DISSERTATION

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany

for the degree of
Doctor of Natural Sciences

presented by
M.S. Alexandre Mendes Fernandes
born in Belo Horizonte, Brazil
Oral examination: June 26, 2012

Phylogeography and Speciation of Selected Passerine Birds of Lowland Amazonia

Implications for Historical Biogeography and Conservation

Referees: Prof. Dr. Michael Wink
Prof. Dr. Thomas Braunbeck

"*During my residence in the Amazon district*
I took every opportunity of determining the limits of species,
and I soon found that the Amazon, the Rio Negro and the Madeira
formed the limits beyond which certain species never passed."

Alfred Russel Wallace, 1852

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Zusammenfassung

Regionaler Endemismus innerhalb des **Amazonasgebietes** scheint in vielen Fällen durch geografische Barrieren in Form **größerer Flussläufe** entstanden zu sein. Die Auswahl der drei hier untersuchten Vogelarten aus der Gruppe der Schreivögel (Suboscines) basiert auf ersten Beobachtungen und Untersuchungen, die darauf hindeuteten, dass der **Artstatus** einiger vor allem durch **kleinere Flüsse** begrenzter Populationen einer Revision bedarf:
Keilschnabel-Baumsteiger (*Glyphorynchus spirurus* Komplex, Furnariidae),
Rotschwanz-Ameisenvogel (*Myrmeciza hemimelaena* Komplex, Thamnophilidae),
Ockerflecken-Ameisenwächter (*Hylophylax naevius* Komplex, Thamnophilidae).

Im Falle von ***G. spirurus*** hat die Untersuchung von zwei mitochondrialen Genen (cyt *b*, ND2) an 134 Individuen durch Bayes-Inferenz (BI) und Maximum Likelihood (ML) mehrere Unstimmigkeiten innerhalb der derzeitigen Systematik auf der Ebene der Unterarten aufgezeigt, in einigen Fällen besteht sogar ein erheblicher genetischer Abstand (bis zu 6%) trotz bislang augenscheinlich identischer morphologischer Merkmale – auch Analysen der Kern-DNA (*BEAST-Bäume der fünf Gene cyt *b*, ND2, ACO, G3PDH Intron, MYO Intron 2) ergaben eine **Paraphylie**. Genomische Fingerprints mithilfe von fünf ISSR Primern zeigen eine klar differenzierte **Populationsstruktur** auf beiden Seiten des Madeira-Flusses.

Für den ***M. hemimelaena*** Artenkomplex ergab die Phylogenie (BI, ML) der mtDNA (cyt *b*, ND2) von 65 Individuen drei klar zu unterscheidende Entwicklungslinien, mit einer neu definierten Unterart, deren Verbreitung auf ein eng begrenztes Areal zwischen den Flüssen Jiparaná und Aripuanã beschränkt ist.

Der dritte untersuchte Artenkomplex von ***H. naevius*** zeigt ebenfalls aufgrund von Phylogenien der mtDNA (cyt *b*, ND2) durch BI und ML sowie anhand von *BEAST-Bäumen dreier Genorte (cyt *b*, ND2, FB5 Intron) von 80 Individuen eine klare **Paraphylie** und eine Unstimmigkeit der bislang gültigen Taxonomie. Es folgt, dass aus der vormaligen Art *H. naevius* eine zusätzliche **neue Art** ausgegliedert werden muss, die durch den Madeira-Fluss und den rechtsufrigen Zufluss Jiparaná begrenzt wird.

Die Gebiets-Kladogramme der drei Artenkomplexe ähneln sich so sehr, dass man annehmen kann, die gleichen **geologisch-ökologischen Ereignisse** hätten bei allen drei Artenkomplexen in gleicher Weise und zu gleicher Zeit gewirkt. Es erfolgte eine **basale Aufspaltung** im **Pliozän**, die die Populationen nördlich und südlich des Amazonas-Flusses voneinander trennte. Danach entstanden phylogeografische Barrieren, die dem Verlauf des Rio Negro und des Madeira-Flusses entsprechen. Eine kleinteilige phylogeografische Untergliederung im Bereich der rechtsufrigen Zuflüsse des Madeira-Flusses (Aripuanã und Jiparaná) aus der Zeit des **Pleistozän** zeigte sich für alle drei untersuchten Artenkomplexe. Die Diversifikation im Gebiet der Madeira-Niederung scheint aber komplexere Ursachen zu haben, da die Abfolge der phylogeografischen Auftrennung der drei Artenkomplexe nicht identisch ist.

Die hier aufgezeigten räumlich-zeitlichen Muster legen nahe, dass die **geologisch-tektonische Restrukturierung** der Amazonas-Niederung vom späten Pliozän bis zum frühen Pleistozän eine entscheidende Triebkraft für die Kladogenese in Amazonien darstellte. Andererseits deuten die hier erbrachten Ergebnisse auf ein komplexes Diversifikationsgeschehen hin, in dem verschiedene ökologische Faktoren eine zusätzlich entscheidende Rolle spielten. Diese und vorangehende Untersuchungen haben gezeigt, dass viele Artenkomplexe in Amazonien nur geringe bis gar keine morphologisch-ökologischen Unterschiede aufweisen, wohingegen **molekular-phylogenetische Daten** das **Vorliegen unterschiedlicher Arten** beweist.

Die Naturschutzbemühungen im Bereich des Aripuanã und Jiparaná in der Madeira-Niederung müssen intensiviert werden. Dies ist **eine der ökologisch vielfältigsten Regionen des Amazonas**, und gleichzeitig eine der meist gefährdeten im Hinblick auf die derzeitige massive Bedrohung durch menschliche Eingriffe. Viele der einzigartigen Lebewesen der Madeira-Niederung, auch jene die bislang keine formelle Anerkennung als biologische Einheit genießen (wie jene hier beschriebenen Vögel), könnten bereits gefährdet oder ausgerottet sein. Wenn **"kryptischer Endemismus"** die Regel ist, dann werden Natur- und Artenschutzmaßnahmen, die diese Vielfalt nicht anerkennen, die **besondere regionale Vielfalt**, vor allem die des **Amazonas**, nicht bewahren können.

Summary

Previous studies focusing on the **geographic distribution** of **Amazonian species** have shown that many taxa are separated from each other by **major rivers**, and that those delimit **areas of endemism**. The three species of passerine birds investigated in this study were chosen primarily because previous evidence had indicated that the **species status** of some of the bird populations, especially those whose distribution is delimited also by **minor rivers** (Jiparaná and Aripuanã), require a **revision**:
Wedge-billed Woodcreeper (*Glyphorynchus spirurus* complex, Furnariidae),
Chestnut-tailed Antbird (*Myrmeciza hemimelaena* complex, Thamnophilidae),
Spot-backed Antbird (*Hylophylax naevius* complex, Thamnophilidae).

In the case of ***G. spirurus***, Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenies generated from two mtDNA genes (cyt *b*, ND2) of 134 individuals shows several incongruences regarding current subspecies taxonomy and, in some cases, high levels of genetic divergence (up to 6%) in absence of morphological differences. The *BEAST tree generated from five loci (cyt *b*, ND2, sex-linked gene ACO, the G3PDH intron, and MYO intron 2) shows **paraphyly** confirming mtDNA data. Genomic fingerprinting using five ISSR primers reveals **population structure** across the Madeira River.

For the second species complex, ***M. hemimelaena***, the mtDNA (cyt *b*, ND2) phylogenies (BI, ML) of 65 individuals reveal three distinct lineages, including a so far undescribed **new taxon** whose distribution is restricted to a small region delimited by the Jiparaná and Aripuanã rivers.

The third species complex, ***H. naevius***, also shows **paraphyly** and discordances with the current taxonomy according to mtDNA (cyt *b*, ND2) phylogenies (BI, ML) and the *BEAST tree generated from three loci (cyt *b*, ND2, FB5 intron) from 80 individuals. The molecular phylogeny of *H. naevius* revealed a **new species** apparently limited by the Madeira River and its right bank tributary, Jiparaná.

Area cladograms of the three species complexes were similar suggesting a **single set of vicariance events** similarly and simultaneously affecting the three taxa. A **basal split** was identified dating from the **Pliocene** which separated the northern and southern Amazon River populations. Subsequent phylogeographic divisions correspond to the limits of the Rio Negro and the Madeira River. Fine-scale phylogeographic structure dating from the **Pleistocene** across right bank tributaries of the Madeira River (the Aripuanã and Jiparaná rivers) was revealed for the three species complexes. However, in this region the sequences of phylogeographical divisions were not identical which suggests a complex history diversification in the Madeira River Basin.

The spatial and temporal patterns presented here suggest that **tectonic activity** leading to major drainage reorganization in the Amazon Basin during the late Pliocene–early Pleistocene was an important driver of cladogenesis in Amazonia. On the other hand, the results indicate a complex diversification scenario in which different vicariance events determined the according differentiation. This and previous studies have shown that the many species complexes in Amazonia commonly feature absence or small variation in morphological and ecological characters, while the occurrence of **distinct species** can be **uncovered by molecular phylogenies**.

Conservation efforts should focus in the regions delimited by the Aripuanã and Jiparaná rivers in the Madeira River Basin. This is one of the **most ecologically diverse areas of the Amazon Basin**, while also being one of the most vulnerable under the current serious anthropogenic pressure. Many taxa from the Madeira Basin, including those yet to be given proper formal scientific names and status (some revelead herein), may now be endangered or possibly already extinct. If **cryptic endemism** is the rule, then conservation policies that do not recognize this endemism will not adequately protect this regional diversity.

This thesis is based on the following manuscripts:

1 – **Fernandes, A.M.**, Wink, M., Aleixo, A. Phylogeography of the Chestnut-tailed Antbird (*Myrmeciza hemimelaena*) clarifies the role of rivers in Amazonian biogeography. Journal of Biogeography. doi: 10.1111/j.13652699.2012.02712.x (in press)

2 – **Fernandes, A.M.**, Gonzalez, J., Wink, M., Aleixo, A. Multilocus phylogeography of the Wedge-billed Woodcreeper *Glyphorynchus spirurus* (Aves, Furnariidae) in lowland Amazonia: Widespread cryptic diversity and paraphyly reveal a complex diversification pattern. Molecular Phylogenetics and Evolution. (under review)

3 – **Fernandes, A.M.**, Wink, M., Aleixo, A. Phylogeography and speciation of the Spot-backed Antbird (*Hylophylax naevius*) in lowland Amazonia. (in prep.)

1 Introduction

1.1 Distributional Patterns of Birds in the Neotropical Region

The Neotropical region supports the World's richest avifauna, constituting nearly a third of all living bird species (Ridgely and Tudor 1989, 1994). But as in other regions the biodiversity is not equally distributed throughout the Neotropics. Cracraft (1985) proposed 33 areas of avian endemism for South America showing that significant distributional pattern exists for birds. The avian species richness in this region increases northwestward as one approaches the Andes particularly along the eastern slope of the Andes of Ecuador (peaking at 845 species) and in southeastern Peru (peaking at 782 species) and southern Bolivia (peaking at 698 species) (Rahbek and Graves 2001). Thus, the richest region of the Neotropics corresponds to Amazonia. Six "hotspots" – areas that contain a large proportion of the World's biodiversity concentrated in a small area of the planet – were proposed for the Neotropical region (Myers et al. 2000). The biodiversity "hotspots" for conservation are also areas with extremely high numbers of endemic species threatened by human development. Thus, endemism has a special role in conservation of Neotropical birds.

The immense biological diversity and high endemism observed in this region is mainly associated to forest formations such as those that cover much of Central America, Amazonia, and the Atlantic coast. Amazonia is the largest such area, with the highest diversity (da Silva et al. 2005) and the majority of its bird species are endemic, i.e., occur only within the Amazon basin (Stotz et al. 1996). However, the distribution of species within Amazonia is also not random. On the contrary, lowland Amazonia is a mosaic of **areas of endemism**. Large geographical areas within the basin with a relatively uniform avifauna are different from other major areas of Amazonia. Individual regions bear very distinctive bird populations and these areas of endemism happen to correspond to the major interfluvia – Amazon-Rio Negro, Rio Negro-Solimões, Madeira-Tapajós (Fig. 1) (Haffer 1974; Cracraft 1985). Different types of birds occur on opposite banks of the large rivers – a phenomenon which is uniquely described for Amazonia.

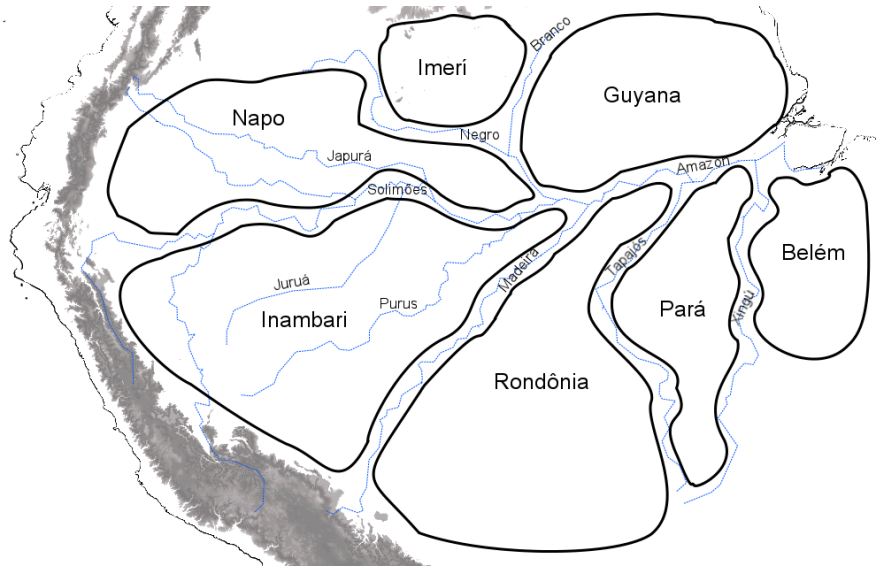
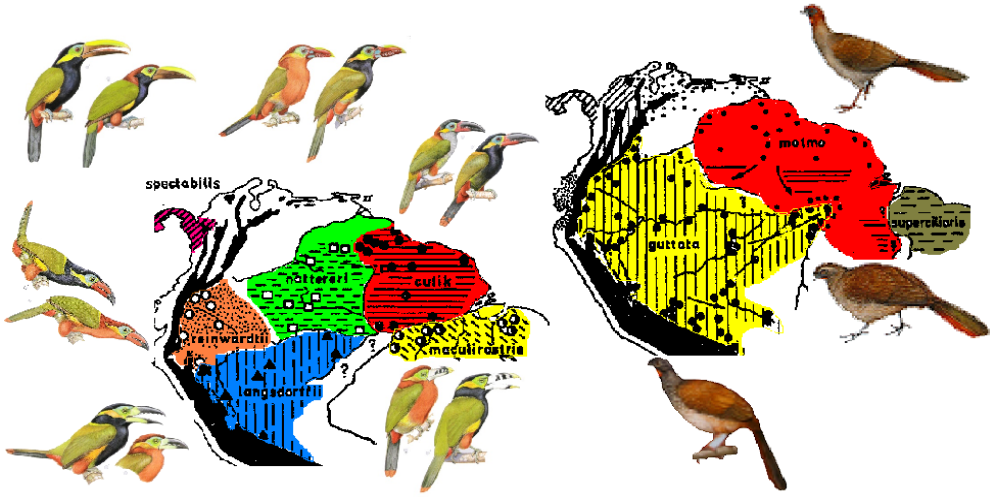


Figure 1 – Generalized localizations of Amazonian areas of avian endemism based on Cracraft (1985) and da Silva (2005). The major rivers are shown by dotted blue lines and their names are indicated.

The Amazon Basin is a promising natural laboratory for studying biogeographic concepts. The different species or subspecies found across the rivers are more closely related to each other than

to any other species/subspecies found on the same river bank. The sister taxa found on opposite banks of Amazonian rivers are often referred to as **superspecies**, suggesting that they are monophyletic (e.g., have a common ancestor) (Haffer 1969, 1974, 1997a) (Fig. 2). This pattern of range distribution offers a great opportunity to study qualitative aspects (e.g., relationships) of biotic areas, knowledge critical for understanding the history of Amazonia.



A toucanet, the *Selenidera maculirostris* superspecies

The chachalaca, *Ortalis motmot* superspecies

Figure 2 – Distribution of the *Selenidera maculirostris* superspecies and the *Ortalis motmot* superspecies (adapted from: Haffer 1969).

1.2 Ornithological Explorations in Amazonia

Lowland Amazonia is home to approx. 1300 species of birds, the highest species richness in the World described to date (Amadon 1973; Pearson 1977; Mittermeier et al. 2003) with roughly 1000 species reported for the Brazilian Amazon alone, which accounts for over 60% of the entire biome's surface (Aleixo 2009). Around 10,000 birds species are known in the world, therefore Amazonia habors 13% of the World's total number of species at only 5% of land. However, this huge diversity is still not well known. In fact, the number of sites in the Amazon Basin that can be regarded as relatively well studied (with lists of species) is minimal. Among these are the *terra-firme* forests of the region north of Manaus (Cohn-Haft et al. 1997), and the of Jau National Park (Borges et al. 2001) also in the state of Amazonas, the area of Alta Floresta in the state of Mato Grosso (Zimmer et al. 1997), and the Amazon National Park in Pará (Oren and Parker 1997).

Despite this general lack of ornithological information in Amazonia, the birds can still be regarded as the best known biological group. The first organized ornithological surveys to Amazonia date from the 19th Century, when foreign expeditions sponsored by western European countries were launched in order to collect bird specimens (Wied-Neuwied 1820; Spix 1824, 1825; Pelzeln 1856). The first half of the 19th Century was the beginning of ornithological explorations in remote areas away from the major two Amazonian cities in Brazil (Belém and Manaus) and the first ornithological collection was established in Amazonia at Belém (Museu Goeldi). Emilie Snethlage and the Ollala family collected birds along the major Amazonian rivers such as the Xingú, Tapajós, Purus, and Juruá (Snethlage 1913). Since then, the ornithological collection has fueled the construction of a well-developed knowledge of the diversity and distribution of Amazonian bird species. The first compilation of the various data was the encyclopedic work of Hellmayr (Cory 1918, 1919; Cory and Hellmayr 1924, 1925; Hellmayr 1929, 1934, 1935, 1936, 1938; Hellmayr and Conover 1948, 1949, 1942) which currently serves

important bibliographic source. Pinto (1938, 1944, 1978) provided the most important and comprehensive information on the synthesis of Brazilian birds. Since then, with the constant increase in the technical literature on Brazilian birds, including Amazonia, there is a more refined sense of geographical distributions.

Recent ornithological surveys have been conducted in sectors of the Brazilian Amazon standing out as the ornithologically least known; most of the results are still unpublished and authored mainly by the two current most active institutions carrying out ornithological surveys in the Brazilian Amazon: Museu Paraense Emílio Goeldi (MPEG) in Belém, and Instituto Nacional de Pesquisas da Amazonia (INPA), in Manaus. The accumulation of recent data on Amazonian birds by the few active researchers in the area has changed the knowledge on the distribution and composition of the bird fauna in the Brazilian Amazon. Recent field studies have expanded the known distributions of numerous species of birds by hundreds or even thousands of kilometers (Whittaker and Oren 1999; Borges et al. 2001; Naka et al. 2006; Aleixo and Poletto 2007; Whittaker 2009). In addition, new species have been validated or discovered (da Silva et al. 1995, 2002; Bierregaard et al. 1997; Whittaker 2002; Whitney et al. 2004; Ribas et al. 2005). Today, there are distribution maps for each species of bird in South America (Ridgely and Tudor 1989, 1994; del Hoyo et al. 1992–2004), including digital files suitable for analysis in systems of geographic information (Ridgely et al. 2003).

1.3 Taxonomy and True Biodiversity

Modern ornithological surveys attempt to obtain detailed information on each specimen collected. The amount of information obtainable from bird specimens has been growing considerably. Field-based information is supplemented by environmental and behavioral characteristics (such as song records employing digital recorders), and molecular analyses.

Such information is critical for expanding the current knowledge on species limits and biogeography of Amazonian birds. Since the 1990s there has been a growing interest in applying the **Phylogenetic Species Concept (PSC)** rather than the Biological Species Concept (BSC) in modern ornithological classification. Using molecular data, the application of PSC has provided an empirical basis for current classifications and have revealed an unexpected diversity in Amazonia linked to a complex biogeographical history. Using a molecular approach, several discrepancies in current subspecies classifications have been described (Cohn-Haft 2000; Aleixo 2004; Cheviron et al. 2005; Nyári 2005; Sardelli 2005; Fernandes 2007; Tobias et al. 2008). Several subspecies are not monophyletic in terms of haplotype relationships (Tavares et al. 2011), some examples including: Ocellated Woodcreeper (*Xiphorhynchus ocellatus*), Buff-throated Woodcreeper (*Xiphorhynchus guttatus*) (Aleixo 2002); Snethlage's Tody-Tyrant (*Hemitriccus minor*) (Cohn-Haft 2000; Sardelli 2005); Blue-crowned Manakin (*Lepidothrix coronata*) (Cheviron et al. 2005); Wedge-billed Woodcreeper (*Glyphorynchus spirurus*) (see Sect. 3.1); and Spot-backed Antbird (*Hylophylax naevius*) (see Sect. 3.3).

Besides of not being monophyletic, the genetic distance among populations within the same subspecies of several Amazonian birds correspond to the divergence found among sympatric species easily distinguishable by morphology, song and ecological characteristics. This possibly may lead, after appropriate analyses, to the recognition of **cryptic speciation**. For example, two populations currently classified as *Hemitriccus minor pallens* have 6% of genetic divergence in a mitochondrial gene (Cohn-Haft 2000; Sardelli 2005). Another similar example is described for

Glyphornchus spirurus inornatus. Populations of this subspecies reveal 5.4% genetic distance in the cytochrome *b* gene across the Aripuanã River, a comparatively small river in Amazonia (Fernandes 2007; see also Sect. 3.1). This high level of mtDNA differentiation corresponds to measures of intergeneric divergence among birds of the temperate zone (Johns and Avise 1998). The taxonomic uncertainty found for several taxa illustrates that the "true" diversity in Amazonia is underestimated which may jeopardize the interpretation of biogeographic patterns. Insights and concepts of global patterns of biogeography and diversification may change with improved taxonomy and molecular phylogeography, especially for tropical regions such as Amazonia (Tobias et al. 2008).

