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Mixing the waters: a linear hybrid zone between two riverine Neotropical cardinals (*Paroaria baeri* and *P. gularis*)

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ABSTRACT

Amazonian rivers have been more frequently conceptualised as barriers rather than as habitats for birds with their own ecological and biogeographic histories. However, many river-restricted bird species have differentiated within the formidable network formed by the Amazon and its tributaries. Here we demonstrate that the riverine-distributed Crimson-fronted Cardinal (*Paroaria baeri*) is narrowly distributed along the middle Rio Araguaia basin, where it comes into contact and hybridises with the geographically widespread Red-capped Cardinal (*P. gularis*). This one-dimensional hybrid zone, which is situated over *ca*.160 km along the Araguaia and Javaés Rivers, appears to be of recent origin. Admixed individuals between the non-sister *P. baeri* and *P. gularis* are phenotypically intermediate between the parental species, and superficially resemble the geographically disjunct and phylogenetically distant Masked Cardinal (*P. nigrogenis*). Two phenotypically admixed specimens were confirmed as such based on sequences of the mitochondrial Cytb and the Z-linked MUSK gene. Field observations and genetic data indicate that *P. baeri* × *P. gularis* hybrids are capable of producing viable offspring, but more data are necessary to confirm hybrid viability and fertility. The non-sister hybridising species *P. baeri* and *P. gularis* last shared a common ancestor 1.8–2.8 mya (uncorrected genetic p-distance of 4%), which corresponds closely to when the Araguaia/Tocantins river basin last discharged directly into the Amazon.

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Distribution; taxonomy; South American birds; speciation

Introduction

Many river-restricted bird species have differentiated within the enormous network comprising the Amazon and its tributaries (Remsen and Parker 1983; Rosenberg 1990). Approximately 15% of the non-aquatic avifauna in the basin is restricted to riverine-created habitats (Remsen and Parker 1983). However, the biogeographic patterns of species restricted to riverine habitats have been little investigated (Aleixo 2006). The role of Amazonian rivers as barriers to dispersal for upland forest birds has received considerably more attention (Haffer 1997; Naka *et al.* 2012), and river width and temporal sequence of river formation are related to the extent of differentiation in numerous bird taxa (Ribas *et al.* 2012; Weir *et al.* 2015).

The long and linear distributions of riverine-restricted species differ dramatically from most lowland birds whose distributions tend to encompass relatively continuous areas (Remsen and Parker 1983; Haffer 1997). This should result in different spatial patterns of gene flow in linear vs. non-linear distributions (Rohlf and Schnell 1971; Felsenstein 1976; Slarkin 1985; Graves 1988). The large perimeter/area ratio makes such linearly distributed populations more sensitive to demographic and ecological stochasticity than two-dimensional populations (Soulé and Simberloff 1986) and this would facilitate fragmentation by natural barriers (Graves 1988). As there are no obvious biogeographic barriers within rivers, present distributional limits appear to be largely determined by ecological factors or relatively recent changes in features of watercourses, including topological relationships between rivers and the soils and habitats dissected by them.

Amazonian rivers have been more frequently conceptualised as barriers to birds than as habitats with their own ecological and biogeographic histories. For

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example, the micro-endemic avifauna of riverine birds in the Araguaia basin has only recently been recognised, despite the fact that it includes the Araguaia Spinetail (*Synallaxis simoni*), an undescribed *Certhiaxis* spinetail (pers. obs.; D. R. C. Buzzetti and A. Whittaker, *in litt*. 2009), the Bananal Antbird (*Cercomacra ferdinandi*; Silva 1997; Silva and Bates 2002) and the Crimson-fronted Cardinal (*Paroaria baeri*). Populations of some Amazonian floodplain forest species lack phylogeographic structure, indicating lack of isolation between different rivers (Aleixo 2006). Consequently, the Araguaia riverine endemics must have evolved in isolation, which implies that the Araguaia was not connected to other rivers with source populations at some point in time.

The genus *Paroaria* comprises six to eight species separated into two ecological groups: the riverine Red-capped Cardinal (*P. gularis*), Crimson-fronted Cardinal (*P. baeri*), Xingu Cardinal (*P. xinguensis*), Yellow-billed Cardinal (*P. capitata*), Bolivian Cardinal (*P. cervicalis*) and Masked Cardinal (*P. nigrogenis*), and the open-forest Red-crested Cardinal (*P. coronata*) and Red-cowled Cardinal (*P. dominicana*) (Dávalos and Porzecanski 2009). Current evidence to recognise *P. xinguensis* and *P. cervicalis* as species is weak (Jaramillo 2011; Remsen *et al*. 2016). Among the riparian species, *Paroaria baeri* is narrowly distributed along the middle Rio Araguaia basin in central Brazil and *P. gularis* is widespread in the Amazonian lowlands with its distribution entering the lower Rio Araguaia (Figure 1). They have been historically considered parapatric or nearly parapatric species (Hellmayr 1907, 1908, 1929; Dávalos and Porzecanski 2009) but recent undocumented records suggest that they are sympatric along the Rio Araguaia (Buzzetti 2000; Pinheiro and Dornas 2009a; 2009b). Despite their marked morphological differences (Figure 2; Table 1) it has been speculated that *P. baeri* and *P. gularis* might intergrade (Ridgely and Tudor 1989). More recently,

