Biogeographic history of the species complex *Basileuterus culicivorus* (Aves, Parulidae) in the Neotropics

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ABSTRACT

The taxonomic status of *Basileuterus culicivorus* complex has been under debate for a long time. There are uncertainties about the relationships among recognized species (*B. culicivorus* and *B. hypoleucus*), and whether *B. culicivorus* can be considered as a single species. In order to elucidate the phylogenetic relationships among these species and their biogeographic history we used a broad geographic sampling from Mexico to Argentina of *B. culicivorus* and *B. hypoleucus*. Using a mitochondrial gene, a nuclear intron and microsatellites we identified highly structured groups according to their spatial distribution. The known subspecies of *B. culicivorus* formed monophyletic groups, except two ones from Brazil and Paraguay. *Basileuterus hypoleucus* sequences did not form a monophyletic clade, being more related to *B. culicivorus* from Brazil and Paraguay. The divergence time analysis indicated that the deep separation of the basal lineages of *B. culicivorus* occurred in Late Pliocene. Contrasting with the other geographically structured populations from Central and South America, the clade of birds from Brazil, Paraguay and Argentina showed a remarkably high population growth starting in the mid-Pleistocene, according to the most plausible evolutionary scenario expected for parulid birds of a range expansion directed southwards.

1. Introduction

The genus *Basileuterus* consists of 24 species inhabiting mainly South America (Curson et al., 1994). The group occurs mostly in forests and woodland undergrowth (Ridgely and Tudor, 1989). The Golden-crowned Warbler, *Basileuterus culicivorus*, has a broad and discontinuous distribution from North Mexico to Argentina and Brazil, and it is often associated with heterogeneous flocks acting as a nuclear species (Maldonado-Coelho and Marini, 2004). It is considered a common species (Curson et al., 1994), and in some forests can be the most abundant bird species (Sick, 2001). It is divided in thirteen subspecies that can be grouped into three allopatric groups ("culicivorus", "cabanisi" and "auricapillus"), which were previously considered as three separate species (Curson et al., 1994; Ridgely and Tudor, 1989). The White-bellied Warbler, *B. hypoleucus*, occurs in the interior of Brazil and Paraguay, and resembles the Golden-crowned Warbler except for its underparts that are typically white, instead of yellow.

Species delimitation between *B. culicivorus* and *B. hypoleucus* is still not well defined, and sometimes they are both referred as part of the *B. culicivorus* complex, whose species division have been often questioned by renowned ornithologists (Pinto, 1944; Ridgely

and Tudor, 1989; Sick, 2001). Presently, the American Ornithologists' Union (AOU) (Emsen et al., 2008) and the Brazilian Ornithological Records Committee (CBRO, 2009) consider the taxa *B. culicivorus* and *B. hypoleucus* as two separate species. Sick (2001) considers the Golden-crowned Warbler and the White-bellied Warbler as a single species (*B. culicivorus*) with two subspecies (*B. c. culicivorus* and *B. c. hypoleucus*). Silva (1992) after an extensive study on the taxonomy and behavior of these two species, concluded that *B. hypoleucus* could be a geographic race of *B. culicivorus*. The AOU mention that *B. culicivorus* and *B. hypoleucus* are almost certainly allospecies (allopatric populations) (Emsen et al., 2008), which could have diverged recently from a common ancestral population. Several authors (Melo-Junior et al., 2001; Robbins et al., 1999) have used morphological characters to indicate a probable hybridization between sympatric populations of *B. culicivorus* and *B. hypoleucus*. Silva (1992) documented an extensive interbreeding in southeastern Brazil, detecting several mixed-species pairs and varying degrees of hybridization, with individuals bearing flanks with different grades of yellow and white parts, and showing that both species responded in play-back records of each other's song. Robbins et al. (1999) recognized also a similar situation for both taxa in Paraguay.

Although a major part of the Parulidae diversity is present in North America, some species have radiated into South America. The genetic studies with three Parulidae genera occurring in the Neotropics, *Parula* (Lovette and Bermingham, 2001), *Phaeothlypis*

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(Lovette, 2004), and *Myioborus* (Pérez-Emán, 2005) have indicated basal lineages in the northern distribution of these taxa. This hypothesis was also presented by Ericson et al. (2003), who suggested that the primary radiation of Oscines took place in North America.

Studies of historical biogeography indicated that the Late Pliocene and Early Pleistocene was an important period for avian differentiation in North America (Pérez-Emán, 2005). However, Lovette (2004) studied six populations of the *Phaeothlypis* complex, a genus positioned within *Basileuterus* according to the wood warbler phylogeny (Lovette and Bermingham, 2002), evidenced that the lineages were separated in a period considerably earlier than the Pleistocene/Pliocene boundary. This is an indication that the genus *Basileuterus* originated long before the Pleistocene/Pliocene boundary, since the *Phaeothlypis* group is nested within the more ancient *Basileuterus* radiation.

In this study, our main goal is to study populations of *Basileuterus culicivorus* and *B. hypoleucus* using nuclear and mitochondrial markers and a broad geographic sampling to address the following questions: (i) do genetic data support the current taxonomic classification?; (ii) how much gene flow is there between *B. culicivorus* and *B. hypoleucus*?; (iii) can we reconstruct the likely scenarios for the origin and expansion of these parulid species found in South America?; (iv) which historical pattern recovered from data can explain the current distribution and divergence among populations? We have used mtDNA and nuclear sequence data to investigate the deep phylogeny, phylogeography and population structuration history of the *Basileuterus culicivorus* complex, and microsatellites to provide some extra information concerning population differentiation and gene flow.

2. Methods

2.1. Sample collection

A total of seven subspecies of *B. culicivorus* was investigated, covering most of the entire range of the species (Fig. 1) and the three allopatric groups (Table 1). Among the seven subspecies, were included: *B. c. basherii*, *B. c. flavescens*, *B. c. culicivorus*, *B. c. occultus*, *B. c. olivascens*, *B. c. azarae* and *B. c. auricapillus*. Each subspecies was identified either in field or according to its defined distribution (Curson et al., 1994; Restall et al., 2005). The subspecies *basherii*, *flavescens* and *culicivorus* are classified within the "culicivorus" group; *occultus* is the only subspecies representing the allopatric group "cabanisi", which is restricted to Venezuela and Colombia; and the subspecies *olivascens*, *azarae* and *auricapillus* are within the "auricapillus" group. The species *B. hypoleucus* was also sampled from most of its range (Table 1 and Fig. 1).

Tissue and DNA samples are deposited at the DNA bank of our University (BD-LBEM/UFMG) (www.icb.ufmg.br/lbem/ddb). Some tissues were borrowed from collections of the Museu Paraense Emilio Goeldi (MPEG), Laboratório de Genética e Evolução Molecular de Aves (LGEMA) from Universidade de São Paulo (USP), University of Kansas Natural History Museum (KUNHM), Field Museum of Natural History (FMNH), and American Museum of Natural History (AMNH). Twenty-six museum skins from the Ornithological Collection, Department of Zoology, from Universidade Federal de Minas Gerais were included only in the microsatellite analysis. In total, 58 out 150 DNA samples are also deposited as specimen vouchers in the Ornithological collections (Table 1).
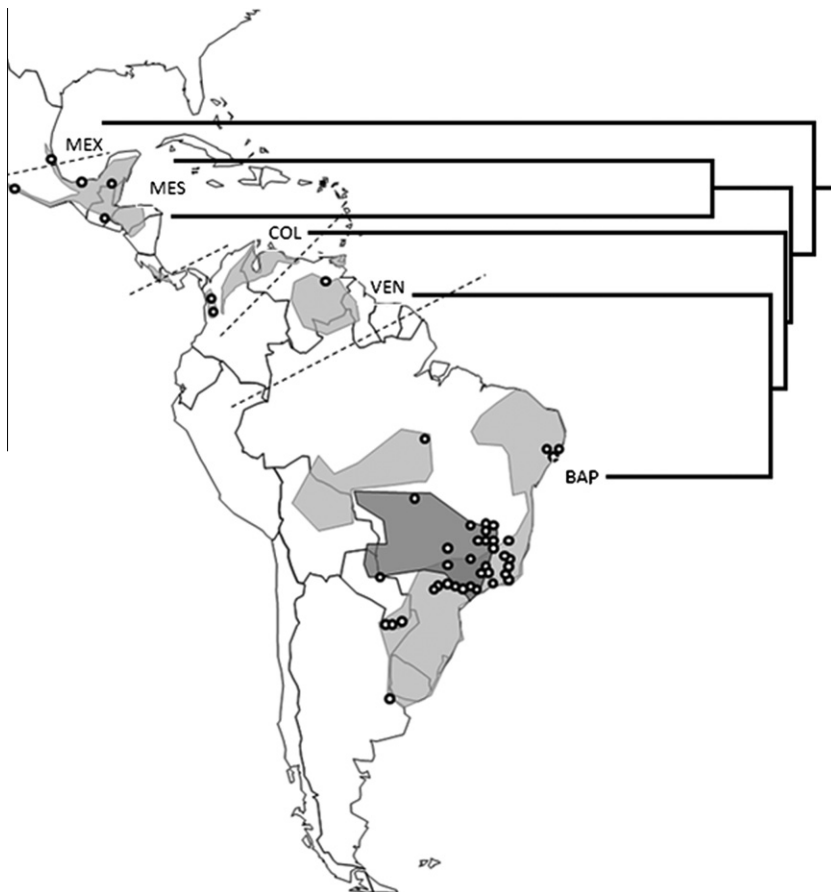

Fig. 1. Distribution map of the species *Basileuterus culicivorus* (light gray) and *B. hypoleucus* (dark gray) with samples used in this study. The dotted lines shows probable genetic breaks of each clade showed in the phylogenetic tree.