1.4 The 'Mini-Interfluvia' between the Rivers Madeira and Tapajós

Studies on birds and primates between the rivers Madeira and Tapajós in southeastern Amazonia have revealed a more complex biogeographic pattern. The tributaries of the Madeira River in the "Rondônia" area of endemism (Fig. 1) seems to constitute a barrier to the distribution of primates (van Roosmalen et al. 1998). The latter authors described geographic substitution in primates (genera *Mico* and *Calicebus*) on opposite banks of small rivers of this interfluvium and described a new species of marmoset, *Mico humilis*, that only occurs on the western bank of the Aripuanã River (Fig. 3). This study highlighted the importance of small rivers in delimiting primate distributions and gave impetus to field work on birds by Cohn-Haft et al. (2007). The latter authors found numerous cases of restricted distributions, opposite bank replacements, and river-delimited vocal variation in birds of this region. For example, the Bar-breasted Piculet (*Picumnus aurifrons*) shows distinct plumage coloration on opposite banks of the Aripuanã River (a tributary of the Madeira River) (Cohn-Haft et al. 2007) and the Warbling Antbird complex (*Hypocnemis* spp.) (Isler et al. 1998; Tobias et al. 2008) has vocally distinct populations with restricted ranges, apparently delimited by the Madeira, and two of its right tributaries, the Jiparaná and Aripuanã rivers. Similar patterns have also been found for butterflies (Hall and Harvey 2002).

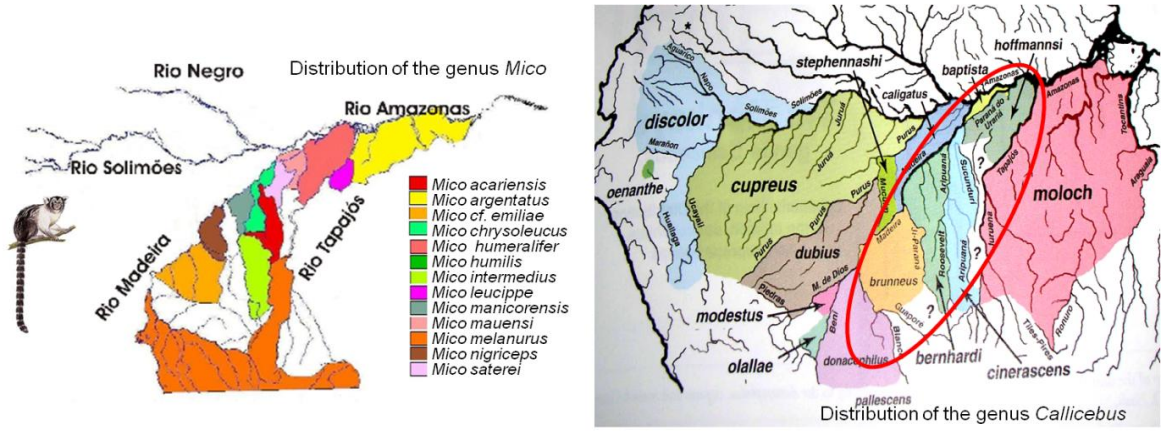


Figure 3 – Distribution of the genera *Mico* and *Callicebus* showing different species across samller rivers in the Madeira-Tapajós interfluvium (adapted from: van Roosmalen et al. 1998).

Molecular studies of birds from the Madeira-Tapajós interfluvium also indicate the relevance of small rivers as geographic barriers. For example, the Snethlage's Tody-Tyrant (*Hemitriccus minor*) (Sardelli 2005) and Wedge-billed Woodcreeper *Glyphorynchus spirurus* (Fernandes 2007) have populations genetically differentiated across the Jiparaná and Aripuanã rivers while being morphologically indistinguishable (see also Chap. 3). This suggests the existence of cryptic endemism in small regions in Amazonia.

Although these studies have created a new awareness of small-scale endemism within the Madeira-Tapajós interfluvium, this region is not yet recognized as having a unique fauna. The Amazonian eco-regions as recognized by WWF (Dinerstein et al. 1995) treat the entire interfluvium as if it were uniform (Fig. 4). However, Mesquita et al. (2007) mentioned the importance of recognizing "mini-interfluvia" in the definition of Amazonian reserves and since 2004 several protected areas were implemented. But this region is still under serious anthropogenic threat (Fearnside 2002, 2006). The southern part of the Madeira-Tapajós interfluvium is located in the "**arc of deforestation**," which is a band along the eastern and southern edges of the Amazonia forest. Deforestation advances from this band towards the center of the region. The deforestation is caused mainly by the advance of agriculture from southern Brazil (export commodities such as soybeans and beef).

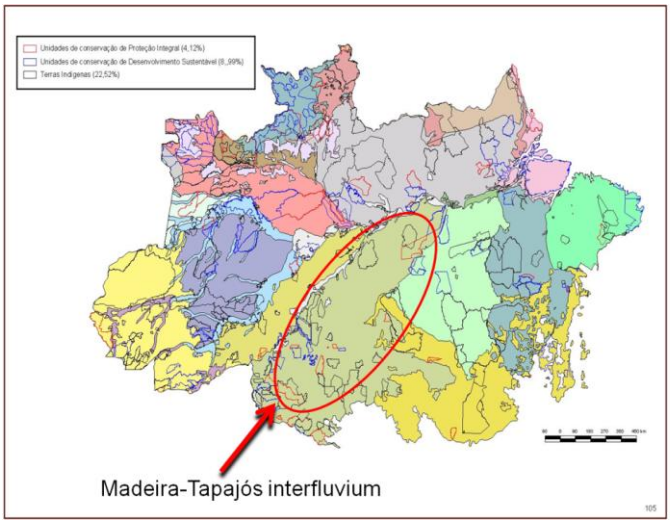


Figure 4 – The Amazonian eco-regions as recognized by WWF (adapted from: Dinerstein et al. 1995). River barriers within the Madeira-Tapajós interfluvium are not recognized.

1.5 Amazonian Historical Biogeography

The development of Amazonian biota has been a long and complex process (Hoorn et al. 2010). Recent advances in the fields of palynology, paleontology, climatology and phylogenetics have provided new insights on the diversification of the organisms and environmental changes in lowland Amazonia (for a review see Hoorn and Wesselingh 2010). While some scientists are using the geological data on Andes uplift to modelate climate patterns others are describing new fossils and pollen records from northern South America (Garzione et al. 2008; Graham 2009; Hoorn et al. 2010). In addition studies based on molecular data are providing information on the time of diversification events (Ribas et al. 2012). The exchange of information and the better communication among experts of these different fields has helped to clearify the complex history of this region (Aleixo and Rossetti 2007; Graham 2009; Hoorn and Wesselingh 2010).

The uplift of the Andes had a profound impact on the diversification of the organisms (Graham 2009; Hoorn and Wesselingh 2010). The drainage reversal of the Amazon river from flowing northwestwards to the modern system that flows to the Atlantic ocean was caused by the northern Andes uplift during the Miocene (Hoorn and Wesselingh 2010; Shephard et al. 2010). This affected climatic conditions in lowland Amazonia by changing the atmospheric circulation and rainfall in the Southern Hemisphere (Poulsen et al. 2010). A high biodiversity in South American is concentrated along the Andes foothills, more precisely in the area alongthe Colombia, Brazil,

and Peru border (Gentry 1982; Rahbek and Graves 2001). The relation of the high diversity and the Andes uplift has also been confirmed by recent phylogenetic studies showing that the Andean orogeny promoted the build-up of biodiversity in lowland Neotropical faunas (Miller et al. 2008; Burney and Brumfield 2009; Patel et al. 2011; Weir and Price 2011a) and flora (Antonelli et al. 2009). These studies showed that both vicariance-based speciation during uplift and dispersal-based speciation following uplift were processes causing diversification in Amazonia. The formation of the modern Amazon river system caused by the orogeny of the Andes had an important role in vicariant speciation on primates and birds (see Chap. 3).

In addition to the Andean orogeny, the Neogene (~23–2.5 Mya) was marked by a braided fluvial systems and wetlands in western Amazonia, prior to the development of the current drainage system as evidenced by paleogeographic and paleontological evidence (Fig. 5) (Webb and Hall 1995; Hoorn and Wesselingh 2010; Latrubesse et al. 2010). During the late Miocene, western Amazonia was dominated by swamps, lakes, internal deltas, and splays; these conditions are related to the tectonic behavior of the Central Andes in which sediments were deposited directly from the Andes in a subsiding basin and in a continental environment (Latrubesse et al. 2010) (Fig. 5). The wetlands could have acted as barriers to dispersal and thereby reproductively isolated populations of terrestrial animals (Aleixo 2004; Ribas et al. 2009) and plants (Antonelli et al. 2009). Aquatic conditions, however, seem to have persisted in western-central Amazonia until at least 7 Mya, when the modern Amazon system was established (Hoorn et al. 2010). Stratigraphic data suggest that this fluvial system extended westward and was limited by the Purus arch, an ancient ridge of western Amazonia that separated the Solimões and Amazon basins and restricted the Amazonian mega-wetland to the west and the paleo-Amazon River to the east (Shephard et al. 2010). The Purus arch is one of several ancient arches formed during uplift of the Andes and that subdivided the Amazon Basin (Graham 2009). Patton et al. (1994, 2000) found that some populations of small mammals, although morphologically similar, differed in having distinct mitochondrial DNA across the supposed locations of the Purus arch.

The subduction of the Nazca Ridge flat during the rise of the central Andes which caused the uplift of the Fitzcarrald Arch in the Pliocene (~4 Mya) also modeled the modern configuration of the Amazon drainage basin. This geological event may be one of the main factors that influenced large-scale modifications of the landscape in the Amazon and consequently induced drastic biota changes in the region since the Pliocene around 4 Mya (Hoorn and Wesselingh 2010; Ribas et al. 2012) (see also Chap. 3). The Amazon River reached its present shape and size from the Pleistocene onwards (Shephard et al. 2010).

The Quaternary (~ last 2.5 Mya) was marked by global cooling and therefore, drier climates in Amazonia. This appears to have caused a reduction of Amazonia, leading to islands of forest surrounded by savanna, especially during the last glacial maxima. Haffer (1969; on patterns of birds distribution; Fig. 2) as well as Vanzolini and Williams (1970; on diversification patterns of the lizard *Anolis chrysolepis*) proposed that populations of forest organisms survived in isolation on those patches of forest (called "Refugia"). This challenges the classical river barrier hypothesis proposed by Wallace in the 19th century. Haffer's explanation, despite being strongly critized, provided an empirical basis for the formulation of new biogeographic hypotheses. Moreover, the geographic patterns he described were used as a basis for delimiting the areas of endemism of Neotropical birds (Fig. 1).

A considerably less complex and more widely accepted explanation is the **river barrier hypothesis**. New studies based on mtDNA sequence variation and molecular dating point out the influence of the rivers in Amazonia in the evolution of Neotropical birds (Ribas et al. 2012; see also Chap. 3). However, the whole diversification process in Amazonia may not be explainable by only one vicariant event, since the history (as described above) was very complex. Several events at different times influenced the diversification of the organisms in this region as suggested by the data on Amazonian birds presented in this dissertation (see Chap. 3).

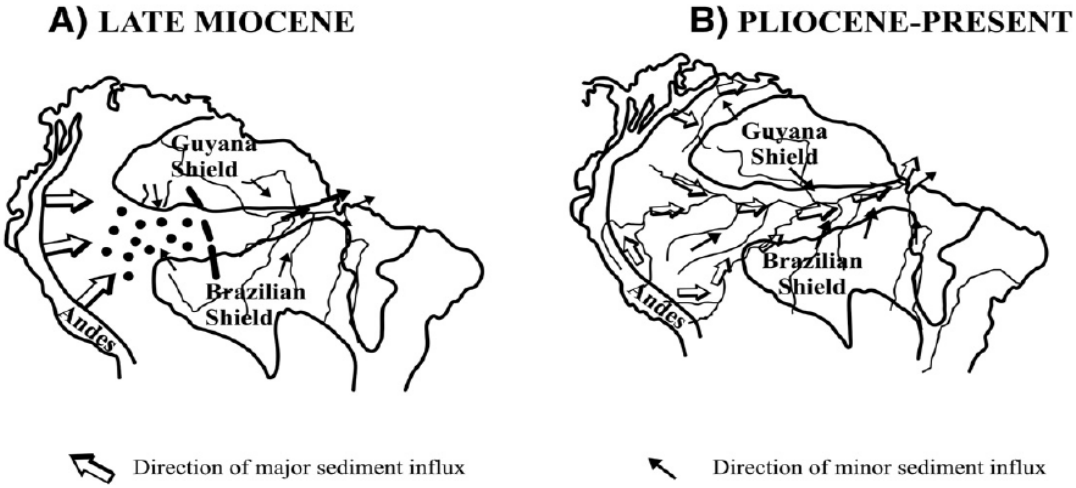


Figure 5 – Paleogeographical reconstruction of sediment and water influx in the Amazon basin during the Late Miocene and from the Pliocene to the present. (**A**) The area was an active sedimentary subsiding basin during the Late Miocene. Western Amazonia received sediments directly from the Andes (*dotted* area). The eastern limit of the Solimões basin is indicated with a *dashed* line. The incomplete Amazon River system was just draining the cratonic-platforms area located to the East. (**B**) After the reorganization of the basin, during the transition between the end of the Miocene and the early Pliocene, the rivers of Southwestern Brazilian Amazonia (Purus, Jurua, and Javarí basins) became lowland rivers without contact with the Andes chain and the Peruvian basins (Ucayali and Madre de Dios) were reorganized into the form as we observe them today. The Amazon system became totally integrated as a transcontinental fluvial basin (cited from: Latrubesse et al. 2010).

1.6 Phylogeography

Biogeography is the science that attempts to document and understand spatial patterns of biological diversity (Lomolino et al. 2010). Since the biological diversity is not equally distributed in time and space, **areas of endemism** are an important concept in biogeography. Throughout the history of biogeography many concepts were proposed to define areas of endemism which are the building blocks of biogeography (Parenti and Ebach 2009).
Most definitions of areas of endemism try to apply phylogenetic methods to biogeography. Thus, its concept is analogous to monophyly in systematic biology and it should not be artificial. Rather it should relate space occupied by a lineage throughout time.

With the advances of molecular biology this concept has been more widely used because molecular phylogenies allow the incorporation of time in spatial analysis. An important application of these concepts and methodologies is the possibility of dating vicariant events and to test hypotheses about paleoclimate and geological events (Ribas et al. 2012). The subdiscipline of biogeography that uses this approach on the intraspecific level is know as **phylogeography**.

Phylogeography studies the historical aspects of the distribution of genetic lineages (Avise 2000). The main aspects emphasized in studies of phylogeography are the description of molecular

variation within and between populations and evolution of lineages through time and space (Avise 2000) (Fig. 6). These studies have widely employed mitochondrial DNA; approx. 70% of phylogeographic studies of animals were performed exclusively or primarily on mtDNA sequences (Avise 2000) (Fig. 7). However, there is a growing concern that the use of only mitochondrial genes may not reflect the evolution of the species but only the gene history (Maddison 1997) (see Sect. 3.1).

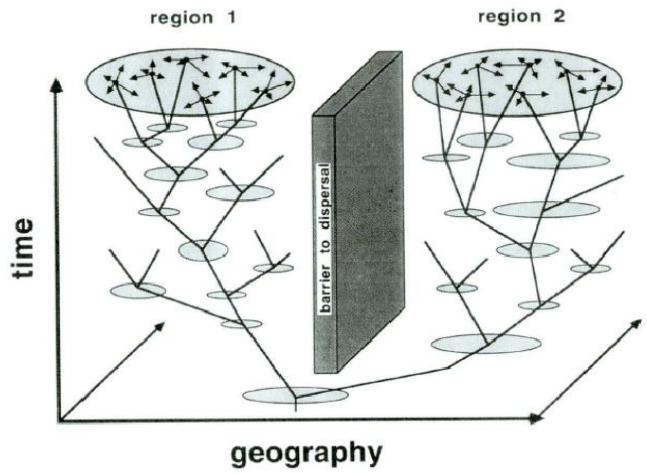


Figure 6 – Hypothetical genealogy for a species displaying restricted gene flow within each of two physically separated regional populations. *Shaded ovals* represent geographic ranges of particular lineages, and *arrow vectors* in the extant populations (*top*) denote spatial magnitudes of contemporary dispersal of individual from their natal sites (cited from: Avise 2000).

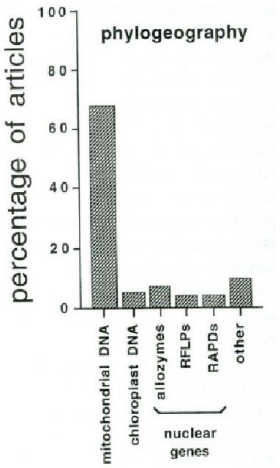


Figure 7 – Widespread use of mtDNA. Breakdown of phylogeographic articles (1987–1998) according to the molecule or assay employed (source: Avise 2000).

The use of mtDNA in studies of population genetics is due to its uniparental origin, usually maternal, having thus haploid DNA, and the according genes are not subject to recombination (Avise 2000). This allows to infer, with a satisfactory accuracy, the past evolution and demographic population history. In addition, these genes have a high mutation rate, favoring intraspecific comparisons. It is believed that the high mutation rate of the mitochondria is due to the following factors: lack of encoding proteins directly related to their replication, lacks of error repair mechanisms, excess of metabolic waste, low-fidelity replication in the mitochondria, and absence of association with histone proteins (Avise 2000). This molecule is also easy to handle due to the large number of copies per cell, small size, and simple organization (Avise 2000) (Fig. 8).

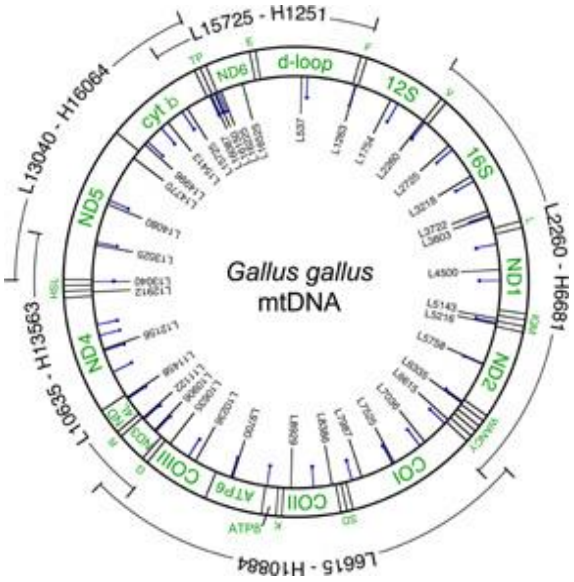


Figure 8 – Schematic diagram of the mitochondrial DNA (source the Sorenson lab, Boston University)

Comparative phylogeography attempts to determine the historical processes of a biogeographic region from cladograms of genealogies of multiple codistributed species (Bermingham and Moritz 1998). Agreement between cladograms of codistributed species reflects a common evolutionary history and, associated with geological and paleontological data, allows to infer the origin and formation of a biogeographic region (Zink 1996).

However, studies of comparative phylogeography in Neotropical regions have shown patterns of incongruent phylogenies among codistributed species. Costa (2003) reported inconsistent phylogeographic patterns among small mammals and suggests that the processes of speciation were unique to each organism, and that "inconsistencies of topologies of phylogenies are a reality for the Neotropical region". For birds there is no evidence of a unique evolutionary history in common that can explain all the biogeographical patterns found. This variation of observed patterns justifies the importance of conducting more comparative studies among co-distributed taxa, since the processes involved in the Neotropical region appear to be complex and quite variable among groups.

1.7 Coalescence

Coalescence was first described at the beginning of the 1980s (Hudson 1983; Tajima 1983). It is the instrument most widely used in the interpretive analysis of population processes that underlie the variation of DNA sequences (Rosenberg and Nordborg 2002). This provides a simple and powerful tool for exploratory data analysis because it has a genealogical approach, modelling the population processes backward in time. The biggest advantage is that it is possible to limit the analysis to the samples that are available (Nordborg 2000). Thus it is not necessary to take into account the entire population (Fig. 9).

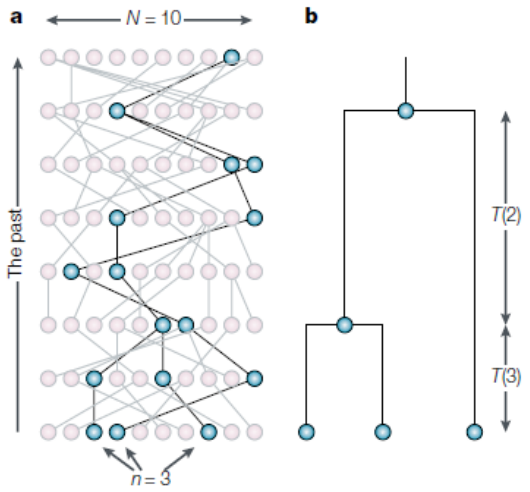


Figure 9 – The genetic composition of a population is completely determined by the group's genealogy and the mutations that occur on it. **(a)** The complete genealogy for a population of ten haploid individuals is depicted by *light dots* (diploid populations of *N* individuals are typically studied using a haploid model with 2*N* individuals). The genealogy of a sample from this generation is shown in *dark dots*. **(b)** In the basic version of the coalescence it is only necessary to keep track of the times between coalescence events (*T*(3) and *T*(2)) and the topology – that is, which lineages coalesce with others. *N*, number of allelic copies in the population; *n*, sample size (cited from: Rosenberg and Nordborg 2002).

Under the coalescence theory, one may consider a population of *N* clonal organisms reproducing according to the neutral Wright-Fisher model (e.g., constant *N*, random mating, lack of selection, discrete generations). Based on the observed polymorphisms of the sampling it is possible to trace the ancestry of a group of individuals of this population back through time. The number of distinct lineages decreases and eventually reaches one, as the most recent common ancestor (MRCA) of the individuals in question is encountered (Nordborg 2000). None of this is affected by neutral genetic differences between the individuals. The MRCA can be estimated based on the observed polymorphisms of the sampling haplotypes and contains information about the unobserved underlying genealogy. Since the average number of nucleotide differences and nucleo-diversity have a large variance, and a large part of this variance is due to stochastic factors, increasing sample size does not help reduce the variance significantly (Tajima 1983; Nordborg 2000). Therefore, it is not necessary to sample the entire population to estimate the MRCA (for a statistical description of the model see Nordborg 2000).

An application of the coalescent analysis is to estimate the demographic history of natural populations (see skyride plots in Chap. 3). Genealogies in an exponentially growing population are less random and will tend to have most coalescences early in the history (Fig. 10).