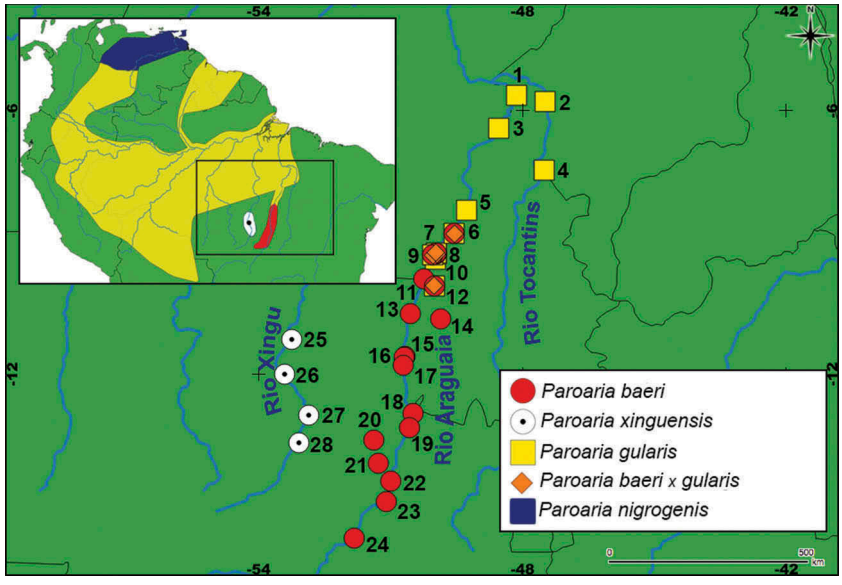

Figure 1. Geographic distribution of Masked Cardinal (*Paroaria nigrogenis*), Red-capped Cardinal (*P. gularis*), Crimson-fronted Cardinal (*P. baeri*), Xingu Cardinal (*P. xinguensis*), and hybrids of *P. baeri* × *P. gularis*. Localities with records of *P. baeri* and *P. gularis* showing the allopatric distribution of *P. baeri* and *P. xinguensis*, the area of overlap between *P. baeri* and *P. gularis* and the localities where *P. baeri* × *P. gularis* hybrids are known. See Figure 2 for photographs. Red circles: *P. baeri*; white circles with black centre: *P. xinguensis*; yellow squares: *P. gularis*; orange diamonds: hybrids of *P. baeri* × *P. gularis*; dark blue squares: *P. nigrogenis*. Inset: approximate geographic ranges of *P. nigrogenis, P. gularis* (nominate and subspecies *cervicalis* shown together), *P. baeri*, and *P. xinguensis* in South America. Modified from Ridgely *et al*. (2007). 1: Araguatins, 2: Itaguatins, 3: Xambioá, 4: Filadélfia/Carolina, 5: Conceição do Araguaia, 6: Araguacema, 7: Rio do Coco/Rio Araguaia, 8: Caseara, 9: Barreira de Campo, 10: Rio Furo da Barreirinha, 11: Fazenda Fartura/Fazenda Barra das Princesas, 12: Rio Javaés close to Centro de Pesquisa Canguçu/UFT, 13: Ilha do Bananal 1 (=Furo das Pedras), 14: Lagoa da Confusão, 15: Ilha do Bananal 2, 16: São Félix do Araguaia, 17: Pousada Kuryala,18: Fio Velasco (12°53′S, 50°29′W), 19: Chapéu do Plano, north of Luís Alves, 20: São Domingos, Rio das Mortes, 21: Rio Cristalino, 22: Boralina (= Cocalina, island close to Cocalinho), 23: Leopoldina, 24: Barra do córrego Ponte Alta, distrito de Registro do Araguaia, Montes Claros de Goiás, 25: Campo de Diauarum (=Acampamento Iauarun, Alto Xingu), 26: Jacaré, baixo Culuene, 27: Rio Culuene, 6.5 km by river upstream from junction with Rio Sete de Setembro, 28: Rio Culuene, 15 km by river upstream from bridge of road MT: 020.