Table 1
Basileuterus samples used in this study.

Species	Subspecies[‡]	Catalogue number	Locality	Cyt-*b*	BF5
Basileuterus culicivorus	*olivascens*	AMNH GFB 2876	Venezuela: Bolivar, Cerro Guanay	GU189067	GU189189, GU189210
Basileuterus culicivorus	*olivascens*	AMNH GFB 2877	Venezuela: Bolivar, Cerro Guanay	GU189068	GU189189, GU189196
Basileuterus culicivorus	*basherii*	AMNH PEP 1241	Mexico: Molango	GU189069	GU189237, GU189238
Basileuterus culicivorus	*basherii*	AMNH PEP 1254	Mexico: Molango	GU189070	GU189230, GU189235
Basileuterus culicivorus	*basherii*	AMNH PEP 1255	Mexico: Molango	GU189071	GU189236, GU189237
Basileuterus culicivorus	*basherii*	AMNH PEP 1279	Mexico: Molango	GU189072	GU189237, GU189238
Basileuterus culicivorus	*olivascens*	AMNH PRS 797	Venezuela: Bolivar, Cerro Guanay	GU189073	GU189189, GU189189
Basileuterus culicivorus	*olivascens*	AMNH PRS 805	Venezuela: Bolivar, Cerro Guanay	GU189074	GU189182, GU189182
Basileuterus culicivorus	*azarae*	AMNH PRS 1098	Argentina: Buenos Aires	GU189075	
Basileuterus hypoleucus		DZ0270	Brazil: Uberlândia, Minas Gerais		
Basileuterus hypoleucus		DZ2896	Brazil: Contagem, Minas Gerais		
Basileuterus hypoleucus		DZ3161	Brazil: Belo Horizonte, Minas Gerais		
Basileuterus hypoleucus		DZ3163	Brazil: Itumirim, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ3584	Brazil: Leme do Prado, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ3595	Brazil: Francisco Sá, Minas Gerais		
Basileuterus hypoleucus		DZ3704	Brazil: São Gonçalo do Rio Abaixo, Minas Gerais		
Basileuterus hypoleucus		DZ3775	Brazil: Perdões, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ3825	Brazil: Grão Mogol, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ3861	Brazil: José Goncalves de Minas, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ3862	Brazil: José Gonçalves de Minas, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ3871	Brazil: Turmalina, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ4071	Brazil: Leme do Prado, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ4266	Brazil: Mariana, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ4347	Brazil: Mariana, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ4352	Brazil: Mariana, Minas Gerais		
Basileuterus hypoleucus		DZ4369	Brazil: Perdões, Minas Gerais		
Basileuterus hypoleucus		DZ4453	Brazil: Nova Lima, Minas Gerais		
Basileuterus hypoleucus		DZ4821	Brazil: Santa Bárbara, Minas Gerais		
Basileuterus hypoleucus		DZ4861	Brazil: Congonhas, Minas Gerais		
Basileuterus culicivorus	*azarae*	DZ4919	Brazil: Divino, Minas Gerais		
Basileuterus hypoleucus		DZ4949	Brazil: Brazilândia de Minas, Minas Gerais		
Basileuterus hypoleucus		DZ4979	Brazil: Chapada dos Guimarães, Mato Grosso		
Basileuterus hypoleucus		DZ4980	Brazil: Chapada dos Guimarães, Mato Grosso		
Basileuterus hypoleucus		DZ4981	Brazil: Chapada dos Guimarães, Mato Grosso		
Basileuterus hypoleucus		DZ5016	Brazil: Felixlândia, Minas Gerais		
Basileuterus hypoleucus		DZ5200	Brazil: Catas Altas, Minas Gerais		GU189191, GU189215
Basileuterus hypoleucus		DZ5215	Brazil: Congonhas, Minas Gerais	GU189076	GU189207, GU189210
Basileuterus hypoleucus		DZ5225	Brazil: Congonhas, Minas Gerais	GU189077	GU189196, GU189198
Basileuterus hypoleucus		DZ5311	Brazil: Gonçalves, Minas Gerais	GU189078	GU189182, GU189217
Basileuterus culicivorus	*azarae*	DZ5641	Brazil: Gonçalves, Minas Gerais	GU189079	GU189182, GU189196
Basileuterus culicivorus x hypoleucus		DZ5642	Brazil: Gonçalves, Minas Gerais	GU189080	GU189182, GU189186
Basileuterus culicivorus[*]	*flavescens*	FMNH 343400	Mexico: Puerto los Mazos, Sierra de Manantlan	GU189081	GU189191,GU189193
Basileuterus culicivorus[*]	*flavescens*	FMNH 343401	Mexico: Puerto los Mazos, Sierra de Manantlan	GU189082	GU189191, GU189204
Basileuterus culicivorus	*culicivorus*	FMNH 343404	Mexico: El Bastonal, Sierra de Santa Martha	GU189083	GU189233, GU189234
Basileuterus culicivorus	*culicivorus*	FMNH 393946	Mexico: Sierra de Santa Martha	GU189084	GU189233, GU189236
Basileuterus culicivorus	*culicivorus*	FMNH 393947	Mexico: Sierra de Santa Martha	GU189085	GU189191, GU189203
Basileuterus culicivorus[*]	*flavescens*	FMNH 393948	Mexico: Puerto los Mazos, Sierra de Manantlan	GU189086	
Basileuterus culicivorus[*]	*flavescens*	FMNH 393949	Mexico: Puerto los Mazos, Sierra de Manantlan	GU189087	GU189193, GU189193
Basileuterus culicivorus	*azarae*	FMNH 395479	Brazil: Boraceia, São Paulo	GU189088	GU189189, GU189224
Basileuterus culicivorus	*flavescens*	FMNH 395798	Mexico	GU189089	GU189192, GU189204
Basileuterus culicivorus[*]	*auricapillus*	FMNH 427306	Brazil: Mata do Estado, Pernambuco	GU189090	GU189191, GU189202
Basileuterus culicivorus[*]	*auricapillus*	FMNH 427307	Brazil: Taquaritinga do Norte, Pernambuco	GU189091	GU189182, GU189189
Basileuterus culicivorus[*]	*auricapillus*	FMNH 427308	Brazil: Taquaritinga do Norte, Pernambuco	GU189092	GU189211, GU189213
Basileuterus culicivorus[*]	*auricapillus*	FMNH 427309	Brazil: Ibateguara, Alagoas	GU189093	GU189206, GU189210
Basileuterus culicivorus[*]	*culicivorus*	FMNH 434208	El Salvador: San Pedro Puxtla	GU189094	GU189191, GU189201
Basileuterus hypoleucus		KU 100	Paraguay: Concepcion, San Luis NP	GU189095	GU189191, GU189191
Basileuterus culicivorus	*culicivorus*	KU 1918	Mexico: Campeche, Calakmul	GU189096	GU189231, GU189232
Basileuterus culicivorus	*azarae*	KU 3269	Paraguay: Misiones, Estancia Santa Ana	GU189097	GU189210, GU189217
Basileuterus culicivorus	*azarae*	KU 3390	Paraguay: Paraguay Neembucu, Cerrito	GU189098	GU189196, GU189210
Basileuterus culicivorus	*azarae*	KU 359	Paraguay: Caazapa, San Rafael NP	GU189099	GU189223, GU189223
Basileuterus culicivorus	*azarae*	KU 360	Paraguay: Caazapa, San Rafael NP	GU189100	GU189210, GU189210
Basileuterus hypoleucus		KU 395	Paraguay: Concepcion, San Luis NP	GU189101	GU189210, GU189217
Basileuterus hypoleucus		KU 396	Paraguay: Caazapa, San Rafael NP	GU189102	GU189189, GU189210
Basileuterus hypoleucus		KU 397	Paraguay: Concepcion, San Luis NP	GU189103	GU189182, GU189196
Basileuterus hypoleucus		KU 398	Paraguay: Caazapa, San Rafael NP	GU189104	GU189182, GU189224
Basileuterus hypoleucus		KU 407	Paraguay: Concepcion, San Luis NP	GU189105	GU189182, GU189196
Basileuterus hypoleucus		KU 410	Paraguay: Concepcion, San Luis NP	GU189106	GU189189, GU189211
Basileuterus hypoleucus		KU 51	Paraguay: Concepcion, San Luis NP	GU189107	GU189195, GU189210
Basileuterus culicivorus	*culicivorus*	KU 5924	El Salvador: Ahuachapan, Canton Concepcion	GU189108	GU189193, GU189200
Basileuterus culicivorus	*culicivorus*	KU 5932	El Salvador: Ahuachapan, Canton Concepcion	GU189110	GU189191, GU189193
Basileuterus hypoleucus		KU 65	Paraguay: Concepcion, San Luis NP	GU189109	GU189196, GU189210
Basileuterus hypoleucus		LBEM B0272	Brazil:Nova Lima, Mata da Piedade, Minas Gerais	GU189111	GU189196, GU189210