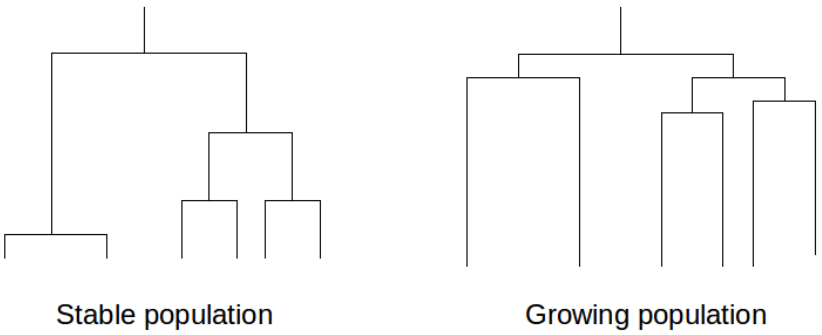


Figure 10 – A constant population is compared to one that has grown exponentially (cited from: Nordborg 2000).

1.8 Molecular Clock

Molecular dating is a technique in molecular biology that uses rates of molecular change to infer the time it took a lineage to radiate in a particular area. The molecular clock approach relates substitution rates of nucleotide or amino acid sequences to divergence times of taxa. Knowing the time of diversification is a key to understanding the evolution of biotic interactions and the evolution of the organisms (Weir 2006). Molecular dating has been used also to estimate rates of speciation and extinction (Weir and Schluter 2007) and determine population demographic history (Vilaça and Santos 2010; Hollatz et al. 2011).

The molecular clock hypothesis was first advanced in the 1960s by Emile Zuckerkandl and Linus Pauling. They calibrated the amino acid substitution rate in mammalian hemoglobins and found them to be proportional to divergence times inferred from paleontological data which suggest that DNA and proteins evolve at an approximately uniform rate. Under this assumption, knowledge of the evolutionary rate allows to date divergence times from sequence data.
In practice, the method they proposed allows biologists to give a temporal dimension to phylogenetic trees constructed from molecular data (Morgan 1998).

The **accuracy of the molecular clock** has long been a subject of controversy. Originally, it was believed that mutation rates are constant across loci and lineages. However, over the years several papers indicated various heterogeneities of the evolutionary rates across nucleotide positions within a codon, among nonhomologous genes within a lineage, among classes of DNA within a genome, among genomes within an organismal lineage and among taxonomic lineages (Avise 2000). By the 1970s, it was well accepted that different proteins evolve at different evolutionary rates (for a historical review see Kumar 2005; Takahata 2007; Wilke et al. 2009. Today it is clear that the universal nature of the molecular clock can be rejected and the calibration of 2% per one million years of animal mtDNA does not hold in many lineages (Kumar 2005). However, new methodologies to deal with the inconstancy of the evolutionary rate were developed and the molecular clock has been revolutionized and reveals new insights into evolutionary biology.

Molecular-dating methods can be divided into population genetics and phylogenetics approaches. In population genetics, a coalescent framework is used to estimate the age of the most recent common ancestor (MRCA) (see Sect 1.7 on Coalescence) of a number of alleles. This approach is applicable for estimating divergence times within a species. For phylogenetic studies several methods have been described. Previous studies used genetic distance matrices to estimate substitution rates but today substitution rates are derived from the phylogenies (Wilke et al. 2009).

Tree-based molecular clocks use the branching topology of a phylogeny together with branch length information to estimate the node depth. Also the total branch lengths between two lineages have been used for estimating divergence times. However, the inconstancy of the evolutionary rates among lineages was the major challenge for molecular dating as the estimation of divergence times from branch lengths (Wilke et al. 2009).
The **relaxed clock model**, which assumes different rates for different branches, has been used to deal with this problem. Some methods are used to test if a strict clock model, which assumes that mutations occur at a single rate along all branches in the phylogeny, can be used in the calibration. The widely used approach for testing the acceptance of a global molecular clock is the likelihood ratio test (LRT) (Felsenstein 1988).

Fossils are one type of source to estimate substitution rates in a given phylogeny. However, due to limitations inherent in the fossil record, especially in Amazonia where the conditions required for fossilization are rare, other techniques have been employed. Moreover, fossils are notoriously difficult to identfy and to classify within a modern DNA-based phylogenetic framework. In the absence of an adequate fossil record, **geological events are often used to calibrate molecular clocks**. The formation of the Isthmus of Panama, which isolated the tropical western Atlantic and eastern Pacific oceans, is one such event that is frequently used to infer rates of nucleotide sequence divergence in the Neotropical region (Weir et al. 2009). However, there is no precise dating for most geological events and calibrations based on biogeographical data suffer from the fact that phylogenetic events may or may not be associated with major biogeographical events (Wilke et al. 2009). Dispersal events linked to geological history are still more complicated.

The origin of volcanic islands can be used to calibrate phylogenies but the problem associated with this method is that island colonization events can occur long after their origin. Calibrations associated with vicariant events are more reliable.

Recently, studies on birds have shown a **constancy in the evolutionary rates of the mtDNA cytochrome *b***. Weir and Schluter (2008) analyzed 90 candidate avian clock calibrations obtained from fossils and biogeographical events. They found constancy in the evolutionary rates (approx. 2.1% per one million years) across 12 taxonomic avian orders over a 12-million-year interval. An elevated substitution rate in cyt *b* appears to be a rare phenomenon in birds, and has been suggested only for one case of an Old World species (*Nectarinia humbloti*; Warren et al. 2003). This strengthens the applicability of **molecular clock approaches in studies of evolution**. In this dissertation, the applied method was that of Weir and Schluter (2008).

1.9 Aims and Scope

This study contributes to an understanding of the **biogeographic history** of **passerine birds** of the **Amazonian forest** by studying their intraspecific diversification. Three species complexes were studied:

Wedge-billed Woodcreeper (*Glyphorynchus spirurus*, Furnariidae)
Chestnut-tailed Antbird (*Myrmeciza hemimelaena*,Thamnophilidae)
Spot-backed Antbird (*Hylophylax naevius*, Thamnophilidae).

The methods and analysis of results included:

- **Sequencing of genes**: mitochondrial (cyt *b*, ND2) and nuclear (ACO, G3PDH intron, MYO intron 2, FB5 intron)
- **Area cladograms** were generated and compared between the three species in order to see whether the birds share the same evolutionary history for mtDNA genes and species trees combining all genes
- **Haplotype networks** constructed with maximum parsimony and median-joining algorithm
- **Dating** divergences based on the mutation rate of cyt *b*
- **Historical population size dynamics** reconstructed by Gaussian Markov random field (GMRF); population genetics analyzed by classical methods (nucleotide diversity π, haplotype diversity *h*, Tajima's *D* and Fu's F_s statistics).
- **Genomic fingerprinting** by means of inter-simple sequence repeats (ISSR) to confirm the obtained population genetic structure.

Background information on laboratory and analytical methods and general laboratory protocols are given in Chap. 2. The results are presented in the three sections of Chap. 3. The individual contributions are summarized in a broader phylogeographic context in Chap. 4.

This thesis shows that molecular phylogenetics can provide convincing evidence of previously unrecognized, distinct and **new species** pointing to the actual extent of **"true" biodiversity** in Amazonia – areas seriously threatened by agricultural and urban development and in need of devoted conservation measures.

2 Materials and Methods

The following provides background information and a rough outline of the procedures used. More detailed information on methods and analysis (i.e., primers, parameter settings, sample information, etc.) will be given in the particular chapters.

2.1 The Three Birds of Interest

The infraorder Furnariides is a large and diverse Neotropical group of about 600 species (Moyle et al. 2009). These birds display a diverse array of morphologies and behaviors (del Hoyo et al. 2002–2004) and are abundant in tropical forests, especially the antbirds (Thamnophilidae) and the woodcreepers (Dendrocolaptidae/Furnariidae). Antbirds and woodcreepers are good models to study evolutionary processes affecting diversification in Amazonia. Several species of antbirds and woodcreepers inhabit the understory of the forest, hence are poorer dispersers and more prone to develop phylogeographic structure than canopy birds and they exhibit morphological, vocal, and genetic differentiation across major geographic barriers such as mountain ranges and rivers (Marantz et al. 2003; Zimmer and Isler 2003).

The three target species were chosen primarily because previous morphological and bioacoustic evidence and molecular data (Fernandes 2007) had indicated that the **species status** of some of the bird populations, especially those whose distribution is delimited by small rivers (**Jiparaná** and **Aripuanã**), **required a revision**. They are relatively common, easy to collect, and widely distributed across the entire Amazon Basin. Although all three species are members of the infraorder Furnariides (suboscine passerines) and are typically understory forest birds, they differ in a variety of autecological attributes and, as such, support the generality of any results found in common. The distribution of these birds spans lowland Amazonia (across rivers and mountains). Studying these birds thus offers a unique opportunity to disentangle the evolutionary processes underlying bird evolution in this region.

2.1.1 Wedge-billed Woodcreeper, *Glyphorynchus spirurus* (Furnariidae)

Glyphorynchus spirurus (Furnariidae) occurs throughout the Amazon basin. This Neotropical bird is one of the most common Amazonian birds and inhabits different types of forest – both *terra-firme* and seasonally flooded forests (Várzea and Igapó). It is a polytypic species widely distributed in Neotropical lowland forests, in Amazonia, Central America, and along the Atlantic coast of Brazil (Fig. 11) (Ridgely and Tudor 1994). Marantz et al. (2003) recognized thirteen subspecies, six of which occur in the Brazilian Amazon. With a body mass ranging from 10.5 to 21 g (typically 12.6–14.8 g in central Amazonia; Bierregaard 1988), this is the smallest woodcreeper (Marantz et al. 2003).

2.1.2 Chestnut-tailed Antbird, *Myrmeciza hemimelaena* (Thamnophilidae)

Myrmeciza hemimelaena is endemic to the Amazon (Fig. 12). The genus *Myrmeciza* is heterogenous and polyphyletic (Brumfield et al. 2007; Moyle et al. 2009) with *M. hemimelaena* belonging to a group of smaller and usually more colorful species. This passerine bird occurs in upland *terra-firme* forest, often in areas where those forests grow on predominantly sandy soils, and sometimes can be found associated with patches of bamboo (pers. obs.). It is not found with mixed flocks. Two subspecies are currently recognized: *M. hemimelaena hemimelaena* (west of the Madeira River and south to the La Paz province in Bolivia) and *M. hemimelaena pallens* (east of the Madeira River; Zimmer and Isler 2003).

2.1.3 Spot-backed Antbird, *Hylophylax naevius* (Thamnophilidae)

Hylophylax naevius is a typical *terra-firme* forest bird. It is distributed throughout most of the Amazon Basin (Fig. 13) (Ridgely et al. 2003). It lives in the undergrowth of humid forest where it is usually found foraging between 1 to 3 meters of the ground. It is not normally associated with mixed flocks nor does it follow ant swarms as a rule. *H. naevius* consists of six subspecies as described on the basis of morphological differences (Zimmer and Isler 2003).

Figure 11 – Geographic distribution of *Glyphorynchus spirurus*

Figure 12 – Geographic distribution of *Myrmeciza hemimelaena*

Figure 13 – Geographic distribution of *Hylophylax naevius*

2.2 Sample Material

DNA tissue samples were collected from living birds. All tissues were obtained either directly in the field or from the following institutions: Academy of Natural Sciences, Philadelphia, USA (ANSP), Field Museum of Natural History, Chicago, USA (FMNH), Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil (INPA), Laboratório de Genética e Evolução Molecular de Aves, São Paulo, Brazil (LGEMA), Louisiana State University Museum of Natural Science, Baton Rouge, USA (LSUMZ), Museu Paraense Emílio Goeldi, Belém, Brazil (MPEG), Universidade Federal de Mato Grosso, Cuiabá, Brazil (UFMT), and Natural History Museum of Denmark, Copenhagen, Denmark (ZMUC); tissue samples were subsequently vouchered together with research specimens (mostly skins). Birds were caught with standard techniques using mist nets or by shotgun. In some cases, birds were recorded with directional microphones and a digital recorder (Sound Devices model 702) and recordings can be used for future bioacoustic studies.

All collecting activities were authorized by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA). In accordance with permit stipulations and Brazilian law, all specimens were deposited at the Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil (INPA).

In total, 134 individuals of *Glyphorynchus spirurus*, 65 of *Myrmeciza hemimelaena*, and 80 of *Hylophylax naevius* were analyzed. For outgroup rooting, sequences of closely related taxa were taken from GenBank. List of samples with voucher numbers, institutions of origin, GenBank accession numbers, and detailed information on localities is given in the particular chapters.

2.3 Apparatus

All instruments employed in laboratory analyses are listed in Table 1.

Table 1 – Analytical instruments used in the present study

Instruments	**Company**
Automated sequencer: ABI 310, ABI 3100	Applied Biosystems
Electrophoresis microcomputer power supply E452	Fröbel
Gel chambers for agarose gel	Heidelberg University
Laboratory scale	Sartorius
Microcentrifuge-Biofuge 13R	Heraeus
Microcentrifuge: Biofuge Fresco	Heraeus
PCR machines: TRIO-Thermoblock and T Gradient	Biometra
PH meter: Pipetman P2, P20, P100, P1000	Gilson
UV-transiluminator II-200-M [312nm]	Bachofer
Vortex: Reax 2000	Heidolf
Incubator	Heraeus
Photometer DU 640	Beckman

2.4 Solutions and Chemicals

A list of chemicals, enzymes, and other materials used in this study is given in Table 2, followed by a list of buffers and solutions in Table 3.

Table 2 – Chemicals, enzymes, and solutions used in this study

Chemicals, Enzymes and other Marials	**Company**
Acetic acid	Merck
Agarose	HYBAID-AGS
Amonium sulfate	Gerbu
Amonium acetate	Merck
Big Dye Terminator kit	Applied Biosystems
Bovine serum albumin	Sigma
Premix Terminator kit	Amersham- Bioscience
Chloroform	Fluka
Ethanol absolute	Merck and J.T Becker
EDTA	Roth
Ethidium bromide	Serva
Formamide	Applied Biosystems
Bromophenol blue	Serva
Guanidine thiocyanate	Roth
Isopropanol	Applichem
ß-Mercaptoethanol	Merck
Nucleotides	Sigma
Phenol	Merck
Proteinase K	Merck
Reaction tubes (0.2, 0.5, 1.5, 2 ml)	Eppendorf
REDTaq™ DNA polymerase	Sigma
Sequagel Solution	Biozym
Sodium dodecyl sulfate (SDS)	Applichem
Sodium acetate	Merck
Sterile filter, 0.22 µm	Sartorius
Taq DNA polymerase	Sigma
dfs-*Taq* DNA polymerase	Bioron
Tris-HCl	Roth
Merck water	Merck
Mineral oil	Sigma

Table 3 – Buffers and solutions used in this study

Stock Solutions	Components
Agarose gel solution	1.3% agarose, 1 µg/ml ethidium bromide, in water
Ammonium acetate	4 M ammonium acetate, in water
Ammonium persulfate	10% solution in water
Chloroform/isoamyl alcohol	Chloroform/isoamyl alcohol in ratio 24:1
EDTA buffer	10% EDTA, 0.5% NaF, 0.5% thymol, 1% Tris (pH 7.5)
Guanidine thiocyanate buffer	4 M guanidine thiocyanate, 0.1 M Tris-HCl, 1% ß-mercapto-ethanol
λ-PST I size standard	DNA cut with PST I restriction enzyme
Lysis buffer	25 mM EDTA, 75 mM NaCl, 10 mM Tris-HCl, pH 7.0
Nucleotide mix	2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 dTTP
PCR buffer (10X)	100 mM Tris, 500 mM KCl, 5% Triton X-100, 15 mM $MgCl_2$,
Phenol/chloroform	Phenol, chloroform, isoamyl alcohol in ratio 25:24:1
SDS solution	20% solution in water
Sodium acetate solution	3 M sodium acetate, acetic acid (pH 4.6)
Sodium chlorid solutium	Sodium chloride in water (saturated)
TAE buffer	40 mM Tris, 1 mM EDTA, acetic acid (pH 8.0)
TBA buffer (10X)	1 M Tris, 89 mM boric acid, 10 mM EDTA, pH 8.6
TE buffer	10 mM Tris, 1 mM EDTA, hydrochloric acid (pH 8.0)

2.5 DNA Extraction, Amplification, and Sequencing

DNA was extracted from breast muscle (approx. 0.2 g) using a standard phenol/chloroform protocol (Sambrook et al. 1989). PCR amplifications were performed with 50 µl reaction volumes containing 1 × PCR buffer (Bioron, Ludwigshafen, Germany), 100 µM dNTPs, 0.2 units of Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 200 ng of DNA, and 5 pmol of primers. Optimal annealing temperature was determined by gradient PCR in a Tgradient thermocycler (Biometra). Thermal cycling was performed under the following conditions: (1) an initial denaturing step at 94°C for 5 min; (2) 35 cycles: 1 min at 94°C, 1 min at 52–60°C, and 1 min at 72°C; and (3) a final 5-min extension at 72°C. PCR products were precipitated with 4 M NH_4Ac and ethanol (1:1:6) and centrifuged for 15 min (13,000 rpm). Sequencing was carried out on an ABI 3730 automated capillary sequencer (Applied Biosystems) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 by STARSEQ GmbH (Mainz, Germany). To confirm observed mutations, both strands of each sample were sequenced.

2.6 Data Preparation

The obtained sequences were aligned manually. Recommended precautions were applied and all sequences confidentally represent mitochondrial DNA for the following reasons: (1) DNA was extracted only from tissue samples, which have high ratios of mitochondria to nuclei relative to blood or skin samples; (2) no stop codons occurred within the mitochondrial genes of any of the sequences; (3) sequences contain no insertions or deletions relative to one another or to other known avian sequences; (4) sequences in both DNA fragments from each individual were identical and unambiguous; (5) in phylogenetic analyses, no samples appeared in unexpectedly basal portions of the tree or had exceptionally short or long branch lengths, both of which, if present, would indicate a fast evolving gene or an early diverged gene (e.g., a pseudogene).

2.7 Phylogenetic Analyses

The phylogenetic analyses of mtDNA marker genes were performed using Bayesian inference (BI) implemented in MrBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) and maximum likelihood (ML) via RAxML GUI vs. 0.93 (Silvestro and Michalak 2011). The evolutionary models were selected with JMODELTEST using the BIC (Bayesian Information Criterion) (Posada 2008). Two independent runs of 5^6 generations each were conducted and trees were sampled every 500 generations. The first 500 samples were discarded as burn-in. Individual genes were analyzed independently and concatenated in a single data matrix. In the combined dataset, a mixed model Bayesian analysis was employed.

Using sequences of the mitochondrial and nuclear genes the species trees were estimated using the method proposed by Heled and Drummond (2010) in *BEAST 1.5.4 (Bayesian Evolutionary Analysis Sampling Trees). The haplotypes of all sequenced genes were grouped in different trait sets, defined by the phylogroups found in the mtDNA analyses. The models were estimated in JMODELTEST (Posada 2008) and used for each partition *a priori*. The model parameters were estimated together with the tree topologies in the same analysis. In addition to the substitution model, the tree topologies were estimated independently for each gene except for the mtDNA genes. Due to a lack of recombination among mitochondrial genes in many organisms including birds, mtDNA genes should be considered as linked in this kind of analyses (Heled and Drummond 2010).

To visualize genealogical relationships among individuals, haplotype networks were constructed with maximum parsimony in the program TCS 1.18 (Clement et al. 2000) or using the median-joining algorithm in NETWORK 4.5.1.0 (Forster et al. 2007). Mean pairwise *p*-distances (Nei 1987) within and among lineages were calculated using MEGA 4.0 (Tamura et al. 2007).

2.8 Molecular Dating

Dating divergences were based on a study by Weir and Schluter (2008), who performed a cross-validation analysis on a dataset that included multiple Neotropical avian lineages of 12 taxonomic Avian orders. They proposed a divergence time mean substitution rate of 0.0105 substitutions/site/lineage/million years for the cytochrome *b* gene. Variation rates of molecular substitutions in the cyt *b* gene among different species appears to be a rare phenomenon in birds. An elevated substitution rate has been suggested only for one case of an Old World species (*Nectarinia humbloti*; Warren et al. 2003).

Estimates of divergence times of main lineages were conducted using data partitions, each with individual models of molecular evolution chosen by JMODELTEST 0.1.1 (Posada 2008). Two runs setting the clock model as relaxed, uncorrelated lognormal were performed. Two independent simultaneous runs of 10^6 generations were performed, sampling one every 1000 trees in BEAST v1.6.1 (Drummond and Rambaut 2007). Posterior probabilities of the nodes were computed for all Bayesian analyses across the sampled trees after burn-in. The number of generations required to reach stationarity of the posterior distribution was determined by examining marginal probabilities plotted as a time series in TRACER 1.5 (Rambaut and Drummond 2007).

2.9 Population Genetics and Historical Demography

For population genetic analyses, groups were defined according to the major clades of haplotypes recovered in the phylogenetic analyses. Calculations of the following population parameters were based only on cyt *b* sequences: nucleotide diversity (π), haplotype diversity (h), Tajima's D, and Fu's F_s statistic were calculated in ARLEQUIN 3.1 (Excoffier et al. 2005). Significance was determined based on 1000 coalescent simulations. To confirm and expand on the results obtained from the phylogenies, *a posteriori* analysis of population genetic structure was performed using AMOVA (Excoffier et al. 1992). The defined groups were also the major clades recovered in the phylogenies.

The historical population size dynamics was reconstructed using GMRF skyride plot (Minin et al. 2008) implemented in BEAST v1.6.1 (Drummond and Rambaut 2007). This is a nonparametric analysis based on the waiting time between coalescent events in a gene tree to estimate changes in effective population size over time. It differs from the related Bayesian skyline plot (Drummond et al. 2005) by not requiring the specification of a user-defined prior on the number of population size changes in the history of the sample (Ho and Shapiro 2011). The defined groups were the clades recovered in the phylogenies for which more than 5 individuals were available.

2.10 Genomic Fingerprinting by Inter-Simple Sequence Repeats (ISSR)

At multiple genomic loci, the inter-simple sequence repeats (ISSR) technique provides a rapid, highly reproducible and inexpensive way of targeting structural changes at the genomic level (Gonzalez et al. 2008; and references therein). ISSR markers present some advantages over SSR methods since no prior knowledge of target sequences is necessary. In birds, this technique has been used for population genetic studies (Gonzales and Wink 2010) and ISSR co-migrating bands may correspond to informative diagnostic characters in intraspecific and closely related species comparisons (Gonzalez et al. 2008).