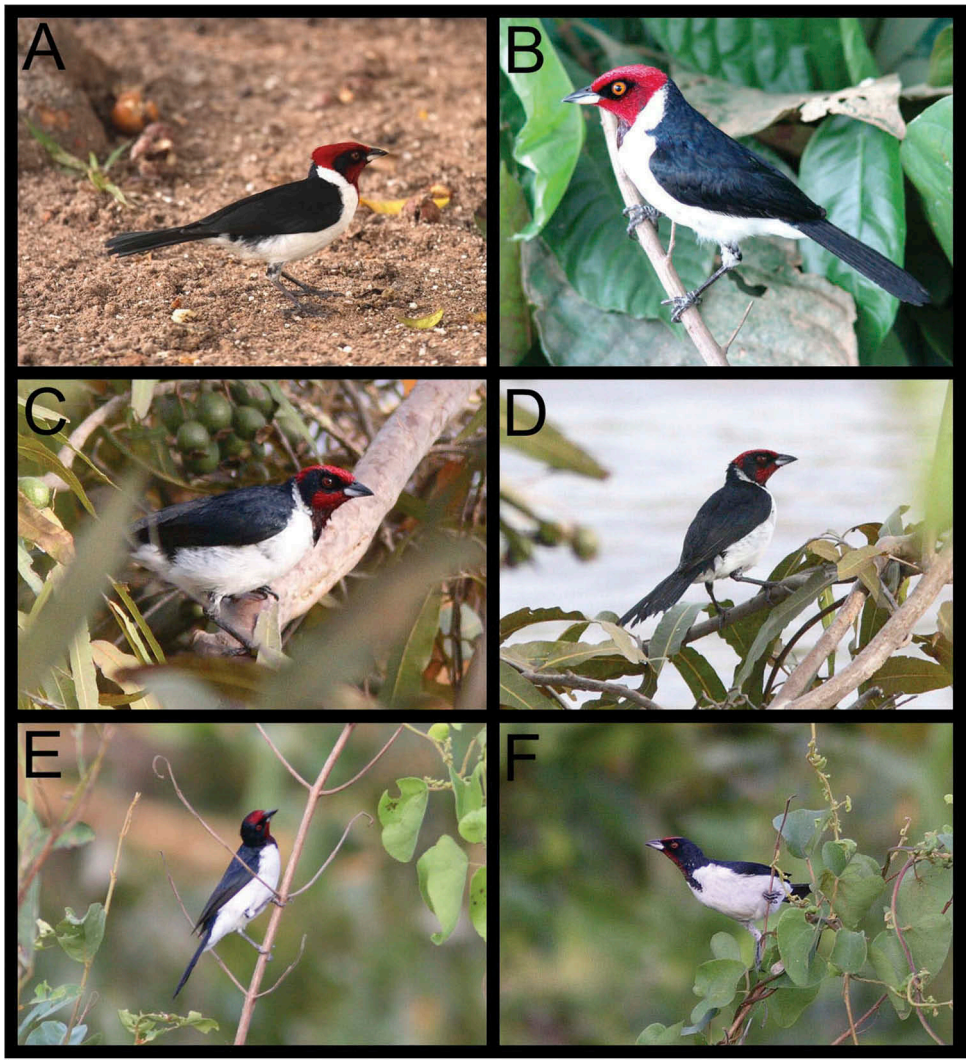

Figure 2. Intermediate phenotypes of *P. baeri* × *P. gularis* hybrids in comparison to pure parental forms (C–D vs. B and E–F), and the similarity between *P. nigrogenis* and *P. baeri* × *P. gularis* hybrids (A vs. C–D). (A) Masked Cardinal (*P. nigrogenis*) (Venezuela, L. Calcaño); (B) Red-capped Cardinal (*P. gularis*) (Parque Estadual do Cantão, Tocantins, Brazil, R. T. Pinheiro); (C) *P. baeri* × *P. gularis* hybrid (Barreira de Campo, Pará, Brazil, W. H. Price); (D) *P. baeri* × *P. gularis* hybrid (Barreira de Campo, Pará, Brazil, W. H. Price); (E) Crimson-fronted Cardinal (*P. baeri*) (Araguacema, Tocantins Brazil, W. H. Price); (F) *P. baeri* (Araguacema, Tocantins, Brazil, W. H. Price).

Table 1. Plumage features and measurements of Crimson-fronted Cardinal (*Paroaria baeri*), Red-capped Cardinal (*P. gularis*), *P. baeri* × *P. gularis* hybrids, and Masked Cardinal (*P. nigrogenis*). Measurements are from specimens in Appendix 1. Data displayed as average ± SD, range in square brackets and sample size in parentheses

	P. baeri	*P. gularis*	*P. baeri* ×*P. gularis*	*P. nigrogenis*
Cap colour	Crimson	Red	Crimson-red	Red
Cap extension	Forehead	Nape	Nape	Nape
Crested appearance	No	No	No	Yes
Auricular area	Black	Red	Black	Black
Neck-side	Black	White	White	White
Throat colour	Crimson	Red	Crimson-red	Red
Bib colour	Bluish-black	Crimson-red	Bluish-black	Red
Wing length (mm)	81.9 ± 2.6 [75.0–85.5] (14)	81.2 ± 2.3 [77.9–85.6] (7)	76.6 ± 4.5 [73.4–79.8] (2)	–
Tail length (mm)	79.6 ± 3.5 [70.5–83.8] (13)	75.9 ± 2.1 [72.0–78.0] (6)	82.5 ± 1.0 [81.8–83.2] (2)	–
Bill length (mm)	12.9 ± 0.7 [12.0–14.0] (14)	12.9 ± 0.7 [11.4–13.8] (7)	12.7 ± 2.0 [11.3–14.2] (2)	–

Jaramillo (2011) and Lopes and Gonzaga (2013) reported possible hybrids based on our unpublished data, which are presented fully here.

Our goals are twofold: to present morphological and genetic evidence indicating that *P. baeri* overlaps and hybridises with *P. gularis* along the Rio Araguaia in central Brazil, resulting in admixed individuals that resemble the more distantly related *P. nigrogenis*; and to discuss the ecological and biogeographic scenarios in which hybridisation occurs between *P. baeri* and *P. gularis*.

Methods

Specimens and field data

We examined 25 study specimens of *P. baeri*, 15 of *P. gularis* and two presumed hybrid *P. baeri* × *P. gularis*. We also examined eight specimens of *P. xinguensis*, because it occurs along the neighbouring Rio Xingu and has been historically considered a subspecies of *P. baeri*. Our sampling includes the type series of *P. baeri* and *P. xinguensis*; unfortunately there is no extant type specimen of *P. gularis* (Linnaeus 1766). We measured wing length (unflattened), tail length (base to tip of central pair of rectrices) and bill length (exposed culmen to the base of the feathers) to 0.1 mm. All specimens examined personally or via photographs are detailed in Appendix 1.

We visited the range of *P. baeri* as follows: between August 2005 and December 2006 and 7–14 July 2008 at the Centro de Pesquisa Canguçu/UFT and Cantão State Park (T.D.), 1–9 September 2010 at the Centro de Pesquisa Canguçu/UFT (J.I.A.), 24–27 January 2001 and 10–12 September 2004, environs of Caseara, Tocantins; 26 December 2009–6 January 2010, Araguaia Valley, from Registro do Araguaia, Goiás, north to Araguacema, Tocantins, and adjacent localities in Mato Grosso and Pará, respectively; 2–14 July 2011, from Registro do Araguaia north to Lagoa da Confusão, at the south-east edge of the Ilha do Bananal, Tocantins; 15–19 November 2011, from Caseara south to the Centro de Pesquisa Canguçu; and 7–12 January 2013, from Araguacema south to Lagoa da Confusão, Tocantins (G.M.K.).

We examined photographs of birds identified as *P. baeri* in search of potentially misidentified hybrids from the following localities (listed south to north): Pousada Kuryala, Mato Grosso (B. W. Davis, archived on www.surfbirds.com; M. Dionizio [WA199171], archived on www.wikiaves.com.br), São Félix do Araguaia, Mato Grosso (A. Alves [WA7810] and M. Moss [WA236891], www.wikiaves.com.br), Lagoa da Confusão, Tocantins (J. Guedes [WA261994] and E. Endrigo [WA262193], www.wikiaves.com.br), Furo de Sambaíba, Tocantins (C. Albano [WA292174], www.wikiaves.com.br), Rio Javaés near the Centro de Pesquisa Canguçu/Universidade Federal de Tocantins (F. Olmos [WA123435], M. A. Crozariol [WA165179] and E. Luiz [WA292174], www.wikiaves.com.br), Caseara, Tocantins (W. H. Price, A. Grosset; and G. Leite [WA43222], www.wikiaves.com.br) and Araguacema, Tocantins (W. H. Price).