Table 1 (continued)

Species	Subspecies[‡]	Catalogue number	Locality	Cyt-b	BF5
Basileuterus hypoleucus		LBEM B0273	Brazil: Nova Lima, Mata da Piedade, Minas Gerais	GU189112	GU189187, GU189210
Basileuterus hypoleucus		LBEM B0274	Brazil: Nova Lima, Mata dos Primos, Minas Gerais	GU189113	GU189205, GU189210
Basileuterus hypoleucus		LBEM B0275	Brazil: Nova Lima, Mata dos Primos, Minas Gerais	GU189114	GU189182, GU189210
Basileuterus hypoleucus		LBEM B0276	Brazil: Nova Lima, Mata dos Primos, Minas Gerais	GU189115	GU189182, GU189239
Basileuterus hypoleucus		LBEM B0277	Brazil: Nova Lima, Mata dos Primos, Minas Gerais	GU189116	GU189202, GU189217
Basileuterus hypoleucus		LBEM B0278	Brazil: Nova Lima, Mata do Jambreiro, Minas Gerais	GU189116	GU189196, GU189196
Basileuterus hypoleucus		LBEM B0279	Brazil: Nova Lima, Mata do Jambreiro, Minas Gerais	GU189118	GU189182, GU189182
Basileuterus hypoleucus		LBEM B0280	Brazil: Nova Lima, Mata do Jambreiro, Minas Gerais	GU189119	GU189191, GU189196
Basileuterus culicivorus	azarae	LBEM B0502	Brazil: Jaboticatubas, Minas Gerais	GU189120	GU189217, GU189221
Basileuterus hypoleucus		LBEM B1473	Brazil: Felixlândia, Minas Gerais	GU189121	GU189182, GU189182
Basileuterus hypoleucus		LBEM B1476	Brazil: Brazilândia de Minas, Minas Gerais	GU189123	GU189184, GU189196
Basileuterus hypoleucus		LBEM B1487	Brazil: Brazilândia de Minas, Minas Gerais	GU189124	GU189182, GU189182
Basileuterus hypoleucus		LBEM B1491	Brazil: Felixlândia, Minas Gerais	GU189122	GU189196, GU189214
Basileuterus hypoleucus		LBEM B1610	Brazil: Felixlândia, Minas Gerais		GU189182, GU189182
Basileuterus hypoleucus		LBEM B1613	Brazil: Felixlândia, Minas Gerais	GU189125	GU189189, GU189196
Basileuterus hypoleucus		LBEM B1624	Brazil: Brazilândia, Minas Gerais	GU189126	GU189189, GU189191
Basileuterus hypoleucus		LBEM B1628	Brazil: Felixlândia, Minas Gerais	GU189127	GU189217, GU189217
Basileuterus hypoleucus		LBEM B1717	Brazil: Brazilândia de Minas, Minas Gerais	GU189128	GU189196, GU189196
Basileuterus hypoleucus		LBEM B1728	Brazil: Brazilândia de Minas, Minas Gerais	GU189129	GU189210, GU189218
Basileuterus hypoleucus		LBEM B2057	Brazil: Brazilândia de Minas, Minas Gerais	GU189130	GU189227, GU189226
Basileuterus culicivorus	azarae	LBEM B2106	Brazil: Bocaiúva, Minas Gerais	GU189131	GU189191, GU189216
Basileuterus culicivorus	azarae	LBEM B2144	Brazil: Turmalina, Minas Gerais	GU189132	GU189182, GU189182
Basileuterus culicivorus	azarae	LBEM B2145	Brazil: Turmalina, Minas Gerais	GU189133	GU189210, GU189212
Basileuterus culicivorus	azarae	LBEM B2146	Brazil: Turmalina, Minas Gerais	GU189134	GU189182, GU189196
Basileuterus culicivorus	azarae	LBEM B2147	Brazil: Leme do Prado, Minas Gerais	GU189135	GU189205, GU189209
Basileuterus culicivorus	azarae	LBEM B2148	Brazil: Leme do Prado, Minas Gerais		GU189189, GU189199
Basileuterus culicivorus	azarae	LBEM B2149	Brazil: José Gonçalves de Minas, Minas Gerais	GU189136	GU189196, GU189217
Basileuterus culicivorus	azarae	LBEM B2150	Brazil: José Gonçalves de Minas, Minas Gerais	GU189137	GU189182, GU189188
Basileuterus culicivorus	azarae	LBEM B2195	Brazil: Bocaiúva, Minas Gerais	GU189138	GU189189, GU189189
Basileuterus culicivorus	azarae	LBEM B2214	Brazil: Bocaiúva, Minas Gerais	GU189139	GU189205, GU189210
Basileuterus culicivorus	azarae	LBEM B2242	Brazil: Bocaiúva, Minas Gerais	GU189140	GU189196, GU189210
Basileuterus culicivorus	azarae	LBEM B2243	Brazil: Bocaiúva, Minas Gerais	GU189141	GU189191, GU189222
Basileuterus culicivorus	azarae	LBEM B2244	Brazil: Bocaiúva, Minas Gerais	GU189142	GU189182, GU189189
Basileuterus culicivorus	azarae	LBEM B2260	Brazil: Bocaiúva, Minas Gerais		GU189189, GU189210
Basileuterus culicivorus	azarae	LBEM B2263	Brazil: Bocaiúva, Minas Gerais		GU189196, GU189196
Basileuterus culicivorus	azarae	LBEM B2272	Brazil: Bocaiúva, Minas Gerais		GU189225, GU189226
Basileuterus culicivorus	azarae	LBEM B2293	Brazil: Bocaiúva, Minas Gerais	GU189143	GU189191, GU189217
Basileuterus culicivorus	azarae	LBEM B2492	Brazil: Turmalina, Minas Gerais	GU189143	GU189196, GU189210
Basileuterus culicivorus	azarae	LBEM B2543	Brazil: Água Doce do Norte, Espírito Santo	GU189145	GU189191, GU189191
Basileuterus culicivorus	azarae	LBEM B2544	Brazil: Alfredo Chaves, Espírito Santo	GU189146	GU189210, GU189217
Basileuterus culicivorus	azarae	LBEM B2547	Brazil: Alto Rio Novo, Espírito Santo	GU189147	GU189182, GU189191
Basileuterus culicivorus	azarae	LBEM B2640	Brazil: José Gonçalves de Minas, Minas Gerais	GU189148	GU189182, GU189182
Basileuterus culicivorus	azarae	LBEM B2673	Brazil: Conceição do Castelo, Espírito Santo	GU189149	GU189182, GU189217
Basileuterus culicivorus	azarae	LBEM B2674	Brazil: Domingos Martins, Minas Gerais	GU189150	GU189191, GU189196
Basileuterus culicivorus	azarae	LBEM B2675	Brazil: Água Doce do Norte, Espírito Santo	GU189151	GU189182, GU189191
Basileuterus culicivorus	azarae	LBEM B2686	Brazil: Turmalina, Minas Gerais	GU189152	GU189182, GU189182
Basileuterus culicivorus	azarae	LBEM B2711	Brazil: Turmalina, Minas Gerais	GU189153	GU189182, GU189217
Basileuterus culicivorus	azarae	LBEM B2712	Brazil: José Gonçalves de Minas, Minas Gerais	GU189154	GU189196, GU189220
Basileuterus culicivorus	azarae	LBEM B2713	Brazil: José Gonçalves de Minas, Minas Gerais	GU189155	GU189182, GU189185
Basileuterus culicivorus	azarae	LBEM B2748	Brazil: Domingos Martins, Minas Gerais		GU189182, GU189210
Basileuterus culicivorus	azarae	LBEM B2920	Brazil: Turmalina, Minas Gerais	GU189156	GU189191, GU189210
Basileuterus culicivorus	azarae	LBEM B2921	Brazil: Turmalina, Minas Gerais	GU189157	GU189182, GU189191
Basileuterus culicivorus	azarae	LBEM B2922	Brazil: Turmalina, Minas Gerais		GU189196, GU189196
Basileuterus culicivorus	azarae	LBEM B3124	Brazil: Viçosa, Minas Gerais	GU189158	GU189182, GU189210
Basileuterus culicivorus	azarae	LBEM B3125	Brazil: Viçosa, Minas Gerais	GU189159	GU189196, GU189196
Basileuterus culicivorus	azarae	LBEM B3127	Brazil: Viçosa, Minas Gerais	GU189160	GU189196, GU189196
Basileuterus culicivorus	occultus	LBEM B3589	Colombia: Neira	GU189161	GU189209, GU189210
Basileuterus culicivorus	occultus	LBEM B3590	Colombia: Yotoco	GU189162	GU189209, GU189209
Basileuterus culicivorus	azarae	LGEMA 10287	Brazil: Cordeiro, Rio de Janeiro	GU189176	GU189182, GU189196
Basileuterus culicivorus	azarae	LGEMA 10288	Brazil: Cantagalo, Rio de Janeiro	GU189177	GU189210, GU189219
Basileuterus culicivorus	azarae	LGEMA 1122	Brazil: Juquitiba, São Paulo	GU189164	GU189191, GU189216
Basileuterus culicivorus	azarae	LGEMA 11423	Brazil: Ortigueira, Paraná	GU189178	GU189182, GU189196
Basileuterus culicivorus	azarae	LGEMA 11442	Brazil: Ortigueira, Paraná	GU189178	GU189191, GU189224
Basileuterus culicivorus	azarae	LGEMA 1158	Brazil: Juquitiba, São Paulo	GU189165	GU189190, GU189196
Basileuterus culicivorus	azarae	LGEMA 1159	Brazil: Juquitiba, São Paulo	GU189166	GU189182, GU189217
Basileuterus hypoleucus	azarae	LGEMA 1242	Brazil: Buri, São Paulo	GU189167	GU189196, GU189197
Basileuterus culicivorus	azarae	LGEMA 1246	Brazil: Buri, São Paulo	GU189168	GU189182, GU189196
Basileuterus culicivorus	azarae	LGEMA 1248	Brazil: Buri, São Paulo	GU189169	GU189182, GU189194
Basileuterus culicivorus	azarae	LGEMA 1272	Brazil: Pinhalão, Paraná	GU189170	GU189196, GU189197
Basileuterus culicivorus	azarae	LGEMA 1430	Brazil: Morro Grande, São Paulo	GU189171	GU189182, GU189217
Basileuterus culicivorus	azarae	LGEMA 1439	Brazil: Morro Grande, São Paulo	GU189172	GU189191, GU189217
Basileuterus culicivorus	azarae	LGEMA 1441	Brazil: Morro Grande, São Paulo	GU189173	GU189183, GU189196
Basileuterus culicivorus	azarae	LGEMA 1765	Brazil: Parque Nacional do Itatiaia, Rio de Janeiro	GU189174	GU189191, GU189191