ISSR-PCR bands were scored as dominant markers, 1 = band present and 0 = band absent. In order to examine the clustering pattern of all individuals a principal coordinate analysis (PCoA) was performed using FAMD 1.25 (Schlüter and Harris 2006) with Jaccard coefficient. The potential number of clusters (K) of individuals was inferred using of a Markov chain Monte Carlo algorithm implemented in STRUCTURE 2.2.2 (Pritchard et al. 2000; Falush et al. 2007). In order to estimate the value of K, a series of five independent runs (with K = 1–10) were performed using a no admixture model and correlated allele frequencies. The proportion of membership to the inferred clusters was assessed for each individual. The burn-in period was set up to 50,000 followed by 500,000 cycles.

3 Research Projects

3.1 Phylogeography and Speciation of the Wedge-billed Woodcreeper *Glyphorynchus spirurus* in Lowland Amazonia

3.1.1 Abstract

Amazonian rivers are important barriers to dispersal of Amazonian birds. Studying the population genetics of populations separated by rivers may help to uncover the dynamics of biological diversification in the Amazon. The phylogeography of the wedge-billed woodcreeper, *Glyphorynchus spirurus* (Furnariidae) in the Amazon basin were reconstructed. Sampling included 134 individuals from 63 sites distributed in eight Amazonian areas of endemism which are separated by major Amazonian rivers. Nucleotide sequences were generated from five loci: two mtDNA genes (1047 bp from cyt *b* and 1002 bp from ND2) and three nuclear genes (647 bp from the sex-linked gene ACO, 319 bp from the intron of G3PDH, and 619 bp of MYO intron 2). In addition, 37 individuals were randomly selected from the Rondônia and Inambari areas of endemism for genomic fingerprinting, using five ISSR primers. The findings reveal allopatric and well-supported lineages within *G. spirurus* with high levels of genetic differentiation (*p*-distance 0.9–6.3%) on opposite banks of rivers. The according multilocus phylogenetic reconstruction reveals several incongruences with current subspecies taxonomy. Within several subspecies, high levels of both paraphyly and genetic differentiation were found, indicating deep divergences and strong isolation that are consistent with species-level differences. ISSR fingerprinting supports the separation between the subspecies *castelnaudii* and *inornatus*. Molecular dating supports an initial vicariation event isolating populations from the Guiana center of endemism during the Upper Miocene/Lower Pliocene, while more recent events subdivided Brazilian Shield populations during the Lower Pleistocene. The results indicate recent population fluctuations during the Pleistocene, supporting an important prediction of the refuge hypothesis.

3.1.2 Introduction

The Brazilian Amazon holds more avian species than almost any other region on Earth (Mittermeier et al. 2003). As in other regions, avian species are not randomly distributed across the Amazon (Haffer 1974). Instead, the evolutionary history of the South American continent has created particular regions known as “areas of endemism” (Haffer 1974; Cracraft 1985), which harbor distinctive assemblages of species and populations whose ranges are delimited by major rivers, such as the Amazon, Rio Negro, Solimões, Madeira, and Tapajós. The finding that different avian assemblages occur on opposite banks of large Amazonian rivers is a phenomenon noted since the first naturalists visited the region over 150 years ago. In fact, the barrier function of rivers is among the earliest hypotheses attempting to account for the diversification of the Amazonian biota (Wallace 1852; Hellmayr 1910; Snethlage 1913).

Although the notion of areas of endemism has contributed over the years to the study of biogeography in Amazonia and elsewhere, other phylogeographic studies challenge the limitations of this approach, particularly concerning the degree of variability among lineages in their responses to common vicariant barriers (Aleixo and Rossetti 2007; Burney and Brumfield 2009; Antonelli et al. 2010). Furthermore, all areas of endemism in Amazonia and elsewhere in the Neotropics have been delimited historically based on the distributions of taxa which were

diagnosed morphologically and without an explicit phylogenetic framework (Haffer 1969, 1974; Cracraft 1985). Thus since the mid-1990s, comparative statistical phylogeography has provided a new paradigm for fundamental questions concerning the diversification of Amazonia and Neotropical biota as a whole (Patton et al. 1994; Costa 2003).

Recent advances in the fields of palynology, paleontology, climatology, and phylogenetics have provided new insights in the diversification of the organisms and environmental changes in lowland Amazonia (for a review see Hoorn and Wesselingh, 2010). For example, evidence of a dramatic change in the course of the Amazonian rivers during the late Pliocene was reported by Espurt et al. (2010) which was governed by neotectonics following the establishment of the Fitzcarrald Arch. This continental-wide drainage reorganization of the Amazon Basin has been reported as the main driver of avian speciation in Amazonia (Fernandes et al. in press; Ribas et al. 2012).

The Wedge-billed Woodcreeper (*Glyphorynchus spirurus*, Furnariidae) has a wide distribution ranging from Central America, west to the Andes, throughout central Amazonia, and south along the Atlantic coast of Brazil (Ridgely and Tudor 1994). It is a common species occurring in different types of lowland habitats, including both *terra firme* and seasonally flooded lowland forests (*várzea* and *igapó*), and up to about 1500 m in the Andes (Stotz et al. 1996). Taxonomically, *G. spirurus* represents a polytypic species with an uncertain number of recognized subspecies, depending upon the authority. The most recent review recognized thirteen subspecies (Marantz et al. 2003), most endemic or associated with particular areas of endemism (Haffer 1974; Cracraft 1985). These characteristics make *G. spirurus* an ideal model organism to investigate the influence of biogeographical barriers (e.g., rivers) on intraspecific diversity, and test hypotheses on the diversity of the Amazonian biota.

(Marks et al. 2002) had already analyzed fragments of three mitochondrial genes of *G. spirurus* to examine the phylogenetic relationships among Neotropical areas of endemism as defined by Cracraft (1985). The authors discovered several inconsistencies between their reconstructed molecular phylogeny and patterns of morphological differentiation providing the basis for subspecific diagnoses in *G. spirurus.* They also documented a more complex pattern of diversification between the Madeira and Tapajós rivers in central Amazonia, whereby smaller rivers in this interfluvium also appear to delimit the ranges of divergent and paraphyletic phylogroups (see also Sardelli 2005; Fernandes 2007 and Fernandes et al. in press). However, some critical nodes of the phylogenetic trees (Marks et al. 2002) were poorly supported, and the methods of phylogeny reconstruction (maximum parsimony and maximum likelihood) showed incongruent tree topologies. Moreover, the historical relationships among Amazonian areas of endemism found by Marks et al. (2002) contrasted strongly with those suggested for other bird lineages (Ribas et al. 2005, 2009; Patané et al. 2009).

Several unlinked genes are required for coalescent theory-based analyses. Superior results can be expected from additional loci (Maddison 1997). Coalescent methods should provide better estimates of speciation times by mitigating the influence of deeply coalescing alleles (Drummond and Rambaut 2007), and directly by providing better estimates of species trees than supermatrix methods (Liu et al. 2009; Heled and Drummond 2010). More genes may also increase the possibility of detecting anomalous loci such as those affected by paralogous gene copies or selection that might otherwise mislead phylogeny and divergence time estimations.

Using a multilocus approach and a larger dataset than previous avian phylogenetic studies in the Amazon, with a special focus on the Amazon basin the phylogeographic and population genetic structure of the Wedge-billed Woodcreeper, *G. spirurus*, were investigated. This study addresses the following questions: 1) Does the current taxonomy accurately reflect genetic structure of *G. spirurus* populations? 2) Do small rivers act to delimit populations within larger interfluvia? 3) Does the genetic structure of populations indicate potential zones of secondary contact? and 4) Which diversification hypotheses best explain the historical relationships revealed by the analysis of *G. spirurus* populations?

3.1.3 Materials and Methods

Taxon sampling

Altogether, tissue samples of *G. spirurus* were obtained from 63 sites distributed in eight Amazonian areas of endemism (sensu Cracraft 1985). Sampling localities encompassed opposite sides of the Andes, all major Amazonian river basins, and the Atlantic Forest on the Brazilian coast (Fig. 14). A total of 134 individuals were sequenced; samples from the Rondônia area of endemism had the largest sample size (see Table 4). Detailed information on specimens, sampling sites, as well as voucher numbers are provided in Table 5.

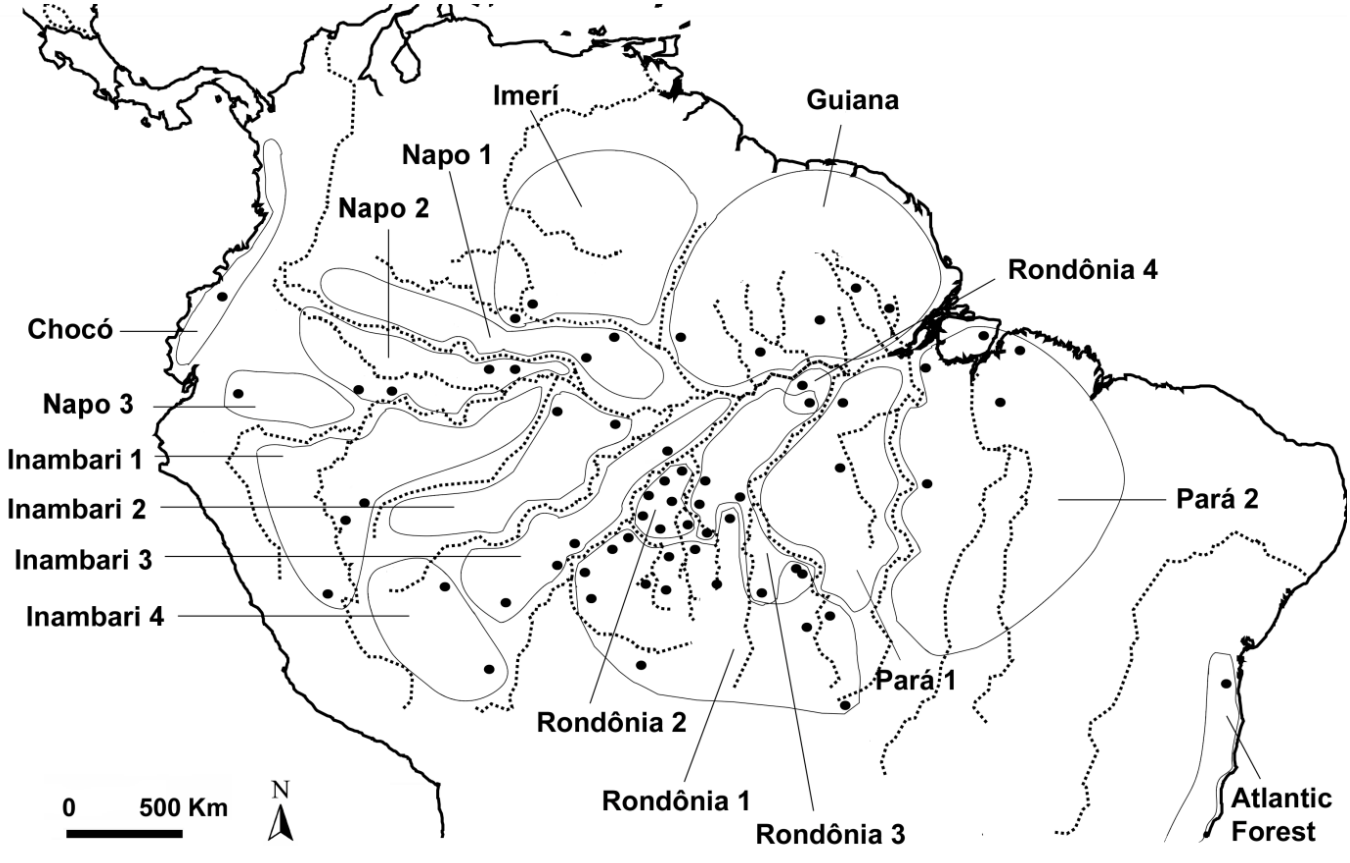


Figure 14 – Geographic distribution of samples (●) of Wedge-billed Woodcreeper (*Glyphorynchus spirurus*) and Neotropical areas of avian endemism based on Cracraft (1985). Outlined areas denote clades of haplotypes as shown in Figs. 15 and 16.

Table 4 – Origins, DNA markers, and sample size of *G. spirurus* analyzed in this study

Region*	Cyt *b* (1047 bp)	ND2 (1002 bp)	ACO1 (647 bp)	G3PDH (319 bp)	MYO (619 bp)
Chocó	2	–	–	–	–
Imerí	6	2	3	2	2
Inambari	16	2	6	4	2
Rondônia	85	16	21	20	18
Pará	8	3	2	2	2
Guiana	6	2	1	1	1
Napo	9	3	2	2	2
Atlantic Forest	1	–	–	–	–
Total	133	28	35	31	27

*Areas of endemism according to (Cracraft 1985).

Table 5 – Endemic region, voucher and GenBank accession numbers

Region	Voucher				
	Cyt *b*	ND2	ACO	G3PDH	MYO
ATLANTIC FOREST	AY089806*				
CHOCÓ	FJ899304* FJ899307*				
GUIANA/Brazil, AM: Viruá National Park, 35 km SSE Caracaraí. 1°29'23"S; 10°00'10"W	INPA1065 INPA1083	INPA1083			
GUIANA/Brazil, PA: Laranjal do Jari, left bank Jari River, Cachoeira Santo Antonio. 00°39'S; 52°30'W	MPEG56939				
GUIANA/Brazil, PA: FLOTA de Faro, ca 70 km NW de Faro. 01°42'S; 57°12'W	MPEG64639				
GUIANA/Brazil, PA: Alenquer, ESEC Grão-Pará. 00°09'S; 55°11'W	MPEG65384	MPEG65384	MPEG65384	MPEG65384	MPEG65384
GUIANA/Brazil, PA: Almeirim, REBIO Maicuru. 00°49'N; 53°55'W	MPEG66199				
INAMBARI1/Brazil, AC: Mâncio Lima, right bank upper Moa River, Ramon stream, PNSD. 7°27'S; 73°46'W	MPEG52733				
INAMBARI1	FJ899303* FJ899306*				
INAMBARI2/Brazil, AM: ca 35 km NW Coari, right bank Solimões River. 3°55'00"S; 63°23'50"W	INPA044	INPA044	INPA044	INPA044	
INAMBARI2/Brazil, AM: right bank of Juruá, RESEX Baixo Juruá, ca. 50 km South of Juruá. 3°45'S; 66°5'W	INPA818		INPA818	INPA818	
INAMBARI3/Brazil, AM: left bank of the Madeira River, ca 350 km SW Manaus, BR 319. 5°12'S; 61°50'W	INPA1495		INPA1495	INPA1495	
INAMBARI3/Brazil, RO: left bank Madeira River, near Jacy Paraná, 45 km SW Porto Velho. 9°10'S; 64°23'W	INPA349 INPA359	INPA349	INPA349 INPA359	INPA349	INPA349
INAMBARI3/Brazil, RO: left bank of the Madeira River, ca. 20 km N Abunã. 9°31'S; 65°21'W	INPA173 INPA191		INPA191		INPA191
INAMBARI3	FJ899296*				
INAMBARI4/Brazil, AC: ESEC Rio Acre, ca. 78 km W Assis Brasil. 11°03'24.5"S; 70°16'16.6"W	MPEG59810 MPEG59828				
INAMBARI4/Brazil, AC: ESEC Rio Acre, Acampamento 2. 11°00'53.4"S; 70°13'02.7"W	MPEG58874				
INAMBARI4	FJ899295* FJ899301*				
IMERÍ/Brazil, AM: left bank upper Rio Negro, 10 km east São Gabriel da Cachoeira. 0°10'S; 66°59'W	INPA1117 INPA1118 INPA1123	INPA1117	INPA1117 INPA1118	INPA1117 INPA1118	INPA1117
IMERÍ/Brazil, AM: right bank of upper Rio Negro, 3 km SW São Gabriel da Cachoeira. 0°8'S; 67°5'W	INPA1153	INPA1153			INPA1153
IMERÍ/Brazil, AM: right bank Rio Negro, 3 km SW São Gabriel da Cachoeira. 0°8'16"S; 67°05'40"W	INPA1143 INPA1159		INPA1143		

Region	Voucher				
	Cyt *b*	ND2	ACO	G3PDH	MYO
NAPO1/Brazil, AM: left bank middle Solimões River; RDS Amanã, Comunidade Nova Canaã, 2°36'S; 64°52'W	INPA224				
NAPO1/Brazil, AM: Barcelos, right bank Cuiuni River. 00°47'43"S; 63°09'53"W	MPEG59460	MPEG59460			
NAPO2/Brazil, AM: Japurá, Acanauí River. 01°56'12.4"S; 66°36'18.8"W	MPEG62654	MPEG62654	MPEG62654	MPEG62654	MPEG62654
NAPO2/Brazil, AM: Japurá, Mapari River. 02°02'31.5"S; 67°17'16.6"W	MPEG62658	MPEG62658	MPEG62658	MPEG62658	MPEG62658
NAPO2 **NAPO3**	FJ899298* FJ899299* FJ899300* FJ899305*				
PARÁ1/Brazil, PA: Belterra, Flona do Tapajós, Base guaipira, BR 163 km 117	MPEG08275				
PARÁ1/Brazil, PA:Satarém, chacará diamantina, estrada da Curva			MPEGA08283	MPEGA08283	
PARÁ1/Brazil, PA: Bom Jardim, ca. Cachoeira da Mucura, left bank Xingu River	MPEG63450	MPEG63450			
PARÁ2/Brazil, PA: Barcarena, Reserve of the Samaúma Hotel	MPEGA07869				
PARÁ2/Brazil, PA: Ourilândia do Norte	MPEGA8392				
PARÁ2/Brazil, PA: Paragominas, Fazenda Rio Capim, CIKEL. 03°40'S; 48°33'W	MPEG58966 MPEG58967				
PARÁ2/Brazil, PA: Ilha do Marajó, Ponta de Pedras, Fazenda Santa Maria. 01°20'08.3"S; 48°57'10.8"W	MPEG57837	MPEG57837	MPEG57837	MPEG57837	MPEG57837
PARÁ2/Brazil, PA: Portel, FLONA do Caxiuanã, Plot PPBIO. 01°57'S; 51°36'W	MPEG61735	MPEG61735			MPEG61735
RONDÔNIA1/Brazil, RO: left bank of lower Jiparaná River, ca. 20 km southeast Calama. 8°14'S; 62°46'W	INPA870 INPA876 INPA886	INPA870	INPA886	INPA886	INPA870 INPA886
RONDÔNIA1/Brazil, RO: right bank of Madeira River, 9.5 km southeast Porto Velho. 8°52'S; 64°0'W	INPA329		INPA329	INPA329	INPA329
RONDÔNIA1/Brazil, RO: right bank of Madeira River, ca. 20 km N Abunã. 9°35'S; 65°21'W	INPA208	INPA208	INPA208		
RONDÔNIA1/Brazil, AM: Floresta Estadual do Sucunduri, 8°34.5'S; 59°08.5'W	INPA845		INPA845	INPA845	
RONDÔNIA1/Brazil, RO: REBIO Jaru, rigth bank Jiparaná River. 10°07'S; 61°54'W	INPA2438 INPA2378 INPA2384		INPA2384	INPA2384	
RONDÔNIA1/Brazil, RO: Entorno REBIO Jaru, left bank Jiparaná River. 10°10'S; 61°55'W	INPA2401 INPA2409 INPA2439		INPA2439 INPA2440	INPA2439	
RONDÔNIA1/Brazil, RO: REBIO Jaru, rigth bank Jiparaná River, Tarumã stream. 9°27'S; 61°40'W	INPA2411 INPA2415 INPA2515				
RONDÔNIA1/Brazil, AM: Guajará-Mirim, Ouro Preto Biological Reserve. 10°50'S; 64°45'W	MPEG54933 MPEG54934 MPEG54937				
RONDÔNIA1/Brazil, MT: Sinop, left bank Teles Pires River, Sítio Chalé Kapp. 11°35.6'S; 55°40.2'W	MPEG57217 MPEG57218 MPEG8435				
RONDÔNIA1/Brazil, MT: Sinop, right bank Teles Pires River, Fazenda Missioneira. 11°36.3'S; 55°40.3'W	MPEG57214 MPEG57215 MPEG 7216				
RONDÔNIA1/Brazil, AM: Manicoré, Rodovia do Estanho, km 137. 08°41'S; 61°24'W	MPEG57581 MPEG57582				

Region	Voucher				
	Cyt *b*	ND2	ACO	G3PDH	MYO
RONDÔNIA1/Brazil, RO: Ji-Paraná, Igarapé Lurdes, Aldeia Gaviões. 10°26'S; 61°39"W	MPEG58183				
RONDÔNIA1/Brazil, MT: Apiacás, Foz do Apiacás	UFMTJB1166				
RONDÔNIA1/Brazil, MT: Aripuanã	UFMTJB638 UFMTJB643 UFMTJB672				
RONDÔNIA1/Brazil, MT: Primavera do Leste	UFMTJB881				
RONDÔNIA1/Brazil, MT: Aripuanã, Serra do Espedito	UFMTJB913 UFMTJB933 UFMTJB934				
RONDÔNIA1/Brazil, MT: SINOPI	UFMTJB1889 UFMTJB1848				
RONDÔNIA1	FJ899297*				
RONDÔNIA2/Brazil, AM: left bank of Aripuanã River, 130 km S Novo Aripuanã. 6°18'S; 60°24'W	INPA461 INPA466 INPA510	INPA461	INPA461 INPA466	INPA466	INPA461 INPA553 INPA562
RONDÔNIA2/Brazil, AM: left bank of lower Roosevelt River, confluence with Aripuanã River. 7°35'S; 60°43'W	INPA906	INPA906	INPA906	INPA906	INPA906
RONDÔNIA2/Brazil, AM: right bank of lower Roosevelt River, confluence with Aripuanã River. 7°38'S; 60°40'W	INPA895	INPA895	INPA895	INPA895	
RONDÔNIA2/Brazil, RO: right bank lower Jiparaná River, ca. 20 km southeast Calama. 8°09'S; 62°47'W	INPA875 INPA877	INPA875	INPA875 INPA877	INPA875 INPA877	INPA877
RONDÔNIA2/Brazil, AM: Humaitá, T. Indígena Parintintin, Aldeia Pupunha. 7°28'S; 62°56'W	MPEG58688				
RONDÔNIA2/Brazil, AM: Humaitá, Território Indígena Ipixuna, Aldeia Canavial, Miriti. 6°33'S; 62°03'W	MPEG59020	MPEG59020			
RONDÔNIA3/Brazil, AM: right bank of Aripuanã, 135 km S Novo Aripuanã. 6°18'S; 60°20'W	INPA535 INPA536 INPA559	INPA561	INPA559 INPA561	INPA559 INPA561	INPA559
RONDÔNIA3/Brazil, AM: right bank of middle Aripuanã, confluence with Roosevelt River. 7°37'S; 60°40'W	INPA890	INPA890	INPA890	INPA890	
RONDÔNIA3/Brazil, AM: Parque Estadual do Sucunduri; right bank of Bararati River. 8°21'S; 58°37'W	INPA852 INPA855	INPA855	INPA852		INPA852
RONDÔNIA3/Brazil, AM: RDS Aripuanã; left bank Aripuanã River; Água Branca stream. 8°25'S; 59°46'W	INPA1282		INPA1282	INPA1282	
RONDÔNIA3/Brazil, AM: left bank Maró River, Lago da Panela, Fé em Deus. 2°51'40"S; 55°40'56"W	MPEG56176				
RONDÔNIA3/Brazil, AC: Carlinda, left bank Teles Pires River, Fazenda Rio da Mata. 09°59.1'S; 55°34.5'W	MPEG57220				
RONDÔNIA3/Brazil, MT: Nova Bandeirante, right bank Juruena River, Fazenda Vale Verde. 10°15.7'S; 58°17.6'W	MPEG57222 MPEG57223				
RONDÔNIA3/Brazil, MT: Apiacás, Foz do Apiacás	UFMT JB1188				
RONDÔNIA3/Brazil, MT: Paranaíta, Fazenda do Apiacás	UFMTJB464 UFMTJB599				
RONDÔNIA4/Brazil, AM: right bank Maró River, Lago da Panela, Fé em Deus. 2°51'15"S; 55°40'36"W	MPEG56179 MPEG56180 MPEG56184	MPEG56179 MPEG56180	MPEG56180	MPEG56179 MPEG56184	MPEG56179 MPEG56180 MPEG56184
RONDÔNIA4/Brazil, AM: left bank Maró River, Lago da Panela, Fé em Deus. 2°51'40"S; 55°40'56"W	MPEG56175 MPEG56177 MPEG56178	MPEG56177 MPEG56178	MPEG56175	MPEG56177 MPEG56178	MPEG56175 MPEG56177 MPEG56178
RONDÔNIA4/Brazil, PA: Juruti, Base Capiranga, Mutum stream. 02º36'S; 56º11'W	MPEG58275 MPEG56618	MPEG58275		MPEG56618	MPEG58275 MPEG56618

Institution acronyms:
INPA – Instituto Nacional de Pesquisas da Amazônia;
MPEG – Museu Paraense Emílio Goeldi;
UFMT – Universidade Federal de Mato Grosso;
*Sequences downloaded from GenBank

DNA extraction, amplification, sequencing and ISSR genomic fingerprinting

DNA was extracted from breast muscle (approx. 0.2 g) and blood using a standard phenol chloroform protocol (Sambrook et al. 1989). Two mitochondrial genes, cytochrome *b* (cyt *b*) and nicotinamide adenine dinucleotide dehydrogenase subunit 2 (ND2), and three nuclear genes, intron 2 of the myoglobin gene (MYO), intron 11 of the glyceraldehyde-3-phosphodehydrogenase gene (G3PDH), and the sex-linked gene for aconitase (ACO) were amplified and sequenced (Table 6).