Genetic analyses

We sub-sampled tissues from 15 specimens archived at the Coleção Ornitológica Fernando C. Novaes of the Museu Paraense Emílio Goeldi, Belém, Brazil (MPEG), and Departamento de Zoologia da Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (DZUFMG). These included eight samples of *P. gularis*, three of *P. baeri* and two of the presumed *P. baeri* × *P. gularis* hybrids (Appendix 2; supplemental material Figure S1). From GenBank we obtained sequences from all species of *Paroaria* (except *P. xinguensis*, which was unavailable to us) (Appendix 2; supplemental material Figure S1). We used sequences of Magpie Tanager (*Cissopis leverianus*) (GenBank accession number EU648033.1) as outgroup (Dávalos and Porzecanski 2009).

We sequenced the entire length of the mitochondrial cytochrome *b* (Cytb) gene, as well as intron 3 of the nuclear Z-linked muscle-specific receptor tyrosine kinase gene (MUSK) (Appendix 2). We extracted total DNA using standard procedures with the phenol-chloroform technique (Sambrook *et al.* 1989). We amplified genes using polymerase chain reaction (PCR); the total reaction volume was 25 µl, containing buffer (1X final concentration), 20 ng genomic DNA, 10 mM (1 µl) dNTPs, 3 mM $MgCl_2$, 1U *Taq* DNA polymerase, and 200 ng (0.5 µl) of each primer. The amplification profile included an initial step of 5 min at 95°C for temperature homogenisation of the block, followed by 35 cycles for 1 min at 95°C, 1 min at 45°C (Cytb) or 50°C (MUSK), 1 min at 72°C, and a final step of 5 min at 72°C. The amplified samples were checked for size through electrophoresis by means of a 1% agarose gel and purified with the Polyethylene Glycol protocol (PEG-8000). Amplification products were cycle-sequenced using the 'Big Dye Terminator Cycle Sequencing Standard Version 3.1' kit and electrophoresed using the Applied Biosystems ABI 3130 sequencer according to the manufacturer's specifications. For Cytb we used the primers L 14841 (sequence 5′ to 3′: GCT TCC ATC CAA CAT CTC AGC ATG ATG) and H 16064 (AAG TGG TAA GTC TTC AGT CTT TGG TTT ACA AGA CC), and for MUSK we used 13F (R) (CTC TGA ACA TTG TGG ATC CTC AA) and 13F (F) (CTT CCA TGC ACT ACA ATG GGA AA).

Nucleotide sequences were manually edited and aligned using BioEdit 7.0.5 (Hall 1999). To reconstruct allelic phases from males from the Z-linked MUSK genotypes, we used the program PHASE 2.1, and accepted the phases having a probability >70% (Stephens *et al.* 2001). A graphic plot of transitions vs. transversions for Cytb genetic distances was prepared using the software Data Analysis in Molecular Biology and Evolution – DAMBE (Xia and Xie 2001) to evaluate possible saturation in substitution rates among taxa. Mean uncorrected genetic divergences (p-distances) were calculated within and between all sequenced *Paroaria* species for which we recovered their phylogenetic position (see below). A mitochondrial gene tree was estimated from the Cytb sequences using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). To determine the best finite-sites model of molecular evolution, we used jModeltest 0.1.1. We ran MrBayes using the best fit model of evolution for Cytb and two independent runs of 5 000 000 generations each (three hot chains and one cold per run), sampling every 500 generations. TRACER 1.4 (Drummond and Rambaut 2007) was used to determine when runs reached stability. Trees (500 000) that were obtained before the Markov chain reached stable and convergent likelihood values were discarded. To determine diversification times of *Paroaria* species we performed a relaxed-clock analysis with Cytb in the program BEAST v 1.8.0 with mutation rate fixed at 2.1% per million years (Weir and Schluter 2008). We used a Yule speciation process and relaxed clock (uncorrelated lognormal) as priors, and ran the analysis for 100 million generations, sampling every 10 000 generations. A haplotype network for the MUSK gene was obtained using Network 4.6.1.2, including samples of *Paroaria gularis, P. baeri* and two specimens of the presumed hybrids *P. baeri* × *P. gularis* (Appendix 2). We sequenced a total of 1505 base pairs (bp) of the mitochondrial Cytb (1011 bp) and nuclear MUSK (494 bp) genes. Saturation was not observed for any of these genes. The best fit models of molecular evolution selected according to the BIC criterion were TPM1uf+I for Cytb and HKY for MUSK.

Results

Geographic distribution

The distributions of *Paroaria baeri* and *P. gularis* overlapped along *ca.*160 km of the Araguaia and Javaés Rivers, from Araguacema (Figure 1: 6) to the Furo de Sambaíba near Centro de Pesquisa Canguçu (Figure 1: 12). Sympatry of *P. baeri* and *P. gularis* is indicated by a photograph of a pure-plumaged *P. gularis* (Figure 2(B)) taken by R. T. Pinheiro at Rio Furo da Barreirinha (Figure 1: 10) in the Parque Estadual do Cantão and another photograph taken by A. De Luca at Araguacema (Figure 1: 6). These two photographs are the only documented records of *P. gularis* for the Rio Araguaia between Araguacema and the southern Ilha do Bananal. However, given what we now know about hybridisation, we cannot exclude the possibility that these 'pure' birds are actually advanced backcrosses (see below). Pure individuals of *P. gularis* have been observed on the banks of the Rio Araguaia *ca.*100 km north of Caseara, albeit rarely (J. F. Pacheco, *in litt.* 2011), and *P. gularis* was the only species found nesting at Xambioá (Buzzetti and Silva 2005; D. R. C. Buzzetti, *in litt.* 2010; Figure 1: 3). The southernmost confirmed locality at which only *P. gularis* occurs along the Rio Araguaia is Conceição do Araguaia (but see below for an undocumented report of hybrids with *P. baeri*).