Table 1 (*continued*)

Species	Subspecies[‡]	Catalogue number	Locality	Cyt-*b*	BF5
Basileuterus culicivorus	*azarae*	LGEMA 2989	Brazil: Buri, São Paulo	GU189175	GU189182, GU189196
Basileuterus culicivorus	*azarae*	LGEMA 824	Brazil: Morro Grande, São Paulo	GU189163	GU189217, GU189217
Basileuterus culicivorus	*auricapillus*	MPEG 61613	Brazil: Querência, Mato Grosso	GU189180	GU189205, GU189210
Basileuterus culicivorus	*auricapillus*	MPEG 61614	Brazil: Querência, Mato Grosso	GU189181	GU189182, GU189191
Basileuterus culicivorus	*auricapillus*	MPEG A8417	Brazil: Ourilândia do Norte, Pará		GU189208, GU189210

[‡] According to the defined subspecies distributions indicated by Curson et al. (1994) and Restall et al. (2005).
[*] These specimens also received a field/museum classification according to the subspecies designation.

2.2. DNA extraction and mitochondrial sequencing

We extracted genomic DNA from blood and tissue samples using standard phenol–chloroform protocol after digestion with proteinase K (Sambrook and Russel, 2001). For museum skin samples, it was added DTT in the proteinase K step. Extracted DNA was quantified in 0.8% agarose gel or in Qubit Starter Kit (Invitrogen). We amplified the entire Cytochrome *b* (Cyt-*b*) with the primers L14841 and H16065 (Sorenson et al., 1999). PCR mixes of 12 μL included 2 μL of genomic DNA, 1 U of *Taq* polymerase (Phoneutria®), 200 μM of dNTPs, 1× Tris–KCl buffer with 1.5 mM MgCl$_2$ (Phoneutria®) and 0.5 μM of each primer. The amplification program consisted of 2 min at 94 °C, followed by 35 cycles of 40 s at 94 °C, 40 s at 45–50 °C, 2 min at 72 °C and a final extension step of 10 min at 72 °C. After amplification, PCR products were run in 0.8% agarose gels and stained with ethidium bromide. Negative controls, where template DNA was omitted, were used in all amplification runs. Only products with a single and well-defined band were used in the sequencing reactions. Before sequencing, PCR products were cleaned by precipitation using 20% polyethyleneglicol with 2.5 M NaCl. Sequencing reactions were performed using the primers L14841, IntR (Brumfield and Edwards, 2007), IntF (Brumfield and Edwards, 2007) and H16065, and were conducted in a final volume of 10 μL containing: 2 μL of purified PCR product, 3 μL of ultrapure water, 1 μL of primer (5 μM) and 4 μL of sequencing kit (ET DYE Terminator Kit, GE Healthcare). The sequencing program consisted of 35 cycles of 95 °C for 25 s, 50 °C for 15 s, 60 °C for 3 min. Then, sequencing products were precipitated with ammonium acetate and ethanol, dried at room temperature, dissolved with formamide-EDTA and run in the automatic sequencer MegaBACE 1000 (GE Healthcare).

To avoid the amplification of nuclear sequences of mitochondrial origin, i.e. *numts* (Sorenson and Quinn, 1998), the following measures were undertaken: (i) we amplified sequences longer than 1000 bp; (ii) amplification primers had degenerate sites and/or had annealing sites in tRNA genes; (iii) for each individual, at least two different PCR products were double strand sequenced until, at least, two high quality and independent sequences could be obtained; (iv) chromatograms were carefully checked for ambiguities, or possible heterozygote positions; (v) Cyt-*b* sequences

produced in the present study were aligned and compared with others obtained from the GenBank, to check for the presence of any start, stop or nonsense codons, as well as alignment gaps.

Consensus sequences were obtained and checked through the programs Phred v. 0.20425 (Ewing et al., 1998), Phrap v. 0.990319 (Ewing and Green, 1998) and Consed 16.0 (Gordon et al., 1998). Alignments were done using the Clustal W algorithm implemented in MEGA 4.0 (Tamura et al., 2007) with manual edition whenever it was necessary.

2.3. Nuclear DNA sequencing

Usually, nuclear introns evolve faster than exons due to the reduced selection acting upon their sequences (Fotheringham et al., 1997). This rapid evolution makes them potentially useful for population comparisons and to evaluate the independent divergence of mitochondrial and nuclear markers. To evaluate the usefulness of nuclear markers for phylogeographic studies in the *Basileuterus* genus we have sequenced the intron 5 of the nuclear β-fibrinogen gene (hereafter BF5). The amplification and sequencing was done with the same protocol used for the mitochondrial Cyt-*b* gene. The primers used for amplification and sequencing were BF5L and BF5H (Brumfield and Edwards, 2007). High quality consensus sequences were obtained through the programs Phred v. 0.20425 (Ewing et al., 1998), Phrap v. 0.990319 (Ewing and Green, 1998), Consed 16.0 (Gordon et al., 1998) and Polyphred (Nickerson et al., 1997). All detected polymorphisms and heterozygote sites identified by Polyphred were also carefully eye checked in the aligned chromatograms in Consed. Haplotype inferences for nuclear data were done using the program PHASE version 2 (Stephens and Donnelly, 2003; Stephens et al., 2001), with analyses run for 100 iterations, 1 thinning interval and a burn-in of 100.

2.4. Microsatellite genotyping

Allelic variation at six microsatellite loci was used to evaluate the population differentiation. All six loci (Table 3) have been previously described for another Parulidae species. The PCR mixes of 12 μL included 2 μL of genomic DNA, 1 U of *Taq* polymerase (Phoneutria®), 200 μM of dNTPs, 1× Tris–KCl buffer with 1.5 mM

Table 2
Estimates for each population: number of haplotypes (*H*), nucleotide diversity (*π*), haplotype diversity (*h*), average number of nucleotide differences (*k*), neutrality tests (Tajima's *D* and Fu's *Fs*) and genetic differentiation (*Φ*$_{st}$) for the mitochondrial and nuclear intron used in this study. *N* refers to the number of chromosomal loci sequenced in each population.

	Cyt-*b*								BF5							
	N	*H*	*h*	*K*	*π*	Tajima's *D*	Fu's *Fs*	*Φ*$_{st}$	*N*	*H*	*h*	*k*	*π*	Tajima's *D*	Fu's *Fs*	*Φ*$_{st}$
MEX	4	4	1.00	12.75	0.01	−0.31	0.56	0.57[**]	8	5	0.85	2.07	0.003	−0.56	−1.113	0.34[**]
MES	12	11	0.98	10.57	0.009	0.28	−2.8	0.57[**]	22	12	0.90	2.82	0.005	−0.29	−5.111[*]	0.33[**]
COL	2	2	1.00	11.00	0.01	0.00	4.33	0.51[**]	4	2	0.50	0.51	0.0009	−0.61	0.172	0.36[**]
VEN	4	3	0.83	3.39	0.003	0.18	0.89	0.58[**]	8	4	0.75	2.02	0.004	−0.63	0.155	0.34[**]
BAP	94	87	0.99	38.34	0.03	0.09	−24.02[**]	0.53[**]	202	41	0.88	2.69	0.005	−1.18	−26.521[**]	0.33[**]

[*] *p* < 0.05.
[**] *p* < 0.001.