Table 6 – List of primers used in PCR amplifications and cycle sequencing

Primers	**Reference or sequences (5′–3′)**
cyt *b* L14990	Kocher et al. (1989)
cyt *b* L15389	Hackett (1996)
cyt *b* H15710	Helm-Bychowski and Cracraft (1993)
cyt *b* HXIPH and L15505	Aleixo (2004)
ND2 L5216 and H6313	Sorenson et al. (1999)
myo2	Slade et al. (1993)
myo3f	Heslewood et al. (1998)
my309l	Irestedt et al. (2006)
myo344h	Irestedt et al. (2006)
[1]G3PDH (f)	CAGCAGCTTTGCTGGAATCCCGTTA
[1]G3PDH (r)	GGCAGGTTCCCATCCACTTCCAATG
[2]ACO Ai15fb	CCCGTGCTAACTACCTAGCCTC
[2]ACO Ai15ra	CCCAGGAATAACATACTGACG

[1] Primers designed particularly for this project ; [2] C. Ribas (pers. comm.).

PCR amplifications were performed 50 µl reaction volumes containing 1 × PCR buffer (Bioron, Ludwigshafen), 100 µM dNTPs, 0.2 units of Taq DNA polymerase (Bioron, Ludwigshafen), 200 ng of DNA and 5 pmol of primers. Optimal annealing temperature was found by gradient PCR in a T-gradient thermocycler (Biometra). Thermal cycling was performed under the following conditions: (1) an initial denaturing step at 94°C for 5 min; (2) 35 cycles of the following: 1 min at 94°C, 1 min at 52°C (for the mitochondrial genes) or 55°C (for nuclear loci) and 1 min at 72°C; and (3) a final 5-min extension at 72°C. PCR products were precipitated with 4 M NH_4Ac and ethanol (1:1:6) and a centrifugation for 15 min (13,000 rpm). Sequencing was carried out using an ABI 3730 automated capillary sequencer (Applied Biosystems) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 by STARSEQ GmbH (Mainz, Germany). In order to distinguish mutations from PCR or sequencing errors, both strands of each sample were sequenced.

Genomic fingerprinting by inter-simple sequence repeats (ISSR)-PCR was performed with 30–60 ng of template DNA in 25 µl reaction volumes containing: 10 pmol of the 5′-anchored microsatellite repeat primer, 0.1 mM of dGTP, dCTP, and dTTP, 0.045 mM dATP, 1 µCi (α-^{33}P)-dATP (Amersham Biosciences), 0.6 units of Taq DNA polymerase (Pharmacia Biotech, Freiburg) and 2.5 µl of 10× amplification buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl and 1.5 mM $MgCl_2$). Thermocycling was performed with a T-gradient thermocycler (Biometra). Following the initial 10 min denaturation at 94°C, the program consisted of 35 cycles of 60 s at 94°C, 60 s at 52–60°C, 120 s at 72°C and 10 min at 72°C for final elongation. DNA fragments were separated by vertical polyacrylamide gel electrophoresis (gel length 40 cm) for 2–3 h at 65 W using a Base Acer Sequencer (Stratagene). After drying, the denaturating gels were exposed for 24 h to X-ray films (BioMax MR Film, Kodak) to produce autoradiograms. The ISSR primer sequences are documented in Table 7.

Table 7 – List of ISSR primers and annealing temperatures used in this study.

Primers	Annealing temperature
(GA)9C	60°C
(GACA)4	52°C
(CT)4(CA)5	54°C
(GA)9T	60°C
CTC(6)3AA	54°C

Phylogenetic analyses based on mtDNA

The phylogenetic analyses of mtDNA marker genes were performed using Bayesian inference (BI) implemented in MrBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) and maximum likelihood (ML) via RAxML GUI vs. 0.93 (Silvestro and Michalak 2011). The evolutionary models were selected with JMODELTEST (Posada 2008). Two independent runs of 10^6 generations each were conducted and trees were sampled every 500 generations. The first 500 samples were discarded as burn-in. The support for nodes in the maximum likelihood tree was assessed using 1000 bootstrap iterations. The mitochondrial genes (cyt *b* and ND2) were analyzed both independently and concatenated in a single data matrix. In the combined data set, a mixed model Bayesian analysis was employed using the GTR + I + G model for ND2 and the HKY + G model for cyt *b*. The genera *Nasica*, *Dendrocolaptes*, *Dendrexetastes*, *Hylexetastes*, *Xiphocolaptes*, *Xiphorhynchus*, *Lepidocolaptes*, *Drymornis*, and *Dendroplex* were used as outgroups (Moyle et al. 2009; Derryberry et al. 2011).

In order to visualize genealogical relationships among individuals, haplotype networks were constructed using the median-joining algorithm in the program NETWORK 4.5.1.0 (Forster et al. 2007). The mean pairwise *p*-distance (Nei 1987) within and among lineages was calculated using MEGA 4.0 (Tamura et al. 2007).

Molecular clock dating based on mtDNA

A molecular dating was carried out using the mtDNA genes with two partitions (cyt *b* and ND2), each with individual models chosen by JMODELTEST 0.1.1 (Posada 2008). The widely used cyt *b* mutational rate of 2.1% sequence divergence per million years (0.0105 substitutions/site/lineage/million years) was applied (Weir and Schluter 2008). Two runs setting the clock model as strict and relax were performed. Two independent simultaneous runs of 10,000,000 generations were performed, sampling once every 1000 trees in BEAST v1.6.1 (Drummond and Rambaut 2007). Posterior probabilities of the nodes were computed for all Bayesian analyses across the sampled trees after burn-in. The number of generations required to reach stationarity of the posterior distribution was determined by examining marginal probabilities plotted as a time series in TRACER 1.5 1 (Rambaut and Drummond 2007).

Multilocus molecular clock dating and species tree

In this study, the species trees were estimated using the method proposed by Heled and Drummond (2010) in *BEAST v1.6.1 (Drummond and Rambaut 2007). This method employs substitution models commonly used in phylogenetics, but also uses coalescence to provide joint inferences of a species tree phylogeny and divergence times from collection of gene trees across a set of closely related species.

The Bayesian haplotype reconstruction for nuclear loci (ACO, G3PDH, and MYO) was performed using PHASE 2.1 (Stephens et al. 2001). As this study is testing the historical relationships among areas of bird endemism of *G. spirurus*, the haplotypes of the five genes (Table 1) were grouped in different trait sets, defined by the endemism area where the populations were sampled. The model that fits the data best was estimated in JMODELTEST (Posada 2008) and used for each gene *a priori*. In addition to the substitution model, the clock model and tree topologies were estimated independently for each gene except for the mtDNA genes. Due to a lack of recombination among mitochondrial genes in most organisms (including birds), mtDNA genes should be considered as linked in this kind of analyses (Heled and Drummond 2010). The same cyt *b* mutational rate used in previous analysis were applied. Setting this mutational rate for the mitochondrial partition allowed to estimate the rate of substitution of the three nuclear partitions.

Two runs were performed setting the clock model as strict and relaxed. Two independent simultaneous runs of 10^6 generations were carried out, sampling once every 1000 trees. Posterior probabilities of the nodes were computed for all Bayesian analyses across the sampled trees after burn-in. The number of generations required to reach stationarity of the posterior distribution was evaluated with TRACER 1.5 (Rambaut and Drummond 2007).

Population genetics and historical demography using cytochrome b sequencing

Population genetics analyzes were carried out using the cyt *b* sequences just for those populations with the largest sampling sizes, i.e., those including two subspecies (*G. s. castelnaudii* and *G. s. inornatus*), two areas of endemism (Inambari and Rondônia), and opposite banks of three Amazonian rivers (Madeira River and two of its right bank tributaries, Aripuanã and Jiparaná). Population groups were defined as major haplotypes recovered in the phylogenetic analyses.

Using the cyt *b* sequences the nucleotide diversity (π) and haplotype diversity (h) among populations in ARLEQUIN 3.1 (Excoffier et al. 2005) were estimated. Tajima's D and Fu's F_s statistic were calculated in order to test population size fluctuations. Significance was determined based on 100 coalescent simulations. To confirm and expand on the results obtained from the phylogenies, *a posteriori* analysis of population genetic structure using AMOVA (Excoffier et al. 1992) were performed.

The historical population size dynamics using Gaussian Markov random field (GMRF) skyride plot method (Minin et al. 2008), implemented in BEAST v1.6.1 (Drummond and Rambaut 2007) were reconstructed. The GMRF skyride plot method is a nonparametric analysis that uses the waiting time between coalescent events in a gene tree to estimate changes in effective population size over time. It differs from the related Bayesian skyline plot (Drummond et al. 2005) by not requiring the specification of a user-defined prior on the number of population size changes (Ho and Shapiro 2011). In this study, the GMRF skyride plots were constructed using the cyt *b* mutational rate of 2.1% sequence divergence per million years (0.0105 substitutions/site/lineage/ million years), time-aware smoothing and strict molecular clock priors. All other parameters were identical to the molecular dating described above.

Clustering of individuals based on ISSR markers

Genomic fingerprints by ISSR-PCR (Gonzalez et al. 2008; Gonzales and Wink 2010) were performed for the populations from Rondônia and Inambari area (the geographic regions with the largest sampling sizes). ISSRs bands were scored as dominant markers, 1 = band present and 0 = band absent. In order to examine the clustering pattern of all individuals, a principal coordinate analysis (PCoA) was performed using FAMD 1.25 (Schlüter and Harris 2006) with a Jaccard coefficient. The potential number of clusters (*K*) of individuals was inferred using of a Markov chain Monte Carlo algorithm implemented in STRUCTURE 2.2.2 (Pritchard et al. 2000; Falush et al. 2007). In order to estimate the value of *K*, a series of five independent runs (with *K* = 1–10) were performed using an admixture model and correlated allele frequencies. The proportion of membership to the inferred clusters was assessed for each individual. The burn-in period was set up to 50,000 followed by 500,000 cycles. In order to evaluate the statistical significance of gene flow between species/subspecies it was used the USEPOPINFO option (Pritchard et al. 2000). The GENSBACK were set to 2 generations and MIGRPRIOR to 0.05.

3.1.4 Results

Phylogenetic analyses based on mtDNA

The Bayesian inference and maximum likelihood from cyt *b* sequences of 134 individuals provided similar tree topologies. Minor differences occurred in the position of the Inambari area of endemism. Both methods of phylogeny reconstruction produced sixteen clearly defined genetic lineages (Figs. 15 and 16).

The expanded Bayesian mtDNA and maximum likelihood (–ln L = 6934.670513) gene trees (based on combined cyt *b* and ND2 sequences; Fig. 17) were identical and consisted of an entirely resolved phylogeny for all cis-Andean populations of *G. spirurus*. With the caveat that this phylogeny includes a slightly smaller subset of individuals and areas than that based solely on cyt *b* sequences, only three nodes are supported by Bayesian posterior probabilities smaller than 0.95, including two with nearly significant posterior probabilities of 0.9 (Fig. 17). A high degree of population subdivision was found in the three areas of endemism: Rondônia (four clades apparently confined, at least in the northern part of their ranges, by the Aripuanã and Jiparaná rivers), Napo (two clades apparently confined by the Japura River), and Inambari (clades apparently confined by the Purus River; Fig. 14).

Subspecies classifications based on morphological characteristics (Marantz et al. 2003) mostly agree with the molecular phylogeny, with some notable exceptions. The subspecies *G. s. inornatus*, a taxon which is thought to be endemic to the Rondônia area of endemism, is not monophyletic. Other subspecies recovered as being non-monophyletic include *castelnaudii*, *paraensis*, and *rufigularis* (Figs. 15, 16, and 17). The mean pairwise genetic *p*-distances from mtDNA between major clades from different areas of endemism ranged from 1.0 to 6.3%, while the mean genetic *p*-distance within major clades varied between 0.0–0.8% (Fig. 15).

The median-joining haplotype networks based on cyt *b* sequences recovered 16 phylogroups that had already been identified in the Bayesian phylogeny. These clades display non-overlapping geo-graphic distributions and are separated by larger-than-average numbers of mutational steps (Fig. 18).

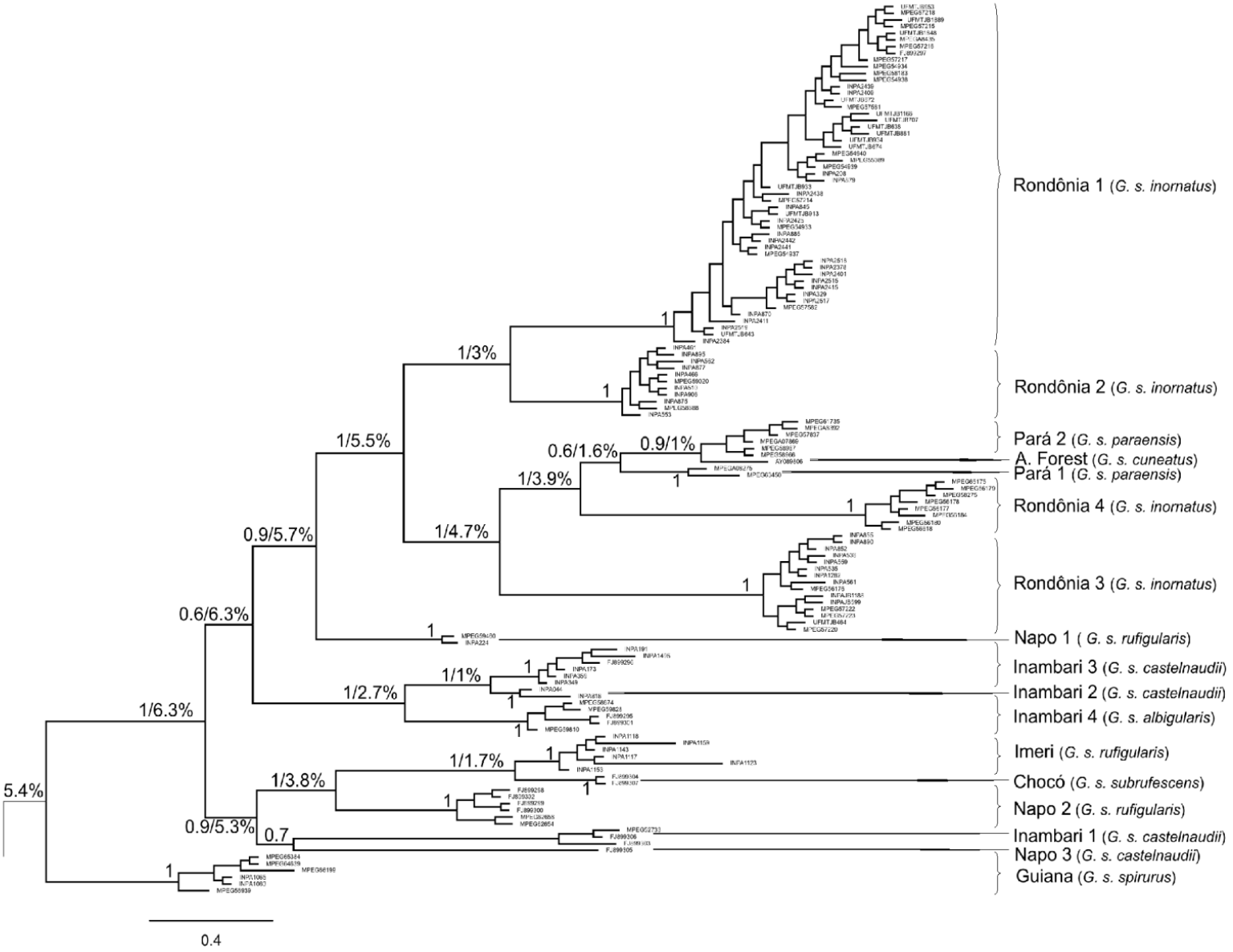


Figure 15 – Bayesian phylogeny based on cyt *b* sequences showing the relationships among different populations of *Glyphorynchus spirurus* distributed throughout the Neotropics. Numbers next to nodes refer respectively to posterior probabilities and uncorrected genetic *p*-distances (%).

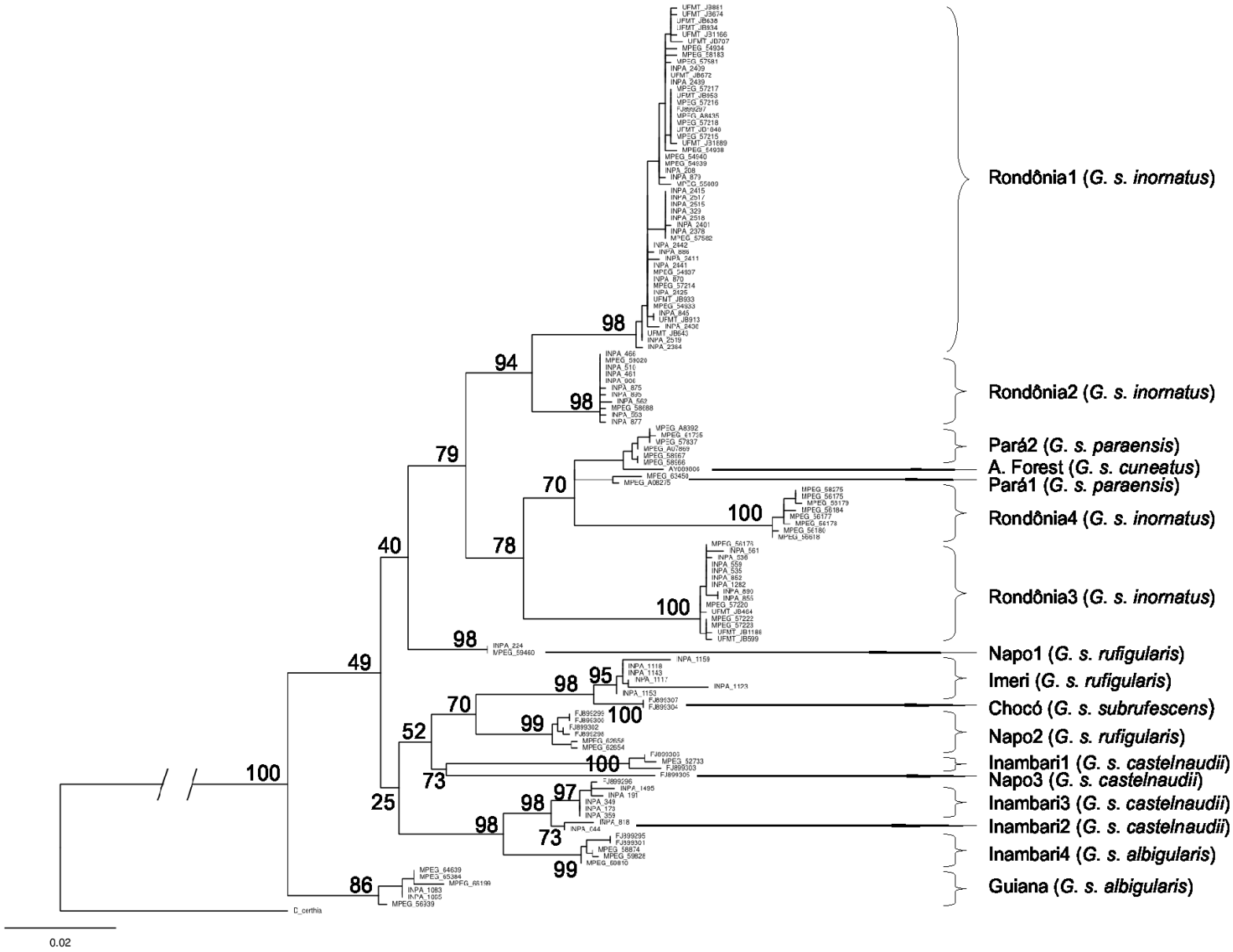


Figure 16 – Maximum-likelihood (–ln L = 6934.670513) tree of *Glyphorynchus spirurus* based on cyt *b* sequences. Numbers at each node represent the bootstrap values (based on 1000 replicates).