Hybridisation

Documented records of birds with plumage features intermediate between those of *P. baeri* and *P. gularis* (Table 1; Figure 2; supporting information Figures S2–S3) come from five localities in which they coexist with presumed pure *P. baeri* and *P. gularis*: Araguacema (Figure 1: 6), junction of the Rio do Coco and Rio Araguaia (Figure 1: 7), Caseara (Figure 1: 8), Barreira de Campo (Figure 1: 9), and near the Centro de Pesquisa Canguçu (Figure 1: 12). We assume all phenotypically intermediate individuals to be *P. baeri* × *P. gularis* hybrids or backcrosses. Two additional undocumented observations of hybrids are from Conceição do Araguaia (A. De Luca, *in litt.* 2014; Figure 1: 5) and the direct environs of Caseara, on the Tocantins side of the river (J. F. Pacheco, *in litt.* 2010; Figure 1: 8). Sightings and photographic records are detailed in supplemental material Appendix S1 (see also Minns et al. [2009]).

Two hybrid specimens from Praia do Sol, at the junction of the Rio do Coco and Rio Araguaia, Tocantins (Appendix 2) were previously misidentified as *P. baeri* by Lopes (2009). The two specimens are characterised by plumage features intermediate between *P. baeri* (supporting information Figure S2A–C) and *P. gularis* (supporting information Figure S2D–F). In the male (DZUMFG-6215; supporting information Figure S3A–D) the crimson extends well behind the eye but narrows to a point on the mid-nape, while the ear coverts are bordered posteriorly by a white crescent mottled with black, which extends from the chest almost as far as the mid-nape. The

throat and malar stripe are both extensively crimson (identical to the crown), but the throat patch becomes extensively mottled black over its lower third. In the female (DZUMFG-6216; supporting information Figure S3E–H) the entire crown and nape are more reddish (thereby more closely resembling *P. gularis*), albeit with some black feathers admixed, especially behind the eyes. Some red feathers are scattered over the throat and malar area, and these are slightly darker than the crown. The specimen also possesses narrow white neck-sides reaching well onto the nape, bordering the posterior ear coverts, but these are difficult to detect due to the specimen's preparation. Most foreneck feathers are missing, making evaluation of their shape impossible. Our small sample of measurements of pure parental and presumed hybrid specimens does not help to clarify the situation, as hybrids fall within the range of variation found in the parental species (Table 1).

The two phenotypically hybrid *P. baeri* × *P. gularis* specimens grouped into different mitochondrial clades despite being collected at the same locality. While the male was recovered with high support in the *P. gularis* clade, the female grouped with high support in the *P. baeri* clade (Figure 3). Conversely, in the MUSK haplotype network, the male shared nuclear alleles with *P. baeri* (H-1 and H-2), while the nuclear allele (H-4) of the hemizygous female was closest to *P. gularis* alleles (H-6; Figure 4). From these patterns we can infer that both the male and female specimens are admixed (Figure 3; Figure 4). We can also infer that the mother of the male specimen was also admixed, because she transmitted a *P. gularis* mitochondrial Cytb sequence and a *P. baeri* MUSK allele to her son (Figure 3; Figure 4). We can conclude that at least some *P. baeri* × *P. gularis* hybrids are capable of producing viable offspring and that post-zygotic reproductive barriers are not complete. More records are needed to better address viability and fertility of hybrids.

In terms of broader phylogenetic results within *Paroaria* the mitochondrial gene tree contained a deep split between open-forest (*P. dominicana* and *P. coronata*) and riparian species (all other *Paroaria*) (Figure 3). Within the riparian clade, *P. nigrogenis* and *P. baeri* were successive sisters to other riparian *Paroaria; P. capitata* was sister to *P. cervicalis* and these two were sister to *P. gularis*. Importantly, *P. baeri* and *P. gularis* were not sister to each other and *P. nigrogenis* was 'basal' to both (Figure 3). Divergence times ranged from 3.7 to 5.0 mya for the split between the open-forest vs. riparian clades, to 0.9–1.6 mya for the *P. cervicalis*/*capitata* split, although both of the extreme events lacked statistical support. *Paroaria baeri and P. gularis* last shared a common ancestor 1.8–2.8 mya, and *P. nigrogenis* last