MgCl$_2$ (Phoneutria®), 0.1 μM of the forward primer with a m13 tail (Schuelke, 2000), 1 μM of the reverse primer and 1 μM of FAM or HEX fluorescent dyes. Laboratory protocols for amplifying these loci followed Winker et al. (1999), Stenzler et al. (2004) and King et al. (2005). After amplification, the products were checked in polyacrylamide gel electrophoresis, and revealed by silver nitrate staining (Dias Neto et al., 1993). For genotyping, the PCR products were eluted in sterilized water and run in the automatic sequencer MegaBACE 1000 (GE Healthcare). All samples were run with the ET 550-R size standard (GE Healthcare). For double checking, 10% of the samples were run more than once and all museum skin samples were genotyped at least twice. The software Fragment Profiler (GE Healthcare) was used to analyze and generate the microsatellite genotypic data.

2.5. Phylogenetic analysis of mitochondrial and nuclear sequence markers

For estimating the evolutionary model of nucleotide change for Cyt-*b* and BF5 sequences we have used Modeltest version 3.7 (Posada and Crandall, 1998) applying the Bayesian Information Criterion (Posada and Buckley, 2004). The selected model for the Cyt-*b* data analysis was the Transversion Model (TVM), which recognizes four separate transversion rates, a single transition rate, and variable base frequencies, with gamma distributed (Γ) rate variation across sites ($\alpha = 0.5085$) and a proportion of invariant sites ($I = 0.6384$). For the BF5 data, the model selected was the Hasegawa–Kishino–Yano model (HKY), which recognizes variable base frequencies, variable transition and transversion frequencies, with transition/transversion ratio ($R = 1.4014$) and a proportion of invariant sites ($I = 0.9050$). Saturation in the DNA sequences was also examined by plotting the number of transition and transversion substitutions against *p*-distances for each pairwise comparison using the program Dambe (Xia and Xie, 2001).

For Cyt-*b*, maximum likelihood (ML) tree analysis was performed in PHYML version 2.4.4 (Guindon and Gascuel, 2003), using the model selected by Modeltest and 1000 nonparametric bootstraps to evaluate the support of the resulted topologies (Felsenstein, 1985). Bayesian phylogenetic (BP) analyses were carried out in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using two independent runs of four Markov chains (1 cold and 3 heated) with 20,000,000 generations and sampling every 100 generations. An initial tree produced by Maximum Parsimony (MP) in MEGA 4 (Tamura et al., 2007) was randomly chosen and used to accelerate the convergence. The first 25% of the sampling trees and estimated parameters were discarded as burn-in. Results of log-likelihood scores were plotted against generation times to identify the point at which log-likelihood values reached an equilibrium state (stationary). For the ML and BP phylogenetic analysis we have used Cyt-*b* sequences retrieved from Genbank as outgroups: *Basileuterus rivularis*, *B. tristriatus* and *B. flaveolus*.

2.6. Population structure analysis

2.6.1. Nuclear and mitochondrial DNA
To calculate the nucleotide diversity (π), haplotype diversity (h) and the average number of nucleotide differences (k) we used the program DNAsp version 4.5 (Rozas et al., 2003). Guided by the phylogenetic results, we used the program Arlequin version 3.11 (Excoffier et al., 2005) to estimate the genetic differences between the groups based on Φ_{st} (an F_{st} analogue that considers allelic differences) with the Analysis of Molecular Variance (AMOVA) approach (Excoffier et al., 1992). We also used Arlequin to calculate Tajima's D and Fu's F_s to assess any departure from neutrality that could indicate a signal of population expansion. In addition, we also used the AMOVA to test if there are any genetic differences that can be

non-randomly associated with the two species *B. culicivorus* and *B. hypoleucus*. For this test, we used individuals from Brazil and Paraguay, the only two countries with samples of both species.

2.6.2. Microsatellites
In order to characterize variation in each locus, we tested the departures from Hardy–Weinberg equilibrium using Arlequin (Excoffier et al., 2005). The frequency of null alleles was estimated according to Brookfield (1996) in the software Microchecker (Van Oosterhout et al., 2004, 2006). AMOVA analysis was also performed in Arlequin, as previously described. Thus, to measure the amount of genetic differentiation attributable among the groups established in the phylogenetic analysis, we estimated the conventional F_{st} that considers only the frequency of alleles and also the R_{st} that takes into account the square differences between allele repeat numbers. The statistical significance was determined by a permutation procedure.

2.7. Molecular clock and divergence time estimates

We used the Bayesian approach implemented in BEAST version 1.4.7 (Drummond and Rambaut, 2007) to calculate the divergence times between phylogenetic groups detected by Cyt-*b* analysis. For the estimation of the time to the most recent common ancestor (T_{MRCA}), we investigated four population models: constant population size, exponential growth, logistic growth and expansion growth. To assess which model was the best fit for the data, the associated Bayes Factors were estimated (Suchard et al., 2001). Since a clock-like evolution was rejected using a likelihood ratio test in Paup (Swofford, 1993) and by the estimation of the parameter ucld.stdev in BEAST, we have applied an uncorrelated log-normal relaxed-clock. Due to the absence of parulid fossil record, it was not possible to calibrate a local substitution rate, and we have used a fixed mutation rate. The Cyt-*b* rate used of 2.07% mutations/million years in Passeriformes was based on the estimates of Weir and Schluter (2008), who used several calibrations points. Because the rate estimated by Weir and Schluter (2008) is associated with an standard deviation, we incorporated the normal distribution to reflect the uncertainty of the original estimation (Ho, 2007). Population size dynamics through time (Drummond et al., 2005) were also estimated using the Bayesian Skyline plot approach in BEAST. In this method, we used two types of analysis to get a better estimation of the evolutionary processes that occurred in each population. First, we analyzed all the haplotypes simultaneously without the assumption of any a priori population structure. Next, the estimation was performed for the monophyletic groups established according to the phylogenies, except the group from Colombia with only two individuals. All analyses in BEAST were run for 70,000,000 generations with a burn-in of 7,000,000. Results were then visualized in Tracer 1.4 (Rambaut and Drummond, 2007). We employed a GTR + I + G model of evolution with six rate categories, and other priors according to the default settings provided by BEAST version 1.4.7. Parameters were sampled every 1000 generations.

3. Results

3.1. Cytochrome b

We obtained a total of 1055 bp for the Cyt-*b* gene from 115 specimens. Neither stop nor nonsense codons were observed. No signal of saturation was observed among the sequences. We identified 107 unique haplotypes with 228 polymorphic sites, from which 172 were parsimony informative and 56 were singleton variable sites. All Cyt-*b* phylogenetic reconstructions identified five

monophyletic groups (Fig. 2). The ML and BP reconstructions found a consistent separation among five clades: (1) Western Mexico (MEX), (2) Eastern Mexico and El Salvador (MES), (3) Colombia (COL), (4) Venezuela (VEN) and (5) Brazil, Paraguay and Argentina (BAP).

The haplotype pairwise differences within these groups varied from 3.39 (VEN) to 38.44 (BAP) (Table 2). The uncorrected sequence divergence among groups ranged from 4.4% (COL vs. VEN) to 6.9% (MEX vs. BAP) with a mean of 5.61%. The Fu's Fs neutrality test, which is useful to search for additional demographic signals, showed a remarkable sign of population expansion in the group of BAP (Table 2). However, the Tajima's D test was not significant for any population. The specific φ_{st} values of each population (Table 2) are homogeneous, indicating that no population contributes differently to the average φ_{st}, and there are no detectable evolutionary constraints in these populations.

The AMOVA analysis found a substantial mtDNA differentiation across the groups ($\varphi_{st} = 0.5138$; $p < 0.001$) with the variation within population (48.62%) and among populations (51.38%) well distributed, which could indicate a significant population structure with a low overall gene flow. When the AMOVA was performed taking into consideration the species $B.$ $culicivorus$ and $B.$ $hypoleucus$ from Brazil and Paraguay, the results were not significant ($\varphi_{st} = 0.006$; $p = 0.54$).

The Bayes Factor favored the model that assumes an exponential growth, thus we rejected the other models and used the Exponential Growth Model for our estimation of the time to the most recent common ancestor (T_{MRCA}). Our analysis with BEAST found a T_{MRCA} for all haplotypes sampled in this study of 2.54 million years ago (MYA) (95% credibility interval: 3.17–1.93 MYA). The T_{MRCA} for each node of the tree is shown in the Fig. 3. When all haplotypes were considered as a single group, the Bayesian Skyline results indicated a rapid population growth starting approximately 500,000 years ago followed by a recent bottleneck (Fig. 4a). When the populations were considered separately, the MEX population maintained a relative stable size (Fig. 4b), the MES showed a recent decline followed by a recovery of the effective size (Fig. 4c), the VEN group showed a constant slow decline in the last 200,000 years (Fig. 4d), and the BAP population indicates a rapid growth in the last 500,000 years (Fig. 4e).