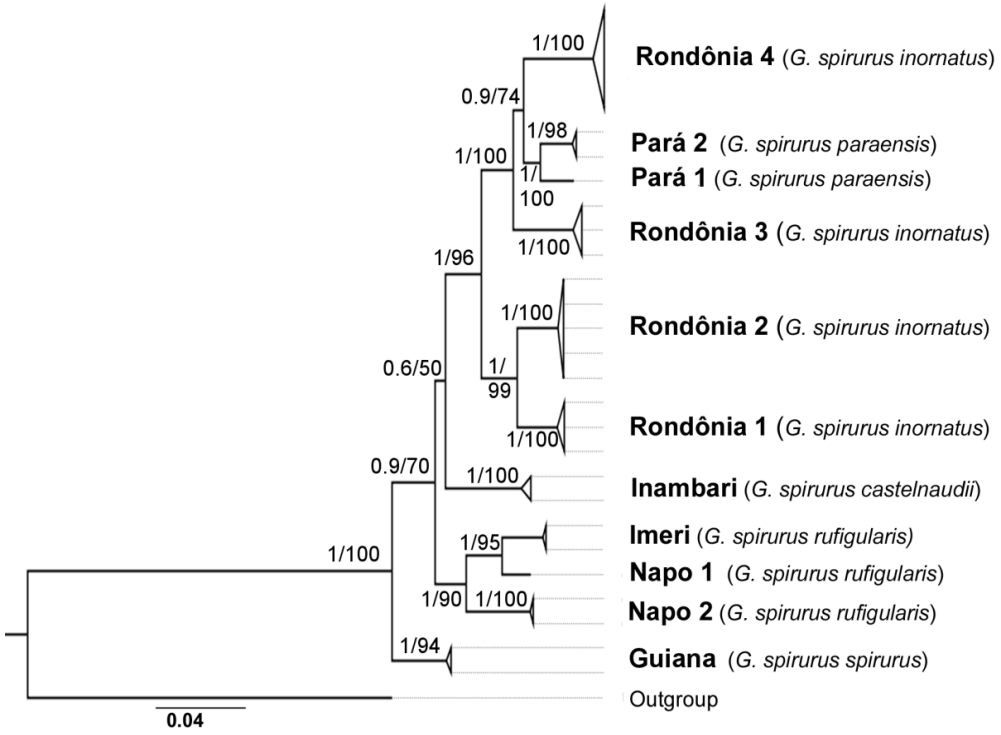


Figure 17 – Bayesian phylogeny based on concatenated ND2 and cyt *b* genes. The areas of avian endemism are indicated for each specimen. Numbers correspond to posterior probability values and the bootstrap values (based on 1000 replicates) from maximum likelihood (–ln L = 4635.368007) analysis.

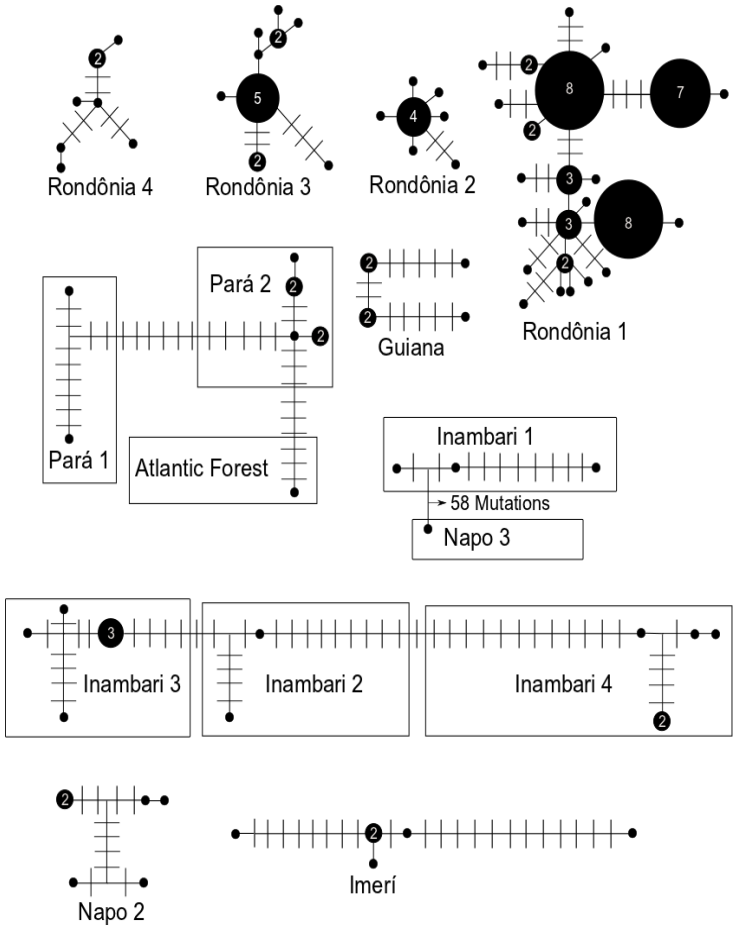


Figure 18 – Haplotype networks based on cyt *b* estimated for different clades within the Bayesian phylogeny for *Glyphorynchus spirurus* (see also Fig. 16). *Black circles* represent different haplotypes and their size is approximately proportional to haplotype frequency. *Numbers* inside the black circles refer to the number of sampled individuals possessing that particular haplotype. The mutational steps are indicated by *short solid lines* on each branch connecting haplotypes.

Molecular clock dating based on mtDNA

The strict and relaxed mtDNA molecular clock models resulted in trees with identical topologies and very similar divergence times. According to the relaxed-clock model the earliest split among *G. spirurus* lineages took place in the Pliocene (5.8–3.4 Mya; mean 4.5 Mya) (Fig. 19). Subsequent splits (Inambari and Rondônia/Pará) occurred in the middle-lower Pliocene (3.6–2.2 Mya; Fig. 19). The latest diversification involved the subspecies from the north of the Amazon River (Napo and Imerí regions) and the subspecies from the Brazilian Shield (Rondônia and Pará) during the Pleistocene.

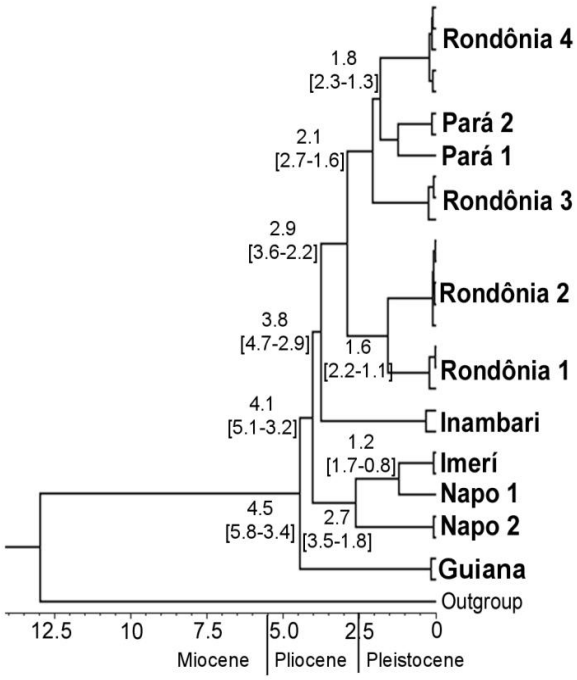


Figure 19 – Bayesian divergence times based on ND2 and cyt *b* genes (relaxed-clock model). *Numbers next to nodes* Bayesian divergence times (Mya). *Numbers within brackets* indicate 95% Bayesian credible intervals.

Multilocus molecular clock dating and species tree

The species tree estimated with *BEAST resulted in a topology with overall lower posterior probability values and more recent divergence time estimates compared to those retrieved from the concatenated mtDNA analysis. In terms of topologies and divergence times, similar results using either strict or the relaxed molecular clock models were obtained. As in the mtDNA dating analysis, the multilocus approach reveals that the areas of endemism from the Brazilian Shield (Pará and Rondônia) cluster together; similarly, the phylogeny also recovered the subspecies *G. s. inornatus* (the taxon endemic to the Rondônia area) as paraphyletic (Fig. 20). However, the position of *G. s. castelnaudii* (Inambari), which clustered together with north Amazonian lineages, differs from the mtDNA phylogeny, but with low support value (0.3)

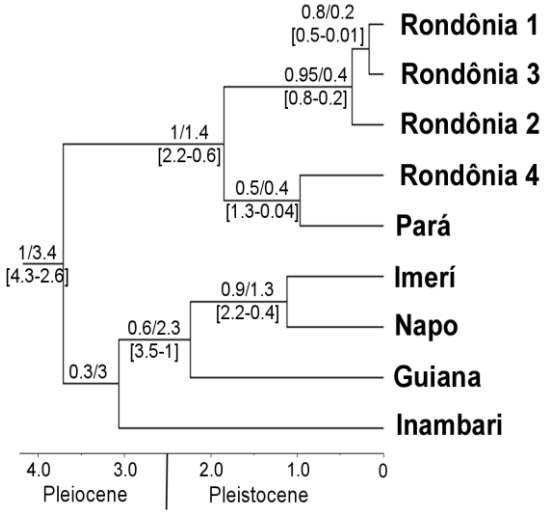


Figure 20 – Species tree based on a relaxed-clock model for *Glyphorynchus spirurus* indicating the relationship among areas of avian endemism. *Numbers next to nodes* refer to posterior probability values and Bayesian divergence times (Mya), respectively. *Numbers within brackets* indicate 95% Bayesian credible intervals.

Population genetics and historical demography using cytochrome b sequencing

The two analyses which were conducted from cyt *b* sequences to detect demographic changes suggest stable demographic histories for Inambari 1, 2, 3 and Rondônia 4, but demographic expansion for Rondônia 1, 2, 3. Values of Tajima's *D* and Fu's F_s statistic were not significantly negative for Inambari 1, 2, 3 and Rondônia 4. Conversely, Tajima's *D* and Fu's F_s statistics were significantly negative for Rondônia 1, 2. Higher values of haplotype diversity (*h*) in comparison to nucleotide diversity (π) were observed in all areas, except Inambari 3, 4, 5 where both parameters were equally high (Table 8). The AMOVA analysis indicates that most of genetic variance is partitioned among groups (90.5%, $P < 0.001$) corroborating the population structure revealed in the phylogenetic analyses.

Table 8 – Genetic diversity and neutrality tests of Inambari and Rondônia populations of *Glyphorynchus spirurus* sampled in this study

Region	***N***	***h***	***Π***	***D***	F_s
Inambari (3, 4, 5)	13	0.95	0.016	1.07	0.50
Rondônia 1	51	0.97	0.0037	–1.45*	–11.03*
Rondônia 2	10	0.87	0.001	–1.83*	–4.52*
Rondônia 3	10	0.95	0.002	–0.57	–3.80*
Rondônia 4	8	1.0	0.003	–0.41	–3.08

* Significant values ($P < 0.05$). *N* = number of individuals sampled; *h* = gene diversity; *Π* = nucleotide diversity; *D* = Tajima's *D* statistic; *F* = Fu's F_s statistic.

The Bayesian Skyride Plots (BSPs) which were estimated for the *G. spirurus* clades agree with the neutrality tests in inferring histories of demographic fluctuations during the Middle and Late Pleistocene. Rondônia 1 presents a population expansion between 0.30 and 0.25 Mya followed by a slight decline and another expansion in the last 0.10 Mya (Fig. 21). Rondônia 2 presents also an expansion-decline-expansion historical demography as Rondônia 1. A small and gradual population expansion was also recovered from Rondônia 3 during the last 0.10 Mya, while the other two clades in western Amazonia (Inambari) and in the northern portion of Madeira/Tapajós interfluvium (Rondônia 4) appeared to have maintained a relatively stable size during the Pleistocene (Fig. 21).

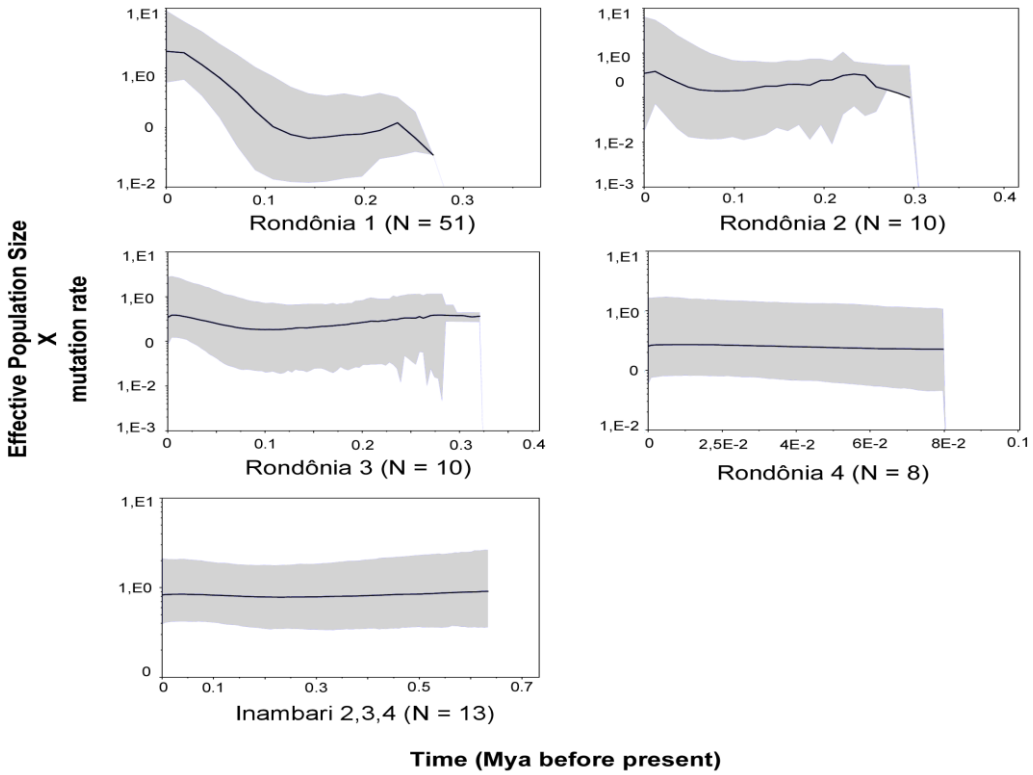


Figure 21 – Bayesian Skyride Plots based on cyt *b* gene showing the demographic history for different lineages of *Glyphorynchus spirurus* (see Fig. 2). The *black line* represents the median value whereas the gray area denotes 95% Bayesian credible intervals. *N* = number of samples analyzed.

Clustering of individuals based on ISSR fingerprints

Genomic fingerprints were performed with 37 individuals randomly selected from Rondônia and Inambari (the most densely sampled regions) including two subspecies (*G. s. castelnaudii* and *G. s. inornatus*) using five different ISSR primers. These primers generated a total of 40 scorable bands.

The PCoA analyses support the current subspecies taxonomy showing a population structure on opposite sides of the Madeira River and no genetic differentiation among phylogeographic provinces within *G. s. inornatus* (Fig. 22). These results were corroborated by the Bayesian probability assignment analyses indicated that the individuals could be partitioned into two main clusters, which captures the major structure in the data (*ln L* = –458.8). Two distinctive clusters include populations from opposite sides of the Madeira River, and one individual among the subspecies *catelnaudii* (Inambari region, individual 2 in Table 9) provides evidence for mixed ancestry with 40% posterior probability (Fig. 23).

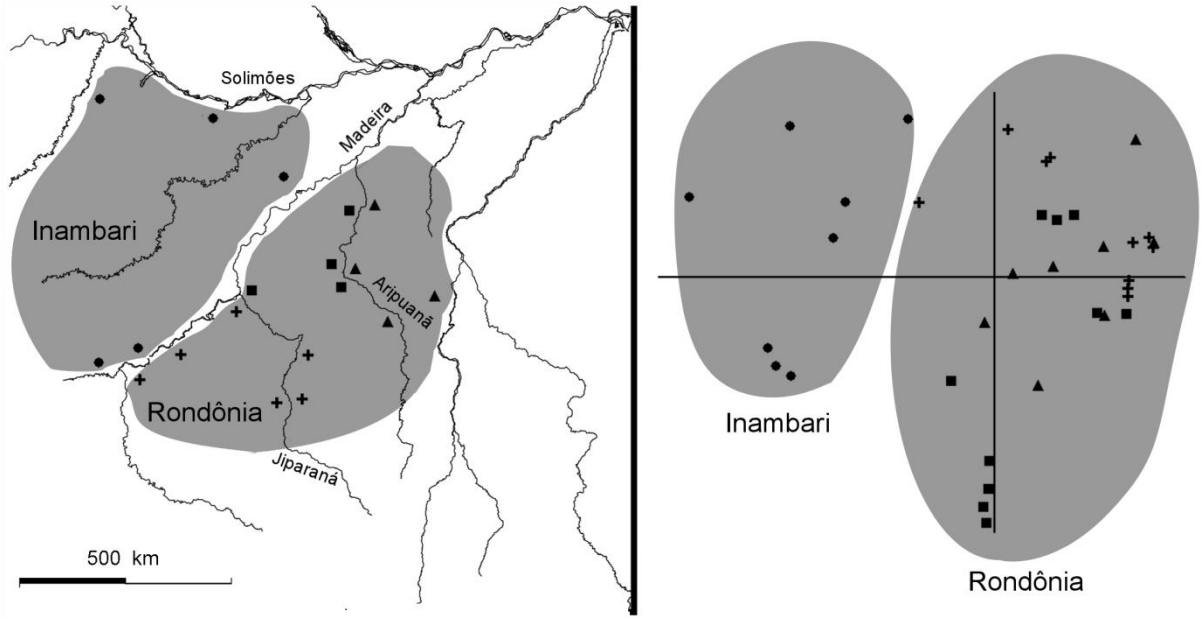


Figure 22 – **(Left)** Geographical distribution of samples used for ISSR fingerprinting. **(Right)** Principal coordinate analysis (PCoA) plot for all possible pairs of ISSR loci.

Figure 23 – Bayesian clustering analysis-based DNA bands generated from ISSR fingerprinting; *black* = mixed ancestry, *dark gray* = Inambari, and *light gray* = Rondônia.

Table 9 – Endemic region, voucher and sampled localities of the samples used in ISSR-PCRs

Numb.	Endemic region: Voucher	Population/Locality
1	Inambari: INPA 044	Brazil, AM: ca 35 km NW Coari, right bank Solimões river, "Cano do Apaurá". 3°55'00"S; 63°23'50"W
2	Inambari: INPA 349	Brazil, RO: left bank of the Madeira river, near Jacy Paraná, ca. 45 km southwest Porto Velho. 9°10'S; 64°23'W
3	Inambari: INPA 359	Brazil, RO: left bank of the Madeira river, near Jacy Paraná, ca. 45 km southwest Porto Velho. 9°10'S; 64°23'W
4	Inambari: INPA 059	Brazil, AM: right bank Solimões River, ca 19 km SSE Codajás, Com. Urucuri, Lago do "Poção". 3° 58,5' S, 61° 57' W
5	Inambari: INPA 173	Brazil, RO: left bank of the Madeira river, ca. 20 km N Abunã. 9°31'S; 65°21'W
6	Inambari: INPA 191	Brazil, RO: left bank of the Madeira river, ca. 20 km N Abunã. 9°31'S; 65°21'W
7	Inambari: INPA 808	Brazil, AM: right bank of lower Juruá, RESEX Baixo Juruá, Rio Andirá, Comunidade Cumaru, ca. 50 km South of Juruá. 3°45'S; 66°5'W
8	Inambari: INPA 818	Brazil, AM: right bank of lower Juruá, RESEX Baixo Juruá, Rio Andirá, Comunidade Cumaru, ca. 50 km South of Juruá. 3°45'S; 66°5'W
9	Inambari: INPA 1495	Brazil, AM: left bank of the Madeira river, ca 350 Km SW Manaus, BR 319. 5°12'S; 61°50'W.
10	Rondônia: INPA 562	Brazil, AM: left bank of Aripuanã river, Arauazinho stream, 130 km S Novo Aripuanã. 6°18'S; 60°24'W
11	Rondônia: INPA 895	Brazil, AM: right bank of lower Roosevelt river, confluence with Aripuanã river. 7°38'S; 60°40'W
12	Rondônia: INPA 466	Brazil, AM: left bank of Aripuanã river, Arauazinho stream, 130 km S Novo Aripuanã. 6°18'S; 60°24'W
13	Rondônia: INPA 461	Brazil, AM: left bank of Aripuanã river, Arauazinho stream, 130 km S Novo Aripuanã. 6°18'S; 60°24'W
14	Rondônia: INPA 875	Brazil, RO: right bank lower Jiparaná river, comunidade Demarcação, ca. 20 km southeast Calama. 8°09'S; 62°47'W
15	Rondônia: INPA 553	Brazil, AM: left bank of Aripuanã river, Arauazinho stream, 130 km S Novo Aripuanã. 6°18'S; 60°24'W
16	Rondônia: INPA 906	Brazil, AM: left bank of lower Roosevelt river, confluence with Aripuanã river. 7°35'S; 60°43'W
17	Rondônia: INPA 875	Brazil, RO: right bank lower Jiparaná river, comunidade Demarcação, ca. 20 km southeast Calama. 8°09'S; 62°47'W
18	Rondônia: INPA 877	Brazil, RO: right bank lower Jiparaná river, comunidade Demarcação, ca. 20 km southeast Calama. 8°09'S; 62°47'W
19	Rondônia: INPA 510	Brazil, AM: left bank of Aripuanã river, Arauazinho stream, 130 km S Novo Aripuanã. 6°18'S; 60°24'W
20	Rondônia: INPA 2515	Brazil, RO: REBIO Jaru, rigth bank Jiparaná river, Tarumã stream. 9°27'S; 61°40'W
21	Rondônia: INPA 208	Brazil, RO: right bank of Madeira river, ca. 20 km N Abunã. 9°35'S; 65°21'W
22	Rondônia: INPA 2384	Brazil, RO: REBIO Jaru, rigth bank Jiparaná river. 10°07'S; 61°54'W
23	Rondônia: INPA 2440	Brazil, RO: REBIO Jaru, left bank Jiparaná river. 10°28'S; 61°55'W
24	Rondônia: INPA 870	Brazil, RO: left bank of lower Jiparaná river, opp. Comunidade Demarcação, ca. 20 km southeast Calama. 8°14'S; 62°46'W
25	Rondônia: INPA 2519	Brazil, RO: REBIO Jaru, rigth bank Jiparaná river, Tarumã stream. 9°27'S; 61°40'W
26	Rondônia: INPA 329	Brazil, RO: right bank of Madeira river, 9.5 km southeast Porto Velho. 8°52'S; 64°0'W
27	Rondônia: INPA 886	Brazil, RO: left bank of lower Jiparaná river, opp. Comunidade Demarcação, ca. 20 km southeast Calama. 8°14'S; 62°46'W
28	Rondônia: INPA 879	Brazil, RO: left bank of lower Jiparaná river, opp. Comunidade Demarcação, ca. 20 km southeast Calama. 8°14'S; 62°46'W
29	Rondônia: INPA 2439	Brazil, RO: Entorno REBIO Jaru, left bank Jiparaná river. 10°10'S; 61°55'W
30	Rondônia: INPA 890	Brazil, AM: right bank of middle Aripuanã, confluence with Roosevelt river. 7°37'S; 60°40'W
31	Rondônia: INPA 561	Brazil, AM: right bank of Aripuanã, Extremo stream, 135 km S Novo Aripuanã. 6°18'S; 60°20'W
32	Rondônia: INPA 535	Brazil, AM: right bank of Aripuanã, Extremo stream, 135 km S Novo Aripuanã. 6°18'S; 60°20'W
33	Rondônia: INPA 559	Brazil, AM: right bank of Aripuanã, Extremo stream, 135 km S Novo Aripuanã. 6°18'S; 60°20'W
34	Rondônia: INPA 536	Brazil, AM: right bank of Aripuanã, Extremo stream, 135 km S Novo Aripuanã. 6°18'S; 60°20'W
35	Rondônia: INPA 852	Brazil, AM: Parque Estadual do Sucunduri; right bank of Bararati river. 8°21'S; 58°37'W
36	Rondônia: INPA 855	Brazil, AM: Parque Estadual do Sucunduri; right bank of Bararati river. 8°21'S; 58°37'W
37	Rondônia: INPA 1282	Brazil, AM: RDS Aripuanã; left bank Aripuanã river; Água Branca stream. 8°25'S; 59°46'W

3.1.5 Discussion

Phylogeographic structure, taxonomy, and species limits

Thirteen of the 16 lineages of *G. spirurus* which were recognized in this study are confined by Amazonian rivers along most of their distribution ranges (Fig. 14), suggesting a major role for rivers as vicariant barriers for diversification in Amazonia. However, our data also show that the extent of drainage limits of major Amazonian river basins are not always in agreement with the distribution of corresponding clades.