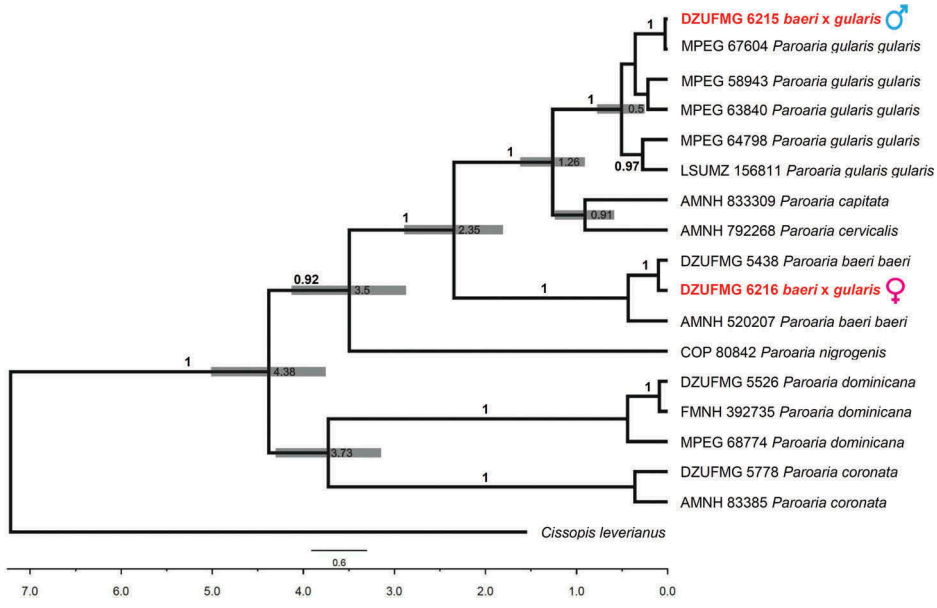

Figure 3. Bayesian tree recovered for the genus *Paroaria* based on 1011 bp of the mitochondrial cytochrome *b* (Cytb) gene. Numbers above branches represent posterior Bayesian probabilities. Numbers in grey bars represent mean divergence time, grey bars denote the confidence interval of the estimated divergence times, and numbers on the time scale below represent millions of years before present. Note the differential placement of the two syntopic *P. baeri* × *P. gularis* hybrids DZUFMG 6215 (male) and 6216 (female) into *P. gularis* and *P. baeri* clades. See Appendix 2 for detailed specimen and sequence information, and supporting information Figure S3 for images of these hybrids.

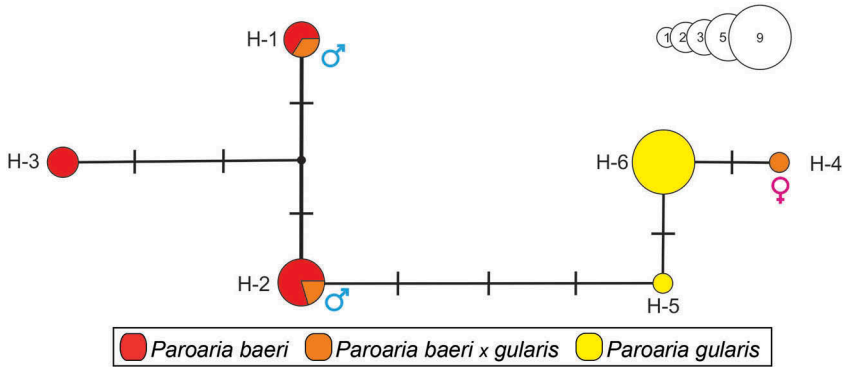

Figure 4. Haplotype (allele) network based on 494 bp of the Z-linked MUSK gene. Circle size denotes number of times each haplotype was found (see references in white circles), and different colours denote haplotypes belonging to different *Paroaria* species or hybrids. Red: *P. baeri* (n = 3 specimens sequenced, with alleles H-1, H-2 and H-3); orange: *P. baeri* × *P. gularis* hybrids (n = 2 specimens, with alleles H-1, H-2 and H-4); yellow: *P. gularis* (n = 8 specimens with alleles H-5 and H-6). Note the clustering of allele H-4 from the hemizygous female *P. baeri* × *P. gularis* hybrid (DZUFMG 6216) with *P. gularis* alleles. Alleles H-1 and H-2 (both in orange) from the heterozygous male *P. baeri* × *P. gularis* hybrid (DZUFMG 6215) are shared with pure *P. baeri* specimens. See Table A1 for specimen information.

shared a common ancestor with these two 2.7–4.2 mya (Figure 3). Levels of uncorrected genetic divergences (p-distances) varied within species from 0.5 to 0.8%, and among species from 2% between *P. capitata* and *P. cervicalis* to 9% between *P. baeri* and *P. dominicana* (supplemental material Appendix S2). The pairwise genetic distance between *P. baeri* and *P. gularis* was 4% (supplemental material Appendix S2).

Discussion

We documented the syntopic occurrence of *P. baeri* and *P. gularis* along the middle Rio Araguaia, Brazil (Figure 1), as well as their coexistence with admixed *P. baeri* × *P. gularis* individuals (Figure 2; Figure 3; Figure 4; supporting information Figures S2–S3). Confirmed and purported hybrids occur on both sides of the Rio Araguaia and are known definitively from five localities: four in Tocantins and one in Pará (Figure 1; Figure 2). The restricted range of the confirmed and presumed admixed individuals precisely where the distributions of *P. baeri* and *P. gularis* overlap on the Rio Araguaia suggests the existence of a hybrid zone between them. The *ca.*160 km-long area of hybridisation encompasses >20% of the distribution of *P. baeri*, while it represents a very small part of the overall range of *P. gularis* (Figure 1). Additional sampling will be needed to better characterise the structure and dynamics of the hybrid zone. Syntopy of admixed individuals with presumed pure individuals in the zone would be consistent with recent contact and hybridisation, infrequent hybridisation between species that have been in longer contact, or strong selection against hybrids.

The genetic data unequivocally indicate the existence of two genetically admixed *P. baeri* × *P. gularis* individuals. Observations of birds having a hybrid phenotype paired with presumed pure *P. baeri* and a presumed mated pair of hybrids also suggest pre-mating isolating mechanisms between the species are incomplete. Our sampling was too limited to formally reject incomplete lineage sorting as an alternative explanation for shared alleles at the MUSK gene, but the geographic distribution of shared alleles close to the contact zone is inconsistent with incomplete lineage sorting. Additional phylogenetic and phylogeographic studies with more thorough genetic sampling of *Paroaria* populations would allow us to better distinguish introgression from incomplete lineage sorting as explanations for the shared variation.

The plumage of the *P. baeri* × *P. gularis* hybrids described here strikingly resembles that of *P. nigrogenis*, whose geographic range is far distant from the *P. baeri* × *P. gularis* hybrid zone in south-east Amazonian Brazil (Figure 1; Figure 2; Table 1). *Paroaria nigrogenis* occurs in the Llanos of Venezuela and Colombia, although it penetrates south as far as the Rio Negro basin in northernmost Brazil (Restall *et al.* 2006). The hybrids differ from *P. nigrogenis* by their darker crimson head, black lower bib, metallic blue upperparts and, in some cases, a shorter crimson cap with no discernible 'crest'. These differences, and the phylogenetic placement of *P. baeri* × *P. gularis* hybrids and *P. nigrogenis*, reject the hypothesis that these hybrids could belong to an isolated population of *P. nigrogenis* in the Araguaia basin (Figure 3; Figure 4). Plumage similarities between the hybrids and *P. nigrogenis* may simply reflect the existence of a

limited palette of colours and patterns in the developmental plan of the genus *Paroaria*.

