation between *W. p. lepidonota* and *W. p. duidae* at 1.46 Mya (1.96–0.93; Fig. 6). Finally, during the last one million years, further splitting events took place within both *W. p. lepidonota* and *W. p. duidae*, including the separation of *W. p. duidae* A, involving the colonization of the western part of the Guiana Shield (Fig. 6). The separation between *W. p. griseiventris* A and *W. p. griseiventris* B also took place during this time frame (1.39–0.41 Mya) and could be related to the most recent cycle of changes leading to the modern conformation of the Madeira drainage (Hayakawa & Rossetti, 2015). The separation among *W. vidua* lineages across the easternmost major tributaries of the Amazon (Tapajós and Xingu) took place only during the last 0.5 Myr, the same time frame related to the differentiation of both *W. p. poecilinotus* lineages across the Trombetas River (Figs 1, 6).

Interestingly, all easternmost populations of *Willisornis* (*W. p. poecilinotus* A, *W. p. griseiventris* B, *W. v. vidua* and *W. v. nigrigula* B) showed consistent signs of population expansion during the Quaternary (last 0.2 Myr), whereas both demographically stable and unstable lineages were found throughout the remaining parts Amazonia (Supporting Information, Fig. S3). This could be an indication that eastern Amazonia was more drastically affected by Quaternary climate change than western Amazonia, and that the diversification of *W. p. poecilinotus* A, *W. p. griseiventris* B, *W. v. vidua* and *W. v. nigrigula* B could have involved episodes of dispersal following the establishment or re-establishment of humid tropical forests in eastern Amazonia, as suggested by Silva *et al.* (2019). However, more explicit tests of this hypothesis should be made with a more representative genomic dataset than the one analysed herein. Because most of the lineages recovered within *Willisornis* exhibited signs of population expansion during the Quaternary, the finding that documented contact zones always involve non-sister lineages (Naka & Brumfield, 2018; Pulido-Santacruz *et al.*, 2018; see also Fig. 1) may be explained by frequent episodes of dispersal and range changes, following cladogenesis mediated by either drainage reorganization patterns, climate change or both factors.

Species limits in *Willisornis*

Even though previous molecular studies have sampled both currently recognized species and several subspecies of *Willisornis* (Brumfield *et al.*, 2007; Fernandes *et al.*, 2014; Weir *et al.*, 2015; Silva *et al.*, 2019; Harvey *et al.*, 2020), herein we obtained the most representative coverage of the genus yet, with the largest number of specimens sampled across all currently recognized taxa (Gill *et al.*, 2022). Therefore, in addition to the possibility of reconstructing the evolutionary history and phylogenetic relationships among all valid taxa in the genus for the first time, we were able also to correlate the genetic and geographical limits between them with previous studies based on morphology and vocalizations (Cory & Hellmayr, 1925; Zimmer, 1934; Isler & Whitney, 2011). While the extensive vocal sampling by Isler & Whitney (2011) showed that significant vocal differences exist between *W. v. vidua* and *W. v. nigrigula* and the remaining taxa grouped under *Willisornis*, differences of smaller magnitude were detected among the other taxa, but these were not statistically significant according to the diagnosability criterion they employed. Other studies have demonstrated that the stringent diagnosability criteria employed by Isler & Whitney (2011) might fail to recognize even some highly vocally differentiated suboscine taxa that differ statistically by only one or two vocal characters (Chaves *et al.*, 2010; Carneiro *et al.*, 2012).