Altogether, only half of the 16 lineages of *G. spirurus* which were revealed in this study are formally recognized taxonomically, indicating a very high degree of cryptic diversity and the need of a thorough taxonomic revision including the description of several new taxa. Additionally, the high number of genetically differentiated populations of *G. spirurus*, in addition to the existence of two main markedly different song types, and several phenotypically distinct subspecies have prompted the speculation that more than a single species is involved (Marantz et al. 2003).

Both the cyt *b* (Figs. 15 and 16) and expanded mtDNA phylogeny (Fig. 17) support the reciprocal monophyly of the following populations and taxa, which fit the definition of species under a plethora of different evolutionary and lineage-based species concepts (de Queiroz 1998): 1) Guiana (*G. [s.] spirurus*); 2) Imeri (*G. [s.] rufigularis*); 3) Rondônia 4 (*G [s.] inornatus*); 4) Inambari 4 (*G. [s.] albigularis*); 5) Pará 2 (*G. [s.] paraensis*); and Atlantic Forest (*G. [s.] cuneatus*). At least 10 additional reciprocally monophyletic mtDNA lineages of *G. spirurus* which would agree with evolutionary species definitions have yet to be described (as discussed in detail below), and thus cannot be treated as species-level taxa herein.

However, when a multilocus species tree is considered (with the caveat that a smaller subset of individuals and areas, and only 5 genes were sampled for this analysis), an overall low statistical support for the mtDNA clades is obtained (Fig. 20). In fact, the multilocus phylogeny provides strong support only for the monophyly of Brazilian shield populations (Rondônia and Pará) with respect to all remaining Amazonian populations, as well as for the split separating Rondônia 2 from Rondônia 1, 3 populations. Thus, under a multi-locus approach, our data allows the recognition of only three species among the Cis-Andean taxa of *G. spirurus*, as follows: *G. spirurus* (the oldest name applicable to sampled populations of the Guiana, Inambari, Imeri, and Napo areas of endemism); *G. inornatus* (the oldest name applicable to populations Rondônia 4 and Pará); and an undescribed taxon **(*Glyphorynchus species novum*)** including populations Rondônia 1, 2, 3. It is expected that because of shared polymorphism and bigger effective population sizes, nuclear genes will sort out latter among monophyletic populations than mitochondrial genes (Heled and Drummond 2010). Thus, the multilocus perspective on intra-specific limits in *G. spirurus* may reflect a conservative estimation of cryptic diversification in this species. However, the mitochondrial phylogenies probably mirror more recent events of gene flow cessation due to isolation by distance or in response to a build-up of new barriers. When different species criteria are considered, the conclusions inferred from a multilocus phylogeny are best translated in terms of biological species, whereas those obtained from mtDNA phylogeny are more easily reconciled with lineage-based species definitions (i.e., the phylogenetic species concept). Considering the different sampling intensities underlying the mitochondrial and multilocus phylogenies, this study have provided a more accurate redefinition of interspecific

limits in *G. spirurus* under a lineage-based rather than biological species concept approach. More extensive future multilocus sampling regimes should provide a better framework for the revision of interspecific limits in *G. spirurus* under a biological species concept perspective. This will allow to reconcile species trees and the mitochondrial phylogenies to support the recognition of distinct evolutionary species.

Comparison with previous studies

The phylogeographic structure recovered here differs in some aspects from the findings of Marks et al. (2002) and contrasts strongly with currently recognized taxonomy and subspecies limits in *G. spirurus* (Marantz et al. 2003). On the other hand, our findings are consistent with a major biogeographic pattern detected for several other avian lineages, whereby populations of the Brazilian shield in south-central Amazonia are reciprocally monophyletic with respect to other populations (Ribas et al. 2005, 2009, 2012; Aleixo and Rossetti 2007; Patané et al. 2009).

Marks et al. (2002) found that the subspecies *G. s. albigularis*, a taxon distributed throughout northern Bolivia and southern Peru in the southern part of the Inambari area of endemism was not monophyletic, sharing haplotypes with *G. s. inornatus*, an endemic taxon to the Madeira/Tapajós interfluvium (Marantz et al. 2003). According to Marks et al. (2002), the Madeira River (the second largest river in the Amazon) did not delimit monophyletic populations of *G. spirurus*, and their mitochondrial DNA phylogeography was inconsistent with patterns of morphological differentiation between these subspecies. However, our results do not support these findings. Based on mitochondrial and nuclear markers, including ISSR fingerprints, a clear genetic border confined by the Madeira River were found, which separates populations from the Inambari and Rondônia areas of endemism in monophyletic lineages. Our larger sampling and multilocus approach showed in fact that the Madeira River represents an effective biogeographical barrier separating monophyletic populations of *G. spirurus*. Similarly, all specimens sequenced which carry the white throat typical for *G. s. albigularis* are monophyletic (MPEG59810, MPEG59828, MPEG58874, FJ899295, FJ899301; Figs. 15 and 16), indicating that this taxon corresponds to a valid and distinct evolutionary unit in *G. spirurus*. In contrast, populations which were thought to belong to *G. s. castelnaudii* proved to be paraphyletic (Figs. 15 and 16). The type locality of *castelnaudii* (Santa Maria, lower Huallaga River in central Peru; Peters 1951) indicates that this name should be applied only to the clade containing Napo 3 and Inambari 1 populations (Figs. 14, 15 and 16). On the other hand, Inambari 2 and 3 populations form a well-supported clade sister to *G. s. albigularis* (Inambari 4; Figs. 15 and 16), but differing from both genetically (2.7% of average cyt *b* uncorrected p-distance) and phenotypically (rusty rather than white throat).

Even though the data presented in this publication are not complete, it has expanded significantly the results of Marks et al. (2002), allowing a re-evaluation of interspecific limits among populations and taxa of *G. spirurus*.

Population structure within larger interfluvia

Our phylogeographic analyzes revealed four non-monophyletic lineages in the Madeira/Tapajós interfluvium (Rondônia area of endemism) (Fig. 14). It was also found that *G. s. inornatus* (the name thought to apply to all populations in this interfluvium; (Marantz et al. 2003) is paraphyletic (Figs. 15, 16, and 17). The type locality of *G. s. inornatus* (Parintins, in the lowermost east bank of the Madeira River in central Amazonian Brazil; Peters 1951) indicates

that this name should be applied only to the northernmost population of the Madeira/Tapajós interfluvium (Rondônia 4). This clade is sister to the lineages distributed to the east of the Tapajós and the Atlantic Forest rather than those immediately to the south in the same interfluvium. Therefore, no name exists that could be applied to the monophyletic populations found in the southern portion of the Madeira/Tapajós interfluvium (i.e, clades Rondônia 1, 2, 3), which represent a legitimate and independent evolutionary lineage (Figs. 15, 16, and 17).

As already suggested by other authors, these patterns reinforce the notion that the Madeira/Tapajós interfluvium has a complex history and holds a rich evolutionary diversity (Willis 1969; Haffer 1997a; Hall and Harvey 2002; Marks et al. 2002; Sardelli 2005; Cohn-Haft et al. 2007; Fernandes 2007; Isler et al. 2007; Tobias et al. 2008). Originally considered to be a single and uniform area of endemism (Cracraft 1985; Dinerstein et al. 1995), the area between the rivers Madeira and Tapajós may contain new phylogeographical provinces delineated by the *G. spirurus* clades recovered in this study as well as other animals (van Roosmalen et al. 1998; Hall and Harvey 2002; Cohn-Haft et al. 2007).

Our dataset also reveals a complex and fine-scale pattern of population differentiation in two additional areas of endemism in Amazonia: Napo and Pará. When a larger dataset was analyzed (concatenated cyt *b* and ND2 sequences), Napo 1 populations became sister to Imeri populations rather than to Napo 2 (Fig. 17). Ribas et al. (2012) also found populations of *Psophia* trumpeters associated with the Napo area of endemism to be paraphyletic, even though their biogeographic affinities with populations from other areas of endemism differed from the pattern recovered for *G. spirurus* herein and by Marks et al. (2002). Populations of *G. spirurus* in the Pará area of endemism (*sensu* Cracraft 1985) are paraphyletic. The populations of *G. s. paraensis* distributed east of the Xingu River (Pará 2) are sister to the disjunct Atlantic Forest population (to which name *cuneatus* apply; Marantz et al. 2003), rather than those populations of *G. s. paraensis* found west of the Xingu in the same area of endemism (Figs. 15, 16, and 17; Marks et al. 2002).

Similarly, Milá et al. (2009) found genetic substructures in the Chocó area of endemism for *G. spirurus*, also suggesting an unrecognized phylogeographic structure and cryptic speciation in this Trans-Andean South American population. Therefore, the number of cryptic lineages in the polytypic *G. spirurus*, which is distributed throughout most of the Neotropics, is yet unknown and will only be revealed by additional phylogeographic and taxonomic studies.

Contact zones

Our data showed that only two of the six areas of endemism which were recognized by Cracraft (1985) in Amazonia contain monophyletic populations of *G. spirurus* (Imeri and Guiana). In contrast, Napo, Inambari, Rondônia, and Pará populations showed extensive paraphyly, with many populations having a closer relationship with clades inhabiting adjacent, rather than the same, areas of endemism (Figs. 15, 16, and 17). Most phylogeographic studies have shown that areas of endemism in Amazonia and elsewhere are often inhabited by non-monophyletic populations (Aleixo and Rossetti 2007), but the degree of paraphyly documented for *G. spirurus* appears to be much higher than that determined for most avian lineages. Some of the few lineages studied so far share the same Trans-Amazonian distribution pattern: *Mionectes oleagineus* (Miller et al. 2008), *Ramphastos tucanus* and *Ramphastos vitellinus* (Patané et al. 2009), and *Psophia* spp. (Ribas et al. 2012). These studies have discovered just a few Amazonian areas of endemism to be inhabited by non-monophyletic populations of those lineages, including Guiana (Miller et

al. 2008), Inambari (Patané et al. 2009), and Napo (Ribas et al. 2012). The concept of areas of endemism does not accurately describe the differentiation patterns of *G. spirurus* which was uncovered in our study. Perhaps these areas of endemism are not cohesive biogeographic units in which lineages became isolated and differentiated in response to a common history (Cracraft 1985) and a different framework for thinking about diversification processes in the Amazon needs to be considered. As shown here for *G. spirurus*, this can result in the recognition of areas of endemism and taxa inconsistent with true patterns of lineage diversification (Aleixo and Rossetti 2007; Burney and Brumfield 2009; Antonelli et al. 2010).

For instance, the Imeri clade distributed mostly to the east of the Rio Negro is also found across this river's western bank and upper course near São Gabriel da Cachoeira in Brazil (Fig. 14). However, the most extreme case involves the Rondônia 1 clade in the southern part of the Madeira/Tapajós interfluvium, whose range is mostly delimited independently of major Amazonian rivers, including both major tributaries of the Tapajós, i.e., Juruena and Teles Pires (Fig. 14). Similarly, the Rondônia 3 clade is also distributed across the Juruena River into the Juruena – Teles Pires interfluvium, where it meets with Rondônia 1 birds in an area not bisected by any major river. Other contact zones apparently not maintained by rivers include those between (1) Rondônia 3 and 4 birds in the northern part of the Madeira/Tapajós interfluvium; (2) Rondônia 1 and Pará 1 birds in the southern portion of the Tapajós/Xingu interfluvium; and (3) Inambari 3 and 4 birds in the southern part of the Inambari area of endemism (Fig. 1). When the mitochondrial data alone are considered, none of these contact zones set away from major rivers discussed above involve sister clades of *G. spirurus*, and thus can be regarded as secondary (i.e., resulting from dispersal of at least one of the lineages involved; Figs. 15, 16, 17). However, when the multilocus species phylogeny is considered (Fig. 20), only the contact zone between Rondônia 1 and 3 birds can possibly be interpreted as primary rather than secondary, but the Bayesian posterior probability associated with this sister relationship is weak (0.8).

The role of rivers

According to the mitochondrial phylogenies, the modern course of the following rivers separate sister clades of *G. spirurus*: lower Amazon (Guiana and all remaining populations; Figs. 15 and 16), mid-lower Rio Negro (Imeri and Napo 1 populations; Figs. 15, 16, and 17), Japurá (Napo 1 and 2 populations; Fig. 16), Marañón (Napo 3 and Inambari 1 populations; Figs. 15 and 16), Purus (Inambari 2 and 3 populations), Madeira (all Inambari and Rondônia/Pará populations; Fig. 17), lowermost Tapajós (Rondônia 4 and all Pará populations; Figs. 15, 16, and 17), and Xingu (Pará 1 and 2 populations; Figs. 15 and 16).

Hence, rivers appear to play an important role as primary diversification barriers in *G. spirurus*, whereas contact zones not coincident with river courses result from secondary contact via dispersal. In *G. spirurus*, the dissociation between riverine barriers and clade limits is present foremost in the Napo (upper Amazon/Rio Negro interfluvium) and Rondônia (Madeira/Tapajós interfluvium) areas of endemism, both known to have suffered major drainage reorganization patterns during the Pleistocene in response to neotectonics (Willis 1969; Latrubesse and Franzinelli 2005; Almeida-Filho and Miranda 2007) and postulated megafans (Latrubesse 2002). According to the mitochondrial data, the confidence interval for the timing of diversification involving Napo populations is entirely in the Pleistocene (0.8–1.7 Mya), whereas those associated with Rondônia populations encompass the Late Pliocene and most of the Pleistocene (1.1–2.7 Mya; Fig. 19). However, when the multilocus species tree is considered, ages associated with

the diversification of Rondônia populations are younger and confidence intervals completely overlap with the Pleistocene (0.01–2.1 Mya), thus supporting the hypothesis of cladogenesis in response to relatively recent drainage reorganization in those two areas of endemism. Interestingly, so far, drainage reorganization patterns in response to tectonism in Amazonia have been described only for a third area of endemism (easternmost Pará) (Rossetti and Valeriano 2007), where local populations of *G. spirurus* were shown to track both spatially and temporally the pattern of drainage change in the lower Tocantins River valley (Faccio et al. unpublished manuscript).

Tectonically mediated mega-drainage capture as documented for the Rio Negro, Madeira, and Tocantins river basins ((Latrubesse and Franzinelli 2005; Almeida-Filho and Miranda 2007; Rossetti and Valeriano 2007) as well as megafans (Latrubesse 2002), can yield diversification patterns in which sister lineages differentiating in response to a river will no longer be separated by this same vicariant barrier over time (Wilkinson et al. 2010). This can apparently explain the observed instances of mismatch between modern drainage limits and the distribution of *G. spirurus* clades in Amazonia, as discussed above. The fact that most, if not all, contact zones away from riverine barriers involve non-sister lineages of *G. spirurus*, and that the opposite is true for the majority of contact zones matching river barriers, provide together strong evidence for the paramount role played by rivers as primary diversification barriers in Amazonia.

Unfortunately, phylogenetic relationships among main *G. spirurus* lineages received low statistical support and are to some extent contradictory among the different phylogeny estimates obtained (Figs. 15, 16, 17, and 19), preventing a more detailed discussion on the spatial patterns of diversification involving those splits. However, confidence intervals associated with the timing of splits involving *G. spirurus* lineages in Amazonia span the Late Neogene (Pliocene; i.e., 5.8 Mya for mitochondrial and 4.3 Mya for multilocus data) through the Quaternary (Late Pleistocene; i.e., 0.8 Mya for mitochondrial and 0.01 Mya for multilocus data) (Figs. 15 and 16). This is consistent with the most recent models based on geological and paleontological evidence which were proposed for the historical development of the Amazon drainage (Espurt et al. 2010; Latrubesse et al. 2010; Mora et al. 2010). According to these models, the continuous subduction of the Nazca Ridge under the South American Plate during the Miocene caused the Amazonian foreland basins located on the eastern Andean foothills in Bolivia, Brazil, Peru, Ecuador, and Colombia to evolve from a depositional to a predominantly erosional state, draining sediments eastward and hence creating the modern transcontinental Amazon drainage flowing towards the Atlantic between the late Pliocene (Espurt et al. 2010; Latrubesse et al. 2010; Mora et al. 2010) and early Pleistocene (Espurt et al. 2010; see also Campbell et al. 2006).

Refuge hypothesis

Based on patterns of present-day bird distributions and paleoclimatic data from the region, Haffer (1969, 1974) proposed that alternating dry and cold periods during the Quaternary caused Neotropical rainforests to expand and contract, with some fragmented “refuges” maintained throughout the entire period. When tropical forests contracted, previously connected populations became isolated in these refugia, where over time they became reproductively isolated and differentiated. When this hypothesis is considered, no spatial prediction exists about the location of splitting events involving lineages isolated in different climatic refuges (Patton and da Silva 1998), but populations under this model of evolution are expected to show signs of demographic fluctuations during the Pleistocene (Zink 1997; Hewitt 1999; Aleixo 2004; Cheviron et al. 2005)

and even earlier (Haffer 1997b), which also contribute to making the temporal predictions of this hypothesis ambiguous. The neutrality tests and Bayesian Skyride Plots suggest that at least three of the five lineages of *G. spirurus* included in population genetics analyses showed clear signs of demographic changes during the Pleistocene (Fig. 21 and Table 7). However, when considering mitochondrial data alone, the intense demographic fluctuations detected for *G. spirurus* during the Pleistocene post-dated their differentiation as separate lineages and have apparently not contributed to most splitting events in the Inambari and Rondônia areas of endemism. On the other hand, when the multilocus species tree is considered (Fig. 20), all splitting events associated with Rondônia populations are entirely within the time frame of the reconstructed historical population dynamics by the Bayesian Skyride Plots. However, this should be viewed with caution given that the Bayesian Skyride Plots were estimated based only on cyt *b* sequences, and thus are more properly contrasted with the chronogram estimated with the mitochondrial data alone. Despite the historical demographic dynamics detected in some *G. spirurus,* there is evidence suggesting that Rondônian populations during the late Pleistocene were accompanied by intense cladogenesis. The fact that demographic changes did not involve all populations in the same area of endemism seems to support a secondary and perhaps more local role for the refuge hypothesis as a diversification promoter in Amazonia. The intensification of the use of multilocus approaches in Amazonian phylogeography should allow for a more accurate reconstruction of the timing of diversification and demographic fluctuations among lineages inhabiting this region.

Conservation implications

The phylogeography of *G. spirurus* corroborates the existence of several cryptic unnamed taxa, most of them restricted to particular sectors of Amazonia, such as several mini-interfluvia of the Rondônia area of endemism. As discussed by Aleixo (2009) and implied by some previous studies (Cohn-Haft et al. 2007; Fernandes 2007; Fernandes, in press), both biogeographic studies and conservation policies should take taxonomic uncertainties into account when respectively delimiting proper analytical and conservation targets. Disregarding the significance of entire cryptically diverse sectors of Amazonia under the current high anthropogenic development pressure may lead to inconsistent interpretations of the diversification history of its organisms and the irretrievable loss of valuable unrecognized species.