Biogeographical aspects of hybridisation

To understand hybridisation among the riparian *Paroaria*, the history of the rivers they inhabit must be considered. The Araguaia/Tocantins Rivers last discharged directly into the Amazon *ca*.1.8 mya, closely corresponding to divergence times between *P. baeri* and the sister clade of *P. gularis*/*cervicalis* and *P. capitata*, and divergence times between the dolphins *Inia geoffrensis* and *I. araguaiensis* (Rosetti and Valeriano 2007; Hrbek *et al.* 2014). These data suggest that separation of the Araguaia/Tocantins river basin from the Amazon isolated populations of riparian *Paroaria* and *Inia* that subsequently differentiated into present-day endemics. As four bird species are endemic to the Rio Araguaia, other isolating phenomena are needed to satisfactorily explain their absence from the Rio Tocantins.

The zone of secondary contact and hybridisation between *P. baeri* and *P. gularis* appears to be of very recent origin. The lack of hybrid specimens in areas historically inhabited by just one species (but where hybrids are apparently frequent at present) supports this hypothesis. For example, although Hellmayr (1929, 1938) reported only pure *P. gularis* from Conceição do Araguaia, presumed sightings of *P. baeri* × *P. gularis* hybrids have been reported from this locality in the present work, suggesting that their geographic overlap is very recent and extends further north than documented records.

Spatial situations analogous to that of *P. baeri* and *P. gularis* probably also occur in other *Paroaria* species. Although *P. nigrogenis* and *P. gularis* occur in close proximity in Colombia (Hilty and Brown 1986) and were reported to occur sympatrically in southwest Venezuela, no hybrids are known from these areas (Restall *et al.* 2006; the sympatry report was based on a misidentified bird *fide* J. Pérez-Emán, *in litt*. 2010). On the other hand, *P. gularis* and *P. cervicalis* may overlap in eastern Bolivia and southwest Brazil, and the type specimen of *P. cervicalis* was suggested to be a hybrid between pure '*cervicalis*' and *P. capitata* (Hellmayr 1938). Species limits appear 'fuzzy' among riparian *Paroaria*, and future studies could provide further insights into the development of breeding barriers and hybridisation in *Paroaria* cardinals. For example, *P. coronata* has hybridised in captivity with *P. nigrogenis* and *P. dominicana* (yielding fertile offspring with the latter), and in captivity with a taxonomically varied suite of species with which it overlaps in nature without hybridising (McCarthy 2006).

The narrow front of the contact zone between *P. gularis* and *P. baeri* in comparison to their long riverine distribution in the Rio Araguaia results in a filiform shape that could behave like a one-dimensional hybrid zone (Figure 1); this contact zone between two mainly parapatric species is thus of considerable theoretical and ecological interest as it would enable study of the effect of hybridisation over a linear space (Woodruff 1973; Graves 1988). Many riverine species respond to flooding events by moving along riparian habitats (Sick 1967; Remsen and Parker 1983), as *P. baeri* does along main rivers and their tributaries (Sick 1950). *Paroaria baeri* nests during the wet season and flooding may cause breeding failures (Sick 1950; Dornas 2008), which can promote further movements. These dynamic processes could push the contact zone between *P. baeri* and *P. gularis* back and forth, facilitating or restricting hybridisation.

We are not aware of other documented one-dimensional hybrid zones involving neotropical riparian species. However, Weir *et al.* (2015) documented hybrid zones among seven parapatrically distributed species/subspecies pairs of Amazonian upland *terra-firme* birds, which come into contact along the headwaters of the Tapajós River in southern Amazonian Brazil. Even though the sampling scale of this study was rather coarse, F1 hybrids and introgressed individuals were found only in a narrow area where lineages met away from major rivers. Overall, these results are consistent with the relatively small geographic area where hybrids and introgressed individuals were documented between *P. baeri* and *P. gularis* in the mid-lower Araguaia River. Interestingly, Weir *et al.* (2015) showed that the sampled lineages continued to hybridise despite 1–4 million years of divergence from a common ancestor, which is within the time frame for the estimated *P. baeri* and *P. gularis* divergence (1.8–2.8 mya), as also estimated based on Cytb sequences. These ages are considerably older than those reported for hybridising avian lineages in the temperate zone and imply that reproductive isolation may evolve slower in tropical when compared to temperate latitudes, as suggested previously (Weir and Price 2011; Lawson and Weir 2014). Finally, instances of hybridisation such as those reported here and by Weir *et al.* (2015) show that lack of complete reproductive isolation does not automatically indicate that the hybridising /introgressed lineages constitute a single species. In fact, these studies indicate that the intensity and impact of gene flow away from the immediate contact zone might be a more accurate measure of the true level of evolutionary independence

and hence a better estimator of inter-specific limits between two hybridising lineages (Gill 2014).

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Appendices

Appendix 1

Specimens examined – acronyms: AMNH (American Museum of Natural History); CM (Carnegie Museum); FMNH (Field Museum of Natural History); MOG (Museu de Ornitologia de Goiânia); DZUFMG (Departamento de Zoologia da Universidade Federal de Minas Gerais); MPEG (Museu Paraense Emilio Goeldi); MNRJ (Museu Nacional de Rio de Janeiro); and MZUSP (Museu de Zoología da Universidade de São Paulo).

Paroaria baeri. AMNH – 520205 [male; holotype], 520206 [male], 520207 [female]. DZUFMG – 5438 [female], 5439 [male], 6214 [female]. FMNH – 356584 [sex?]. MNRJ – 14813 [female], 14814 [male], 14815 [male]. MOG – nn

[nn]. MPEG – 49191 [male]. MZUSP – 17558 [female], 17559 [male], 35324 [male], 35235 [male], 35236 [male], 35237 [male], 34259 [female], 35328 [female], 35330 [female], 88304 [male], 88305 [female], 88306 [male], 85420 [female].