When the current accepted species limits in *Willisornis* are considered (Isler & Whitney, 2011; Gill *et al.*, 2022; Pacheco *et al.*, 2021; Remsen *et al.*, 2022), our data did not support consistently the reciprocal monophyly between *W. poecilinotus* and *W. vidua* (Figs 1, 2). While the concatenated multilocus phylogeny recovered *W. poecilinotus* and *W. vidua* as reciprocally monophyletic, the mitochondrial and multilocus species trees did not (Figs 1, 2). However, the posterior probability associated with the node supporting the monophyly of *W. poecilinotus* in the concatenated multilocus tree was low, whereas all estimated phylogenies recovered with high statistical support the monophyly of *W. vidua* (Figs 1, 2). Finally, our coalescent multilocus species tree recovered with strong statistical support a paraphyletic *W. poecilinotus*, whereby *W. p. duidae*, *W. p. griseiventris* and *W. p. lepidonota* clustered in the same clade as *W. vidua*, to the exclusion of *W. p. poecilinotus* and *W. p. gutturalis* (Fig. 2). These conflicting results resemble those of previous studies, which have either supported the reciprocal monophyly between *W. poecilinotus* and *W. vidua* (Harvey *et al.*, 2020) or not (Isler *et al.*, 2014; Silva *et al.*, 2019). In the case of the Harvey *et al.* (2020) study, even though it was based on the largest molecular dataset available for *Willisornis* and several other suboscine lineages, it lacked samples of the genetically divergent *W. p. duidae*, *W. p. gutturalis*, *W. p. lepidonota* and *W. v. nigrigula*, and, therefore, a sparser taxon sampling could have influenced the recovered topologies. Given our dense taxon sampling, added to the fact that coalescent approaches usually outperform concatenated phylogenetic analyses when multilocus datasets are considered (Liu *et al.*, 2009), we regard our species tree as the best estimate of the *Willisornis* phylogeny, and discuss interspecific limits based on it. As mentioned above, strong statistical support was obtained by the coalescent species tree for the sister-relationship between *W. vidua* and a clade uniting *W. p. duidae*, *W. p. griseiventris* and *W. p. lepidonota* to the exclusion of *W. p. poecilinotus* and *W. p. gutturalis*, which grouped together as sister-taxa, albeit with low statistical support (Fig. 2). Therefore, the paraphyly recovered for *W. poecilinotus* with respect to *W. vidua* is at odds with currently recognized species limits in the genus (Pacheco *et al.*, 2021; Gill *et al.*, 2022, Remsen *et al.*, 2022), which can be revised now according to our multi-character analyses.

Our unguided species delimitation tests supported a total of 13 reciprocally monophyletic lineages recovered by the Bayesian analyses as highly coalesced entities, possibly indicating independent species statuses (Fig. 3). However, BP&P results have to be interpreted with caution given that they might over-split lineages (Sukumaran & Knowles, 2017), particularly those with ongoing gene flow (Jackson *et al.*, 2017), as is clearly the case with *Willisornis* (Fig. 5; Supporting Information, Figs S4, S5; Weir *et al.*, 2015; Pulido-Santacruz *et al.*, 2018). Therefore, instead of interpreting our BP&P results by their face value, we suggest the recognition of new interspecific limits in *Willisornis* based on the congruency between deeply coalesced lineages and consistent plumage diagnoses verified among taxa. As such, the coalescent multilocus species tree recovered all seven currently recognized taxa in *Willisornis* as monophyletic and deeply coalesced groups (*duidae*, *griseiventris*, *gutturalis*, *lepidonota*, *nigrigula*, *poecilinotus* and *vidua*), which are also, for the most part, highly distinct from each other, considering female and male plumages (see also: Isler & Whitney, 2011). The only exception pertains to *duidae* and *lepidonota*, which are apparently diagnosed by plumage only according to the intensity of the rufous colour in females. Unfortunately, our sampling of *lepidonota* skins was too small to allow for a thorough analysis of plumage variation in this taxon, but results from Isler & Whitney (2011) indicate that *lepidonota* and *duidae* were the only taxa in *Willisornis* not fully diagnosed by at least one plumage character. Furthermore, the genetic data presented herein showed a clear mismatch between plumage and genetic patterns of variation found between these taxa in north-eastern Peru, with populations historically treated under *lepidonota* based on plumage characters (Isler & Whitney, 2011) grouping genetically in the same clade as *duidae* populations (see Results above). This lack of full diagnosis in plumage characters, coupled with their sister-relationship, parapatric ranges and an apparent mismatch in patterns of plumage and genetic variation, suggest a possible wide zone of intergradation where their ranges abut in north-eastern Peru, indicating that *lepidonota* and *duidae* may be more strongly connected to each other through gene flow than any other taxon pair in *Willisornis*. Hence, in this particular case, our BP&P results may be reflecting only extensive population structure within a widespread set of lineages still connected through either past or current significant amounts of gene flow, which would favour their treatment as subspecies rather than independent species.