3.2. β-Fibrinogen intron 5

For the intron 5 of the nuclear β-fibrinogen gene (BF5), we obtained a total of 572 bp. We found 58 haplotypes and 42 polymorphic sites, with no indel detected, and no signal of recombination. Of the 58 haplotypes identified, four were observed only in the group MEX, 10 in the group MES and 36 were exclusive of the BAP group. The Fu's Fs test found a significant population expansion for the groups MES and BAP and Tajima's D found no significant result (Table 2). The AMOVA results suggest, as for Cyt-b, a highly structured population ($\varphi_{st} = 0.3315$; $p < 0.001$) with a twofold higher variation within populations (66.85%) than among populations (33.15%). The highest φ_{st} values were observed among the MEX and VEN populations ($\varphi_{st} = 0.70$; $p < 0.001$) and the lowest among BAP and VEN ($\varphi_{st} = 0.01$; $p = 0.35$). When we considered the differences between $B.$ $culicivorus$ and $B.$ $hypoleucus$, the AMOVA results were similar as the ones from Cyt-b, with a φ_{st} of -0.006 ($p = 0.81$).

3.3. Microsatellites

The number of alleles obtained for the loci used ranged from 12 to 29. Of the six loci, only the locus DkiD123 was in Hardy–Weinberg equilibrium when considered the entire sample. When analyzed separately in the five geographic groups, most of the loci
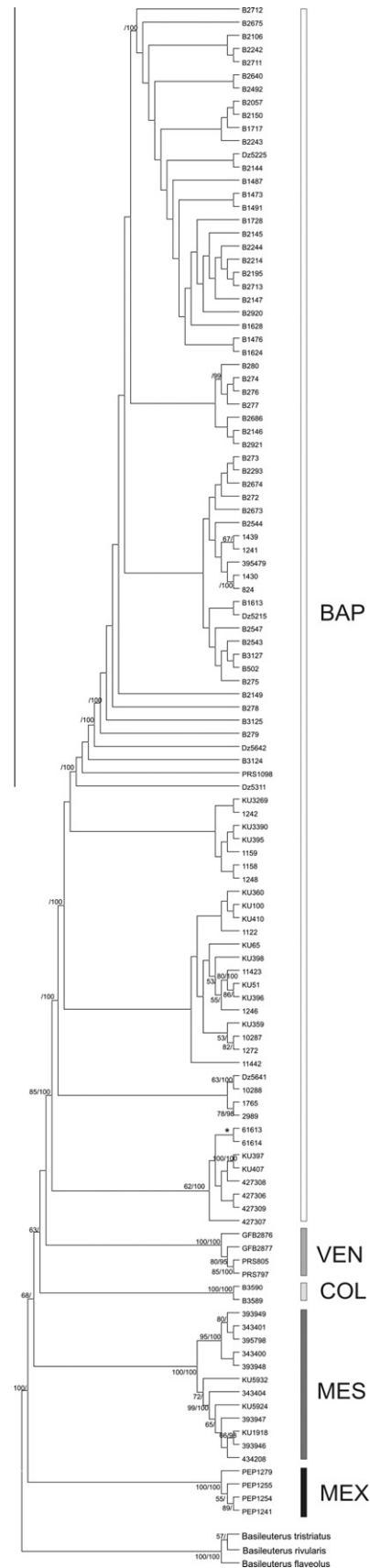

Fig. 2. Maximum Likelihood and Bayesian tree using all 115 Cyt-b sequences of $B.$ $culicivorus$, $B.$ $hypoleucus$ and three outgroups. Only bootstrap values >50 (before slash) and posterior probabilities >95 (after slash) are shown. The asterisk indicates the only clade that showed a difference in topology between ML and BP analysis.

Table 3
Microsatellite loci used for genotyping, including number of samples genotyped (N), number of observed alleles (A), observed (H_o) and expected (H_e) heterozygosities, significance level of Hardy–Weinberg equilibrium (p) and the frequency of null alleles (N_a).

Locus	Fluorescence	All samples						BAP					VEN					MEX					MES					COL				
		N	A	H_o	H_e	p	N_a	n	A	H_o	H_e	p	n	A	H_o	H_e	p	n	A	H_o	H_e	p	n	A	H_o	H_e	p	n	A	H_o	H_e	p
VeCr02‡	FAM	136	13	0.25	0.414	0.00	0.1217	119	9	0.252	0.370	0.000	–	–	–	–	–	4	2	0.500	0.429	1.00	10	7	0.200	0.821	0.00	–	–	–	–	–
VeCr04‡	FAM	144	12	0.437	0.637	0.00	0.1211	124	10	0.418	0.560	0.000	4	4	0.750	0.786	1.00	4	3	0.00	0.714	0.031	12	8	0.667	0.848	0.156	–	–	–	–	–
DkiD120§	FAM/HEX	144	21	0.743	0.886	0.00	0.0737	122	17	0.721	0.865	0.000	4	6	1.00	0.9285	1.00	4	6	0.75	0.893	0.445	12	11	0.833	0.819	0.331	2	3	1.00	0.833	1.00
DkiD126§	FAM	144	20	0.861	0.897	0.012	0.0167	123	18	0.854	0.888	0.042	4	8	1.00	1.00	1.00	4	5	1.00	0.893	1.00	11	10	0.812	0.887	0.116	2	4	1.00	1.00	1.00
DkiD123§	FAM	142	15	0.803	0.832	0.07	0.0222	122	14	0.828	0.821	0.281	4	6	1.00	0.893	1.00	4	5	0.25	0.893	0.011	10	9	0.600	0.800	0.076	2	4	0.00	1.00	1.00
Lsw18♥	HEX	146	29	0.781	0.903	0.00	0.0659	124	27	0.814	0.901	0.029	4	6	1.00	0.929	0.33	4	8	1.00	1.00	1.00	12	10	0.4166	0.891	0.000	2	2	0.00	0.667	0.001

* We could not amplify the locus VeCr02 for the populations of VEN and COL and the locus VeCr04 for COL.

‡ Stenzler et al. (2004).

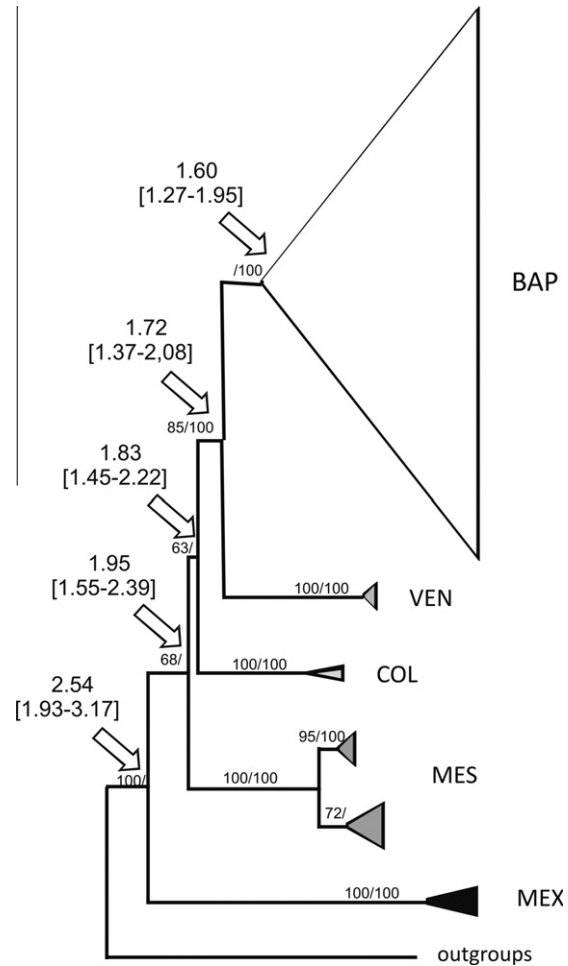§ Dawson et al. (1997).

♥ Winker et al. (1999).

Fig. 3. Condensed tree of the Cyt-*b* gene, exhibiting the times of divergence of each clade in million years ago (MYA). The number within brackets shows the 95% confidence interval for each estimate.