Paroaria xinguensis. AMNH – 802297 [male], 802298 [female]. MNRJ – 26230 [male; holotype], 31209 [female; paratype], 31210 [male; paratype], 31211 [female; paratype], 31256 [male; paratype], 31259 [male; paratype].

Paroaria gularis. FMNH – 63646 [female?], 63647 [male]. MOG – 721 [sex?]. MPEG – 20673 [male], 20674 [male], 21974 [male], 21975 [female]. MZUSP – 53117 [female], 53118 [female], 53119 [male], 53120 [female], 53121 [male], 53122 [male], 81152 [unknown]. CM – 101762 [female].

Hybrids *P. baeri* × *P. gularis*. DZUFMG – 6215 [male], 6216 [female].

Appendix 2.

Tissue samples sequenced in this study for the mitochondrial cytochrome *b* (Cytb) gene and the intron 3 of the nuclear gene muscle-specific receptor tyrosine kinase (MUSK), linked to the Z sexual chromosome in birds. GenBank mitochondrial cytochrome *b* (Cytb) sequences used in molecular analyses

Voucher/accession number[a]	Taxon	Locality
DZUFMG 5438	*Paroaria baeri*	Brazil, Goiás, Montes Claros de Goiás, Barra do córrego Ponte Alta (15° 44′ 02″ S, 51° 49′ 40″ W)
DZUFMG 5439	*Paroaria baeri*	Brazil, Goiás, Montes Claros de Goiás, Barra do córrego Ponte Alta (15° 44′ 02″ S, 51° 49′ 40″ W)
DZUFMG 6214	*Paroaria baeri*	Brazil, Tocantins, Caseara, Praia do Sol, margem direita do Rio do Coco (09° 17′ 45″ S, 49° 57′ 47″ W)
AMNH 520207/FJ715683	*Paroaria baeri*	Brazil, Goiás, Rio Araguaia
DZUFMG 6215	*Paroaria baeri* × *P. gularis*	Brazil, Tocantins, Caseara, Praia do Sol, margem direita do Rio do Coco (09° 16′ 27″ S, 49° 58′ 20″ W)
DZUFMG 6216	*Paroaria baeri* × *P. gularis*	Brazil, Tocantins, Caseara, Praia do Sol, margem direita do Rio do Coco (09° 14′ 56″ S, 49° 58′ 43″ W)
COP 80842/FJ715679	*Paroaria nigrogenis*	Venezuela, Bolívar, Río Caroni, Isla El Hornero
DZUFMG 5778	*Paroaria coronata*	Brazil, Mato Grosso, Cáceres, Fazenda Baía de Pedra (16° 27′ 59″ S, 58° 09′ 06″ W)
AMNH 833850/J715656	*Paroaria coronata*	Uruguay, Río Negro, Estancia Las Flores, E of Ruta 3 (270 km), 13 km along Ruta 20 to Pueblo Greco
DZUFMG 5526	*Paroaria dominicana*	Brazil, Minas Gerais, Januária, Fazenda Três Irmãs, Refúgio da Vida Silvestre Rio Pandeiros (15° 39′ 59″ S, 44° 37′ 59″ W)
FMNH 392735/FJ715664	*Paroaria dominicana*	Brazil, Sergipe, Caninde do São Francisco, Curituba, Fazenda Serrote
AMNH 833309/FJ715667	*Paroaria capitata*	Bolivia, Santa Cruz, Velasco, near localidad El Tuná, 300 m N of Rio Mercedes
AMNH 792268/FJ715678	*Paroaria cervicalis*	Bolivia, El Beni, Río Itenez
LSUMZ 156811/FJ715676	*Paroaria gularis*	Peru, Ucayali, west bank of Río Shesha,65 km ENE of Pucallpa
MPEG 78101	*Paroaria gularis*	Brazil, Tocantins, Araguaína, Ilha dos Cavalos, Rio Araguaia (7° 37′ 27.77″ S, 49° 22′ 52.77″ W)
MPEG 78111	*Paroaria gularis*	Brazil, Tocantins, Araguaína, Ilha dos Cavalos, Rio Araguaia (7° 37′ 27.77″ S, 49° 22′ 52.77″ W)
MPEG 63840	*Paroaria gularis*	Brazil, Acre, Feijó, Rio Envira, Novo Porto, Foz do lg. Paraná do Ouro (8° 27′ 35.5″ S, 70° 33′ 22.9″ W)
MPEG 58943	*Paroaria gularis*	Brazil, Acre, ESEC Rio Acre, Acampamento 1 (11° 03′ 05.2″ S, 70° 12′ 59″ W)
MPEG 63669	*Paroaria gularis*	Brazil, Acre, Porto Acre, AC 010 linha 07, Reserva Humaitá (09° 45′ 47.8″ S, 67° 36′ 32.9″ W)
MPEG 64798	*Paroaria gularis*	Brazil, Pará, FLOTA de Faro, *ca*.70 km NW of Faro (01° 42′ S, 57° 12′ W)
MPEG 67604	*Paroaria gularis*	Brazil, Mato Grosso, Paranaíta, Rio Teles Pires (ilha) (9° 24′ 00.5″ S, 56° 33′ 53.9″ W)
MPEG 68774	*Paroaria gularis*	Brazil, Piauí, Castelo do Piauí, Fazenda Bonito, ECB (5° 12′ 42.1″ S, 41° 42′ 13.3″ W)

[a]Collection acronyms: American Museum of Natural History, New York, USA (AMNH); Colección Ornitológica Phelps, Caracas, Venezuela (COP); Departamento de Zoologia da Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (DZUFMG); Field Museum of Natural History, Chicago, USA (FMNH); Louisiana State University Museum of Natural Science, Baton Rouge, USA (LSUMZ); Museu Paraense Emílio Goeldi, Belém, Brazil (MPEG).