In contrast, the remaining deeply coalesced *Willisornis* lineages corresponding to monophyletic taxa according to our multilocus species tree (Figs 2, 3), and which are also diagnosed from each other by at least one plumage character (i.e. *griseiventris*, *gutturalis*, *nigrigula*, *poecilinotus* and *vidua*; see also: Isler & Whitney, 2011), can be treated as independent species, with genetic data demonstrating significant population structure within them usually bound by major Amazonian tributaries. As shown for at least some of these taxa, they are still capable of reproducing with each other (Fig. 5; Supporting Information, Figs S4, S5) and even producing fertile offspring (Weir *et al.*, 2015; Pulido-Santacruz *et al.*, 2018) in areas where their ranges connect to each other, but no widespread intergradation has been documented between them (see also: Isler & Whiney, 2011). Possibly, strong selection against hybrids (shown at least for the *griseiventris*/*nigrigula* contact zone; Pulido-Santacruz *et al.*, 2018; see also: Cronemberger *et al.*, 2020) may be the ultimate factor preventing these taxa from merging with each other, following their recent secondary contact (as indicated by their non-sister relationships) after a period of separation, where reproductive barriers might have evolved. Hence, based on their degree of evolutionary independence, congruence between genetic and phenotypic characters and even apparent lack of extensive hybridization, we recommend the recognition of the following species-level taxa in *Willisornis* based on the general lineage species concept (de Queiroz 2005), as follows:

Willisornis poecilinotus (Cabanis, 1847) – distributed in the Guiana Shield in Brazil (north of the Amazon and east of the lower Rio Negro) to French Guiana, Suriname, Guyana and southern Venezuela (Bolívar and north-eastern Amazonas; Fig. 1; Supporting Information, Fig. S1). This species and the distantly related *W. lepidonota duidae* (see below) meet along the Orinoco in Venezuela (state of Amazonas) and the middle-upper Rio Negro–Rio Branco interfluve in the Brazilian states of Roraima and Amazonas, with some possible documented hybrids (Zimmer, 1934; Isler & Whitney, 2011), but no indication of widespread intergradation (see also: Naka & Brumfield, 2018). Our data recovered two deeply coalesced lineages in *W. poecilinotus* replacing each other in the western and eastern parts of the Guiana Shield, and whose ranges appear to be limited in the south by the Rio Trombetas (Fig. 1; Supporting Information, Fig. S1).

Willisornis gutturalis (Todd, 1927) – distributed in a relatively small area between the south and west bank of the upper Amazon/Solimões and Ucayali rivers in Brazil and Peru and the left bank of the Juruá River in the Brazilian state of Amazonas. As shown here, the distantly related *W. gutturalis* and *W. griseiventris* meet somewhere along the Juruá River (see also: Del-Rio *et al.*, 2021), where at least some limited hybridization occurs (Fig. 5; Supporting Information, Fig. S4).

Willisornis vidua (Hellmayr, 1905) – distributed east of the Xingu River in the Brazilian states of Mato Grosso, Maranhao, Pará and Tocantins. Our data did not support the existence of a contact zone between *W. vidua* and *W. nigrigula* in the upper Xingu River (Fig. 1; Supporting Information, Fig. S1), so it remains speculative whether these taxa meet in the sector of the Xingu–Tocantins interfluve (Isler & Whitney, 2011). Our sampling indicates that a contact zone between this species and *W. griseiventris* (see below) may also exist in the upper reaches of the Xingu River in the north-eastern part of the Mato Grosso State (Fig. 1; Supporting Information, Fig. S1).

Willisornis nigrigula (Snethlage, 1914) – distributed between the northern part of the Madeira–Tapajós interfluve and the western bank of the Xingu River in the Brazilian states of Amazonas, Mato Grosso and Pará (Fig. 1; Supporting Information, Fig. S1). The Tapajós River delimits two highly structured and divergent populations in this species (Figs 1, 2), which appear not to be fully diagnosable by any phenotypic characters (see also: Isler & Whitney, 2011). Both of these populations are in direct contact with *W. griseiventris* in the middle sector of the Tapajós–Madeira interfluve and the upper reaches of the Tapajós and Xingu rivers, where they hybridize (Supporting Information, Fig. S5), although selection against highly introgressed hybrids indicates the existence of post-mating reproductive barriers between them, causing their hybrid zone to be extremely narrow, at least in upper Tapajós drainage (Pulido-Santacruz *et al.*, 2018).

Willisornis griseiventris (Von Pelzeln, 1869) – distributed south of the Amazon and east of the Ucayali rivers eastward to the upper reaches of the Xingu River, encompassing Peru (departments of Madre de Díos, Ucayali and southern Loreto), Bolivia (Beni, La Paz, Pando and Santa Cruz) and the Brazilian states of Acre, Amazonas, Rondônia and Mato Grosso (Fig. 1; Supporting Information, Fig. S1; Isler & Whitney, 2011). Limited gene flow exists between this species and both *W. gutturalis* and *W. nigrigula* in different parts of its range (see above). The Madeira River bounds two structured and divergent populations of this species (see also: Fernandes *et al.*, 2014), which are not consistently distinguished by plumage or vocal characters (Isler & Whitney, 2011).