were in equilibrium (see Table 3). The software Microchecker detected a significant frequency of null alleles for the loci VeCr 02, VeCr 04, DkiD120, and LsW18, when the entire population was considered. We used the Brookfield's (1996) method, which assumes that heterozygote deficiencies are mainly due to null alleles and not population structure. Although the microsatellite loci were not isolated in the species under study (which could lead to high levels of null alleles), this assumption is not reasonable, since there is a significant population structure that could lead to a Wahlund effect, and in the separated geographic groups most loci show equilibrium. Moreover, the two loci that showed the highest estimates of null alleles (VeCr 02 and VeCr 04), also showed this pattern in another study for the warblers *Vermivora chrysoptera* and *V. pinus* (Vallender et al., 2007). The analysis of variance in allele repeat numbers over the populations recovered in the phylogeny yielded a weighted estimate of R_{st} = 0.19 ($p < 0.001$). A similar analysis, taking under consideration only the frequency of alleles, estimated an F_{st} of 0.07 ($p < 0.001$). This discrepancy could be due to the fact that conventional F_{st} often provide less biased estimates of differentiation than R_{st} when the number of scored loci is low (Gaggiotti et al., 1999), and therefore the further considerations will only take into account the estimates done with F_{st}. The only significant F_{st} values were observed between the BAP and MES clades (F_{st} = 0.09; $p < 0.001$), BAP and MEX (F_{st} = 0.08; $p = 0.02$), MES and VEN (F_{st} = 0.08; $p = 0.02$), and MEX and MES (F_{st} = 0.08; $p = 0.03$). When the two species (*B. culicivorus* and *B. hypoleucus*)
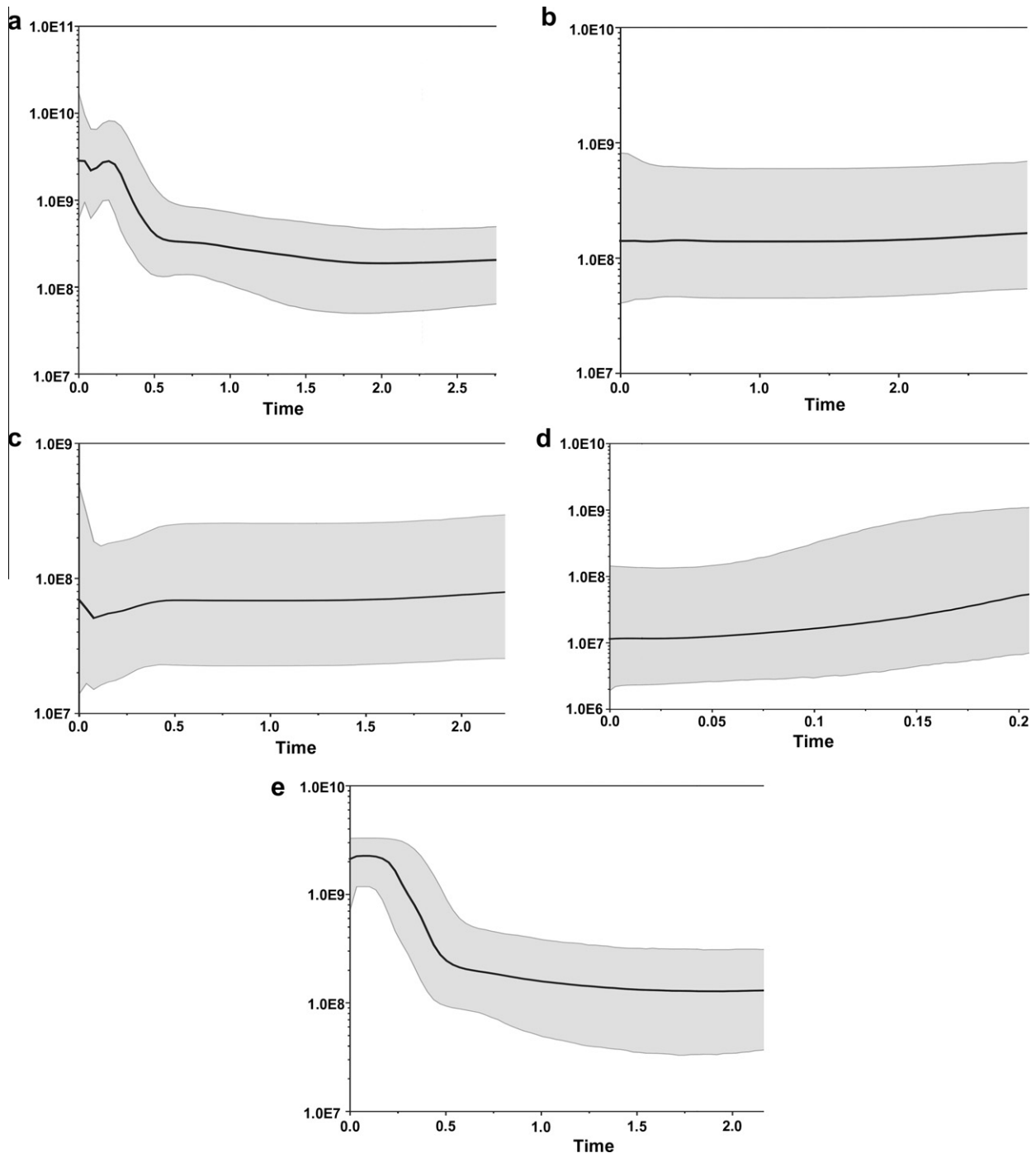
Fig. 4. Bayesian Skyline Plot for Cyt-*b* sequences with a log-normal relaxed clock and a generation time of 1 year (Milot et al., 2000). The *y* axis is the effective number of females. The thick solid line is the median estimate and the thin line (grey) show the 95% highest posterior density limits. The *x* axis is scaled as million years ago (MYA). (a) all clades, (b) MEX clade, (c) MES clade, (d) VEN clade, (e) BAP clade.

were compared, the results were similar as the ones from mito-chondrial and nuclear markers, presenting a non-significant F_{st} of 0.003 ($p = 0.12$).

4. Discussion

4.1. Historical biogeography

The Late Pliocene–Pleistocene was a highly active period both geologically and environmentally in South America (Colinvaux et al., 1996). The final closure of the Panama Isthmus took place approximately 2.8 MYA (Coates and Obando, 1996) and the temperature shifts during this period probably affected both indig-enous and immigrant fauna. Our data suggest that the Pliocene–Pleistocene boundary was an important time for the differentiation of *B. culicivorus*, since their T_{MRCA} indicates a basal divergence at 2.54 MYA. On the other hand, a major part of bird diversification in North America appeared to have taken place before the Pliocene–Pleistocene boundary (Bermingham et al., 1992; Garcia-Moreno and Fjeldsa, 2000). Species of the genus *Myioborus*, which is closely related to the genus *Basileuterus*, have diverged

in the Pliocene between 3.6 and 3.9 MYA (Pérez-Emán, 2005). Lovette (2004), studying the radiation of *Phaeothlypis*, found that the intraspecific divergence of this group occurred also between 3.0 and 3.8 MYA. These results associated with the estimated dates that we have found, corroborate the fact that the two parulid genera that radiated into South America had their initial diversification during the Late Pliocene. However, Johnson and Cicero (2004) argued that the Pleistocene was also a significant period for bird speciation, since many pairs of species have diverged during this period. Within the Parulidae family, species of *Dendroica* have diversified during the Pleistocene (Johnson and Cicero, 2004). This period was also an important period for diversification of *B. culicivorus* as it coincides with the divergence of several clades within South America.

The colonization of South America by many Oscine birds precedes the final closure of the Panama Isthmus, since studies with birds demonstrated that species of northern origin first came to South America when the continent was still separated (Barker, 2007; Lovette, 2004; Pérez-Emán, 2005; Weir et al., 2009). In a recent analysis, Weir et al. (2009) showed that a family with a northern origin (Icteridae) presented a higher dispersal rate to South America before the formation of the land bridge in Panama, contrasting with bird families of southern origin, which required a complete land bridge to cross between the two continents. Interestingly, Mayr (1964) affirms that the *Basileuterus* colonization of South America "surely preceded the closing of the Panama gap", but the time was uncertain. Contrasting with previous analyses and ideas, our results indicate that *B. culicivorus* expanded into South America in a time interval posterior to the closure of the Panama Isthmus. This fact indicates a more recent history of southern dispersion for *B. culicivorus*, when compared to other Oscine birds. Additionally, it reveals that although this warbler has a great capacity of flight, which would be an advantage in order to cross the ocean waters, it likely arrived in South America flying over connected lands along adjacent forest habitats.

It is believed the Parulidae originated in the northern Central America, and the two parulid genera endemic in the Neotropics, *Basileuterus* and *Myioborus*, have radiated into South America after a northern origin (Curson et al., 1994; Mayr, 1964). This hypothesis was confirmed for the *Myioborus* genus (Pérez-Emán, 2005) and we also found similar results for *Basileuterus*. In this study, the first and second most basal clades of *B. culicivorus*, the groups MEX and MES are from the Mexico and El Salvador, the northernmost extreme of the species distribution. The general branching pattern consists of a paraphyletic clustering, whose southern clades are nested within northern located clades. This indicates that the center of origin for this species is in the north of its distribution, with a subsequent spread into the south. All five mtDNA clades show a remarkable population structuring, which is supported by the high values of bootstrap and φ_{st} analysis. For nuclear intron BF5, a high structuration is also apparent, with several private alleles observed among the populations. The high geographic resolution exhibited by nuclear and mtDNA analysis suggests that all five groups became isolated at different times, due to a single and continuous colonization event from northern ancestral populations through a southward route.

An indication of structuration has also been drawn from microsatellite data in the comparative analysis of the separated populations inferred from the phylogeny. However, microsatellites have not shown to be as informative as mtDNA and BF5 markers, probably because of their higher mutation rates and levels of homoplasy that could explain the lower F_{st} values among deeply diverging phylogroups.