Willisornis lepidonota (Sclater & Salvin, 1880) – includes two subspecies: *W. lepidonota lepidonota*, distributed at least from the southern/western bank of the Napo and western bank of the Ucayali rivers in Amazonian Ecuador and Peru (departments of Loreto, Amazonas, San Martín, Huánuco, Pasco, Junín, Ayacucho, Apurímac, southward to Cusco); and *W. lepidonota duidae* (Chapman, 1923), distributed from the western bank of the Orinoco in Venezuela (state of Amazonas) and the middle-upper Rio Negro–Rio Branco interfluve in the Brazilian states of Roraima and Amazonas to Colombia (both banks of the Caquetá; Isler & Whitney, 2011). The Japurá/Caquetá, Putumayo/Icá and Marañon rivers delimit deeply coalesced lineages of *W. lepidonota* for the molecular markers sequenced in this study (Figs 1, 2). Both subspecies meet between the Napo and Caquetá rivers in north-eastern Peru and Colombia, and may intergrade, as indicated by the mismatch between patterns of genetic and morphological variation documented in this study and in Isler & Whitney (2011).

CONCLUSION

Herein, we have established the phylogenetic relationships amongst all recognized *Willisornis* taxa, relating them to known patterns of plumage variation in the genus. The molecular data supports the existence of 13 independent genetic lineages in the genus and the monophyly of all currently recognized basal taxa (*sensu* Gill *et al.*, 2022). Of these, six are also distinguished by at least one discrete plumage character found in males and females, and are proposed herein to constitute distinct species. Although now ample evidence exist for gene flow between some of the species recognized herein, in at least one instance (i.e. *W. griseiventris* and *W. nigrigula*), introgression was shown to be limited and apparently hampered by at least some degree of reproductive isolation acquired during periods of allopatry (Pulido-Santacruz *et al.*, 2018). From a biogeographic perspective, the evolutionary history of the genus *Willisornis* has been tightly associated with patterns of drainage evolution and climate change cycles across Amazonia since the Plio-Pleistocene, with a recent boom in diversification over the past 1 million years.

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DATA AVALIABILITY

The data underlying this article are available in the GenBank Nucleotide Database under unique identifiers OM792201–OM792768.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Detailed information on *Willisornis* specimens examined for both molecular and plumage analyses. GenBank accession numbers for the different specimens and genes sequenced are provided. Taxonomy follows Gill *et al.* (2022). Voucher specimen and tissue numbers belong to the following institutions: Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil (INPA); Louisiana State University Museum of Natural Science, Baton Rouge, USA (LSUMZ); Museu Paraense Emílio Goeldi, Belém, Brazil (MPEG); and Natural History Museum of

Denmark, Universty of Copenhagen, Copenhagen, Denmark (SNM). GenBank numbers associated with each gene sequence / specimen are also provided.
Table S2. Primers and annealing temperatures used for each gene.
Table S3. Uncorrected pairwise genetic distances (*p*-distance) calculated between (above) and within (below) lineages recovered in phylogenetic analyses. The colours correspond to those used in Figure 1.
Figure S1. Distribution of specimens sampled for the analyses of plumage characters.
Figure S2. Phylogenetic hypotheses obtained through Bayesian inferences (BI) based, respectively, on *BF5* and *MUSK*.
Figure S3. Bayesian skyline plots (EBSP). Demographic histories of *Willisornis* lineages based on nDNA (*MUSK* and *BF5*) and mtDNA (*ND2* and *Cytb*) sequences. Black solid lines represent median values and dashed lines correspond to 95% confidence intervals. The X-axis corresponds to time in million years before present, while the axis corresponds to the effective population size (*Ne*).
Figure S4. Likely hybrid individual between *Willisornis poecilinotus gutturalis* and *W. p. griseiventris*, located in the headwaters of the Juruá River (specimen CAM021).
Figure S5. Hybrid individuals between *Willisornis poecilinotus griseiventris* and *Willisornis vidua nigrigula*, located in the River Teles Pires contact zone. Specimens identified as hybrids by Weir *et al.* (2015).