In our dating approach we expect some population events to precede coalescence of lineages within groups, e.g. the T_{MRCA} for the monophyletic group COL + VEN + BAP (Fig. 3) should be more recent than the split of Central American populations originating South American ones. Therefore, we consider here the T_{MRCA} dates

for monophyletic geographic groups as minimal time estimates for ancestral population splits. However, some T_{MRCA} estimates can also work as an upper limit for the split of derived populations, e.g. the T_{MRCA} estimate joining MES (Central America) and COL + VEN + BAP (South America) should precede the population split between both regions. We found that the most basal divergence of the *B. culicivorus* lineages occurred in the Late Pliocene. However, its diversification through South America occurred in a period between 2.22 and 1.45 MYA, which is the period corresponding to their T_{MRCA} in Colombia (COL + VEN + BAP). Next, *B. culicivorus* colonized Venezuela and took a southern route of colonization, arriving to Brazil, Argentina and Paraguay. *Basileuterus culicivorus* probably used a route through northern-northeastern Brazil since haplotypes from this region belong to the most basal clade within the BAP group, which is congruent with the Coastal Corridor that connected southern and northern patches of savannas during the Pleistocene (da Silva and Bates, 2002; Quijada-Mascareñas et al., 2002). This north–south migration pattern is congruent among several birds (da Silva and Bates, 2002; Remsen et al., 1991), mammals and reptiles (Quijada-Mascareñas et al., 2007), which show a remarkable resemblance in their distribution areas. Wüster et al. (2005), studying the rattlesnake *Crotalus durissus*, a species complex with a similar distribution to *B. culicivorus*, found an analogous pattern of sequential migration across North, Central and South America, with very similar dates of arrival in the southern continent.

The Bayesian Skyline Plot of geographic groups indicated that each population is evolving differently according to demography. After the *B. culicivorus* population first arrived in Brazil, Paraguay and Argentina, they experienced a rapid growth that expanded its population by several orders. It started 500,000 years ago and ended 250,000 years ago, a period corresponding to the Middle Pleistocene. The repeated glaciations during the Pleistocene that caused the periodical retractions of the Amazon and Atlantic rain forests (Haffer, 1969, 1993; Quijada-Mascareñas et al., 2007) could have benefited *B. culicivorus*. A retraction of the Amazon forest have allowed the establishment of a corridor of more open vegetation from the northern South America to southern habitats during the Middle Pleistocene that could have lasted long enough to permit the population growth. Although there is some controversy about the Amazon forest shrinkage during the Early and Middle Pleistocene (Colinvaux et al., 1996; Wüster et al., 2005), other studies confirm that such corridors might have existed (da Silva and Bates, 2002), which is also plausible according to the documented Milankovich cycles that occurred before the Quaternary glaciations (Bartlein and Prentice, 1989; Bennett, 1990; Haffer, 1993). Even though *B. culicivorus* can be currently found in some open areas of the Amazon region, particularly close to the Cerrado (Brazilian savannah), it does not seem to occur in the typical rainforest biome, probably because it cannot compete with locally adapted species, including other *Basileuterus* or even other migratory Parulidae. Thus, the retraction of the rain forests to small fragments separated by dry forests and savannas during the Pleistocene have likely promoted a new route of migration to *B. culicivorus*, favoring its population expansion into South America.

In conclusion, our results enlighten the dynamics of *Basileuterus* populations in South America and provide new evidences for Amazon fragmentation and connection with the Cerrado (Brazilian savanna) during the Pleistocene, demonstrating the potential usefulness of phylogeographical studies of common species with wide distributions to elucidate the dynamics of processes in a continental scale.

4.2. Phylogeny and systematics

In this study, haplotypes of *B. culicivorus* and *B. hypoleucus* were not recovered as reciprocally monophyletic, suggesting an

incomplete lineage sorting or an extensive gene flow. Although these two recognized species cannot be differentiated genetically, we can distinguish five well supported clades within *B. culicivorus* with a strong geographical correlation. The few subspecies represented by samples identified in the field or deposited as vouchers can also be recognized as monophyletic clades, *B. c. flavescens* and *B. c. auricapillus*, the former within the group MES and the latter within the major group BAP. The only two individuals classified as *B. c. culicivorus* were also grouped within the clade MES. Considering the geographic occurrence of thirteen *B. culicivorus* subspecies (Curson et al., 1994; Restall et al., 2005), we could delineate their possible correlation with other monophyletic clades. The MEX group is formed by the subspecies *B. c. basherii* and the COL group by *B. c. occultus*. Within the MES clade that includes individuals from El Salvador, all should belong to the *culicivorus* subspecies according to its geographical distribution, supporting the monophyly of this race. The other clade within the MES group is formed by individuals of the subspecies *flavescens*. In the VEN clade, all individuals were collected in the Venezuelan region where the *B. c. olivascens* subspecies occurs. Within the BAP group, two subspecies are recognized: *B. c. auricapillus* and *B. c. azarae*. Even though the *auricapillus* individuals from northeastern Brazil grouped together, in this clade we also have individuals from Paraguay, which should belong to the *azarae* race. Thus, the lack of a robust separation between these two subspecies could be resulted either from incomplete lineage sorting of mtDNA lineages or from an incipient morphological differentiation used to define both subspecies. However, since the BAP population experienced a recent population growth, the short divergence time may be not sufficient to reach mtDNA coalescence within each subspecies.

Considering haplotype divergence estimates, the mean intraspecific divergence observed in *Basileuterus culicivorus* (5.61% for mtDNA) was consistent with the values (3.6–6.8%) found for another related species of the genus (Lovette, 2004). However, it is a high divergence if we compare with other Parulidae: *Parula americana – P. pitiayumi*, 1.0% (Lovette and Bermingham, 2001); *Dendroica townsendi – D. occidentalis*, 0.9% (Lovette and Bermingham, 1999); *Dendroica nigrescens – D. graciae*, 1.5% (Lovette and Bermingham, 1999); *Dendroica coronata – D. auduboni*, 0.4% (Lovette and Bermingham, 1999). This low divergence pattern observed among other parulid species contrasts strikingly with the high geographic structuring and genetic diversity of the *Basileuterus* genus, as already highlighted by Lovette and Bermingham (2001) and also detailed in this study.

Silva (1992) already noticed that several specimens of *B. hypoleucus* displayed variations in the intensities and distribution of the yellow color in museum skins. Even though white color is observed in their underparts, the yellow is frequently present in specimens classified as *B. hypoleucus*. We also observed this pattern in the museum skins analyzed for this study. Silva (1992) also demonstrated that in regions where these two species are sympatric, the intensity of yellow in *B. hypoleucus* is higher, which could indicate elevated levels of admixture. Therefore, it also suggested that in the allopatric areas of occurrence of *B. hypoleucus* there should be less introgression of genes from *B. culicivorus*.

If we consider the BAP group phylogeny, *B. hypoleucus* Cyt-*b* haplotypes are represented by the most basal lineages (KU407 and KU397). Thus we can infer that this taxon (or some individuals) probably differentiated in the past, when the first migrants arrived in the countries of Brazil and Paraguay. However, our results indicate that there is no present reproductive barrier between these recognized species. Although it is not currently possible to distinguish genetically between these two species, the restricted area of occurrence of *B. hypoleucus* could suggest either that this is an incipient species in the process of differentiation or the white color is a restricted polymorphism of a major taxon, *B. culicivorus*. Our re-

sults and the observation that these recognized species do not own differences in vocalization or morphometry (Silva, 1992), might be a strong indication that these belong to a single species.

Following the unified species concept (de Queiroz, 1998, 2005, 2007) a lineage to be considered a different species has to be evolving separately from other lineages. Considering other species' concepts, as the biological (which takes into consideration reproductive isolation), isolation (which considers isolating mechanisms), recognition (mating and fertilization compatible), monophyletic (reciprocal monophyly) and genealogical (coalescence of alleles) (see de Queiroz, 2007, for a review of species concepts) each property of different concepts should only be seen as an evidence of lineage separation. Considering the arguments above, *B. culicivorus* and *B. hypoleucus* do not retain any characteristics that could distinguish them as separate species. However, the lack of characteristics that could corroborate their separation does not mean that they are not separate species, since "the farther along lineages are in the process of divergence, the larger the number of differences they can be expected to have acquired relative to one another, and therefore the easier it should be to find evidence of separation" (de Queiroz, 2007). Since we have used different markers (mitochondrial and nuclear) and our findings are corroborated by another morphological study (Silva, 1992), we consider that the hypothesis of existence of two species is not supported, and we propose that these two taxa should be lumped in a single species *Basileuterus culicivorus*.

Regarding the five sub-clades or phylogroups recovered in the phylogenetic tree, we have not observed any grouping according to the three allopatric divisions suggested by Curson et al. (1994). The phylogeny also failed in separating some subspecies, indicating that they could be evolving as a single taxon (e.g. *auricapillus × azarae*). Thus, supported by our data and by distinct morphological characteristics that define each subspecies or species (Curson et al., 1994), the identified clades should be considered in further studies as possible independent taxa: the groups MEX (*basherii*), MES (with likely two sub-taxa: *flavescens* and *culicivorus*), COL (*occultus*), VEN (*olivascens*), and BAP (with likely three sub-taxa: *azarae*, *auricapillus* and *hypoleucus*). Whether these clades can be defined as five different subspecies or even species, demands further studies in a larger sampling.

Finally, this detailed phylogenetic analysis of the Golden-crowned Warbler (*B. culicivorus*) depicts the importance of the study of common bird species to understand patterns of range expansion and colonization in the Neotropics. Moreover, it illustrates an interesting biogeographic scenario that could be mirrored by many other species colonizing South America after the closure of the Panama Isthmus, which were likely subjected to Pleistocenic vicariant events.

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