3.2 Phylogeography and Speciation of the Chestnut-tailed Antbird (*Myrmeciza hemimelaena*) in Lowland Amazonia

3.2.1 Abstract

Patterns of spatial and temporal diversification of the Amazonian endemic chestnut-tailed antbird, *Mymeciza hemimelaena* (Thamnophilidae), were examined to evaluate the diversification of a widespread avian taxon across rivers that potentially represent major natural barriers. Sequences of the mitochondrial *ND2* and cytochrome *b* genes were investigated from 65 individuals distributed throughout the entire range of *M. hemimelaena*, including the two currently valid subspecies *M. h. hemimelaena* and *M. h. pallens.* Based on a combination of phylogeographic tools, molecular dating, and population genetic methods, it was reconstructed a spatio-temporal scenario of diversification of *M. hemimelaena* in the Amazon. The data revealed three genetically divergent and monophyletic groups in *M. hemimelaena*, which can also be distinguished by a combination of morphological and vocal characters. Two of these clades correspond to the previously described taxa *M. h. hemimelaena* and *M. h. pallens*, which are separated by the upper Madeira River, a main Amazon tributary. The third clade is distributed between the middle reaches of the Madeira River and the much smaller tributaries Jiparaná and Aripuanã and although currently treated as *M. h. pallens*, clearly constitutes an independent evolutionary lineage probably deserving separate species status. Molecular clock and population genetic analyses indicate that diversification in this group occurred throughout the Pleistocene, with demographic fluctuations assumed for *M. h. hemimelaena* and *M. h. pallens*. The findings implicate rivers as barriers driving diversification in the *M. hemimelaena* complex. Levels of mtDNA divergence and associated morphological and vocal traits support its division into at least three separate species with comparatively small ranges. The **existence of a previously unrecognized lineage** in the *M. hemimelaena* complex, and the high degree of population structuring found in *M. h. hemimelaena*, underscore the pervasiveness of cryptic endemism throughout Amazonia and the importance of DNA-based taxonomic and phylogeographic studies in providing accurate estimates of diversity essential for conservation planning.

3.2.2 Introduction

Amazonian landscape evolution was probably influenced by a combination of tectonic events that mould river attributes and orbital induced climatic changes (Haffer and Prance 2001; Rossetti et al. 2005). Thus, both river formation and glacial cycles have historically been considered major drivers of biotic diversification in Amazonia (Haffer 1997b). Originally proposed by Wallace (1852), the *riverine barrier hypothesis* states that closely related taxa on opposite river banks are assumed to have diversified due to the riverine barrier effect (Wallace 1852; Hellmayr 1910). Thus, one of its predictions is that given sufficient time, isolation will generate reciprocally monophyletic sister populations/species across major Amazonian rivers. In contrast to this hypothesis, and despite the fact that major rivers are the primary distributional boundaries for a large proportion of Amazonian vertebrates, the importance of river dynamics as a cause of allopatric speciation has not gained wide acceptance, mainly because some major rivers located in western Amazonia (e.g. the Juruá and Purus) do not appear to have acted as primary diversification barriers for some *terra-firme* vertebrate taxa (Antonelli et al. 2010). However, recent molecular phylogenies of Neotropical birds revealed splits consistent with the establishment of major Amazonian rivers during the Pliocene–early Pleistocene (Tobias et al. 2008; Patel et al. 2011; Ribas et al. 2012). Therefore, phylogeographic data amassed so far for

several lineages of Amazonian vertebrates provide mixed support for the riverine barrier hypothesis (Antonelli et al. 2010).

The competing *refuge hypothesis* postulates that forest cover in Amazonia was reduced and fragmented during glacial maxima throughout the late Pliocene and Pleistocene (Haffer 1969; Vanzolini and Willians 1970). Savanna and seasonally dry forest blocks are assumed to have functioned as barriers separating isolated populations of humid forest species, thus facilitating allopatric speciation (Haffer 1969, 1997b). However, phylogeographic studies addressing predictions derived from the refuge hypothesis (namely, frequent and recurrent episodes of population contraction and expansion during the Pleistocene; see Hewitt 1999; Moritz et al. 2000) are still very limited (Aleixo 2004; Cheviron et al. 2005; Ribas et al. 2012). Additionally, the paucity of fully resolved, densely sampled time-calibrated phylogenies for Amazonian organisms renders it difficult to assess the generality of any diversification hypothesis proposed so far for the region, underscoring the need for additional phylogeographic studies aimed at evaluating the role of Amazonian landscape evolution and climatic oscillations on diversification of the local biota.

Antbirds (Aves: Thamnophilidae) are particularly good models for studying diversification in the Neotropics because they (1) are usually common; (2) inhabit the forest understory and hence are poorer dispersers and more prone to develop phylogeographic structuring than canopy birds (Burney and Brumfield 2009); and (3) exhibit morphological, vocal, and genetic differentiation across major geographic barriers such as mountain ranges and rivers (Zimmer and Isler 2003).

The Amazonian endemic chestnut-tailed antbird, *Myrmeciza hemimelaena* Sclater 1857, is one of the few species that has already been the focus of experimental studies as a model system for understanding Amazonian diversification (Seddon and Tobias 2007). It occurs on opposite river banks of different sizes south of the Amazon/Marañon/Solimões rivers from eastern Peru eastward to the western bank of the Xingu River in Brazil, reaching as far south as northeastern Bolivia in eastern Santa Cruz province (Zimmer and Isler 2003). Two subspecies are currently recognized: *M. h. hemimelaena* (west of the Madeira River and south to the La Paz province in Bolivia) and *M. h. pallens* (east of the Madeira River; Zimmer and Isler 2003). *Myrmeciza castanea*, its sister species, ranges from southern Colombia through eastern Ecuador and northeastern Peru and was originally described as a subspecies of *M. hemimelaena* (Zimmer 1932), but later recognized as a distinct species based on bioacoustics and morphometrics (Isler et al. 2002; Zimmer and Isler 2003). Both *M. hemimelaena* and *M. castanea* occur in the same overall habitat type throughout their ranges, i.e. upland *terra-firme* forest, often in areas where those forests grow on predominantly sandy soils (Zimmer and Isler 2003).

More restricted bird distributions and new endemic species have recently been found to be confined to the Jiparaná/Aripuanã interfluvium in the Madeira River basin (Sardelli 2005; Fernandes 2007; Isler et al. 2007; Tobias et al. 2008), showing the existence of smaller Amazonian areas of endemism in what was referred to as “mini-interfluvia” (Cohn-Haft et al. 2007). Several avian lineages inhabiting this area are represented by closely related taxa replacing each other on opposite banks of comparatively small rivers (Cohn-Haft et al. 2007; Isler et al. 2007). Molecular studies have shown the existence of genetically distinct populations with restricted ranges bounded by the Jiparaná and Aripuanã rivers (Sardelli 2005; Fernandes 2007). Such discoveries have important implications for defining and recognizing new areas of endemism in the Amazon Basin.

A putatively new species of *Myrmeciza* was observed and collected on several field trips to the Jiparaná/Aripuanã interfluvium area in 2003–2004 and evidence for species-level divergence based on vocal and morphological features was reported by B. Whitney and M. Cohn-Haft (INPA, pers. comm.; see also Isler et al. 2002; Whittaker 2009), details of which are to be communicated formally. Our task has been to provide a comparative molecular analysis to evaluate the existence of a purported new species in the context of a broader phylogeographic study focused on the *Myrmeciza hemimelaena* complex. The samples of the proposed new species investigated in this study were from specimens collected in the Jiparaná/Aripuanã interfluvium area by (Cohn-Haft et al. (2007) and A. Fernandes in 2009 (deposited at the Instituto Nacional de Pesquisas da Amazônia) and the staff of the Museu Paraense Emílio Goeldi (MPEG) in 2004 (deposited therein; see Appendix S1 in Supporting Information).

Here, it is presented phylogeographic and population genetic analyses of the *M. hemimelaena* complex to elucidate its evolutionary history in lowland Amazonia. It was sought to evaluate the role of putative riverine barriers and climatic oscillations on its diversification. Finally, it was revised current interspecific limits in this group by delimiting genetically differentiated populations known also to differ morphologically and vocally, including the purported new taxon from the Jiparaná/Aripuanã interfluvium.

3.2.3 Materials and Methods

Sampling design

A total of 65 individuals were sampled from 47 localities covering the entire range and all currently described subspecies of *M. hemimelaena* (Fig. 24, Table 10). All tissues were either collected directly in the field or obtained from the following institutions: Field Museum of Natural History, Chicago, USA (FMNH), Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil (INPA), Laboratório de Genética e Evolução Molecular de Aves, São Paulo, Brazil (LGEMA), Louisiana State University Museum of Natural Science, Baton Rouge, USA (LSUMZ), Museu Paraense Emílio Goeldi, Belém, Brazil (MPEG), Universidade Federal de Mato Grosso, Cuiabá, Brazil (UFMT), and Natural History Museum of Denmark, Copenhagen, Denmark (ZMUC); tissue samples were subsequently vouchered together with research specimens (mostly skins). Included in the analysis are samples collected close to the type localities of *M. hemimelaena hemimelaena*, *M. hemimelaena pallens* and *M. castanea castanea* (used as outgroups; Fig. 24). As additional outgroups it was employed sequences of *Cercomacra serva* and *Cercomacra tyrannina* downloaded from GenBank, because the most complete phylogeny estimated so far for the Thamnophilidae had, with high statistical support, recovered these two species as the immediate sister clade to *M. hemimelaena* (Brumfield et al. 2007).

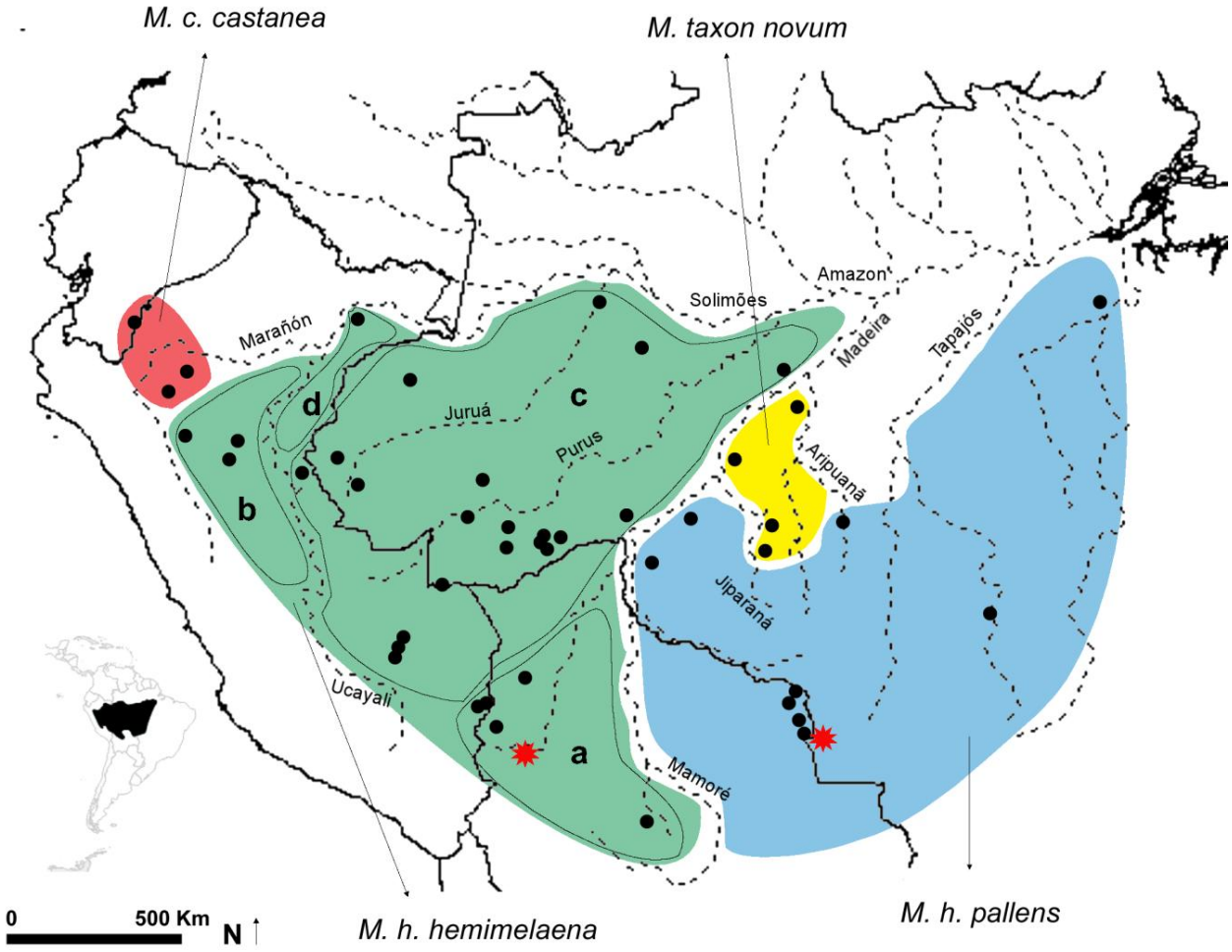


Figure 24 – Origin of samples sequenced across the range of the *Myrmeciza hemimelaena* complex in Amazonia and distribution of phylogroups recovered in the Bayesian phylogeny (Isler et al. 2002; Zimmer and Isler 2003). Red asterisks depict the type localities of *M. hemimelaena* (Mapiri, La Paz, Bolivia; Gyldenstolpe 1945) and *M. h. pallens* (Vila Bela da Santíssima Trindade, Mato Grosso, Brazil; Berlepsch and Hellmayr 1905); a, b, c, and d are clades of *M. h. hemimelaena*.

Table 10 – Collection locality, voucher number, institutions of origin, and GenBank accession numbers for *Myrmeciza hemimelaena* and *M. castanea* samples used in this study.

Taxon	**Locality**	**Voucher**[1]	**Institution***	**Cyt *b***	***ND2***
M. castanea	Peru, Loreto, *c*. 3 km SE Jeberos	42168[1]	LSUMZ	HM637196	HM637149
M. castanea	Peru, San Martin, *c*. 33 km NE Florida	44678	LSUMZ		JQ080441
M. castanea	Ecuador, Cord. del Condor	126372	ZMUC	JQ080440	JQ080440
M. h. hemimelaena (a)	Bolivia, La Paz, Puerto Araona	391141	FMNH	JQ080454	JQ080454
M. h. hemimelaena (a)	Bolivia, La Paz, Prov. B. Saavedra	22742	LSUMZ	JQ080536	JQ080457
M. h. hemimelaena (a)	Bolivia, Sajta, Cochabamba	144634	ZMUC	JQ080482	JQ080482
M. h. hemimelaena (a)	Peru, Puno, below Putina	58358	LSUMZ	JQ080456	JQ080456
M. h. hemimelaena (a)	Peru, Puno Dept, San Juan del Oro	58365	LSUMZ	JQ080455	JQ080455
M. h. hemimelaena (b)	Peru, Loreto, 79 km WNW Contamana	27904	LSUMZ	JQ080458	JQ080458
M. h. hemimelaena (b)	Peru, San Martin, Quebrada Upaguiha	46036	LSUMZ	JQ080460	JQ080460
M. h. hemimelaena (b)	Peru, Loreto, E bank of upper Pauya	40555	LSUMZ	JQ080459	JQ080459
M. h. hemimelaena (c)	Peru, Ucayali	20237[1]	UAM	EF639965	EF640032
M. h. hemimelaena (c)	Peru, Madre de Dios	398061	FMNH	JQ080487	JQ080487
M. h. hemimelaena (c)	Peru, Madre de Dios, Moskitania	433436	FMNH	JQ080490	JQ080490
M. h. hemimelaena (c)	Peru, Madre de Dios, Moskitania	433441	FMNH	JQ080491	
M. h. hemimelaena (c)	Peru, Cuzco, Paucartambo, Consuelo	433437	FMNH	JQ080488	
M. h. hemimelaena (c)	Peru, Cuzco, Paucartambo, Consuelo	433442	FMNH	JQ080450	

Taxon	Locality	Voucher[1]	Institution*	Cyt *b*	*ND2*
M. h. hemimelaena (c)	Peru, Cuzco, Paucartambo, Consuelo	433435	FMNH	JQ080489	
M. h. hemimelaena (c)	Brazil, RO, left bank of the Madeira	203	INPA	JQ080494	JQ080444
M. h. hemimelaena (c)	Brazil, AM, left bank of middle Madeira	419	INPA	JQ080495	JQ080445
M. h. hemimelaena (c)	Brazil, AM, right bank of lower Juruá	820	INPA	JQ080496	JQ080442
M. h. hemimelaena (c)	Brazil, AM, right bank Moaco River	1417	INPA	JQ080497	
M. h. hemimelaena (c)	Peru, Ucayali, SE slope Cerro	11217	LSUMZ	JQ080531	JQ080447
M. h. hemimelaena (c)	Brazil, AM, Tefé, Base Petrobras/Urucu	57134	MPEG	JQ080503	JQ080443
M. h. hemimelaena (c)	Brazil, AM, Tefé, Base Petrobras/Urucu	57135	MPEG	JQ080504	
M. h. hemimelaena (c)	Brazil, AM, Tefé, Base Petrobras/Urucu	57136	MPEG	JQ080505	
M. h. hemimelaena (c)	Brazil, AC, ESEC Rio Acre	59847	MPEG	JQ080509	
M. h. hemimelaena (c)	Brazil, AC, Porto Acre, Reserva Humaitá	60013	MPEG	JQ080515	JQ080446
M. h. hemimelaena (c)	Brazil, AC, Porto Acre, Reserva Humaitá	60014	MPEG	JQ080516	JQ080449
M. h. hemimelaena (c)	Brazil, AC, Rio Branco	60015	MPEG	JQ080517	
M. h. hemimelaena (c)	Brazil, AC, Rio Branco	60016	MPEG	JQ080518	
M. h. hemimelaena (c)	Brazil, AC, Rio Branco	60017	MPEG	JQ080519	
M. h. hemimelaena (c)	Brazil, AC, Rio Branco	60018	MPEG	JQ080520	
M. h. hemimelaena (c)	Brazil, AC, Senador Guiomard	60696	MPEG	JQ080511	JQ080452
M. h. hemimelaena (c)	Brazil, AC, Bujari, Fl. Est. do Antimary	60697	MPEG	JQ080512	JQ080448
M. h. hemimelaena (c)	Brazil, AC, Plácido de Castro	60698	MPEG	JQ080513	
M. h. hemimelaena (c)	Brazil, AC, Tarauacá, Fl. Est. do Mogno	60699	MPEG	JQ080514	
M. h. hemimelaena (c)	Brazil, AC, Rio Branco, AC-090, km 70	61310	MPEG	JQ080522	
M. h. hemimelaena (c)	Brazil, AC, Porto Walter	62088	MPEG	JQ080523	
M. h. hemimelaena (c)	Brazil, AC, Mâncio Lima, Est. do Barão	62089	MPEG	JQ080524	
M. h. hemimelaena (c)	Brazil, AC, Rio Branco, Est. do Quixadá	63537	MPEG	JQ080525	
M. h. hemimelaena (c)	Brazil, AC, Rio Branco, Est. do Quixadá	63538	MPEG	JQ080526	
M. h. hemimelaena (c)	Brazil, AC, Santa Rosa, left bank Purus	63357	MPEG	JQ080527	
M. h. hemimelaena (d)	Peru, Loreto, south Amazon River	4576	LSUMZ	JQ080529	JQ080461
M. h. hemimelaena (d)	Peru, Loreto, south Amazon River	4641	LSUMZ	JQ080530	JQ080462
M. h. pallens	Bolivia, SC, Velasco	12654	LSUMZ	JQ080532	JQ080478
M. h. pallens	Bolivia, SC, Velasco	12957	LSUMZ	JQ080533	JQ080475
M. h. pallens	Bolivia, SC, Serrania de Huanchaca	14434	LSUMZ	JQ080534	JQ080479
M. h. pallens	Boliva, SC, Serrania de Huanchaca	14771	LSUMZ	JQ080545	JQ080480
M. h. pallens	Bolivia, SC, PN. Noel Keonpff	18392	LSUMZ	JQ080535	JQ080477
M. h. pallens	Brazil, RO, Guajará-Mirim, RBOP	36800	LSUMZ	JQ080538	JQ080474
M. h. pallens	Brazil, RO, Guajará-Mirim , RBOP	54990	MPEG	JQ080510	JQ080471
M. h. pallens	Brazil, RO, right bank of Madeira River	313	INPA	JQ080483	JQ080476
M. h. pallens	Brazil, RO, right bank of Madeira River	314	INPA	JQ080484	JQ080470
M. h. pallens	Brazil, RO, right bank of Madeira River	328	INPA	JQ080485	JQ080472
M. h. pallens	Brazil, PA, Torrão, left bank Xingu River	65297	MPEG	JQ080528	
M. h. pallens	Brazil, MT, Sinop	1955	UFMT	JQ080500	JQ080481
M. h. pallens	Brazil, MT, Sinop	1954	UFMT	JQ080501	
M. h. pallens	Brazil, MT, P. L. e Vila Bela da S. Trind.	520	LGEMA	JQ080544	JQ080473
M. h. pallens	Brazil, MT, Aripuanã	455	LGEMA	JQ080543	
M. taxon novum	Brazil, AM, left bank of Aripuanã River	532	INPA	JQ080493	JQ080463
M. taxon novum	Brazil, AM, left bank of Aripuanã River	548	INPA	JQ080502	JQ080464
M. taxon novum	Brazil, RO, REBIO Jaru, r. bank Jiparaná	2428	INPA	JQ080499	
M. taxon novum	Brazil, RO, REBIO Jaru, r. bank Jiparaná	2374	INPA	JQ080498	JQ080465
M. taxon novum	Brazil, AM, Humaitá, TI Parintintin	58730	MPEG	JQ080506	JQ080469
M. taxon novum	Brazil, AM, Humaitá, TI Parintintin	58732	MPEG	JQ080507	JQ080466
M. taxon novum	Brazil, AM, Humaitá, TI Parintintin	58733	MPEG	JQ080508	JQ080467
M. taxon novum	Brazil, AM, Humaitá, TI Parintintin	58734	MPEG	JQ080521	JQ080468

*Institution acronyms: FMNH – Field Museum of Natural History; INPA – Instituto Nacional de Pesquisas da Amazônia; MPEG – Museu Paraense Emílio Goeldi; LSUMZ – Louisiana State University Museum of Natural Science; UAM – University of Alaska Museum; UFMT – Universidade Federal de Mato Grosso; LGEMA – Laboratório de Genética e Evolução Molecular de Aves; ZMUC – Natural History Museum of Denmark. [1] **Sequences downloaded from GenBank.**

DNA extraction, amplification, and sequencing

DNA was extracted from breast muscle (0.2 g, approximately) using a standard phenol/chloroform protocol (Sambrook et al. 1989). The mitochondrial cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 2 (*ND2*) genes were amplified using published mitochrondrial DNA (mtDNA) primers: (1) for cyt *b*: L14993 and H16064 (Fernandes 2007); and (2) for *ND2*: H6313 and L5215 (Brumfield *et al.* 2007). Two additional primers:

forward 5′-CCCTAGGCGGTTGAGCCGGA-3′

reverse 5′-TTGTGTTGAGAGTAAGGAAGATGGGGA-3′

were designed particularly for this project. Population genetic analyses of *M. hemimelaena* were based on sequences of cyt *b* (*c*. 984 bp) for 65 individuals and *ND2* (*c*. 1041 bp) for 41 individuals. Phylogeographic analyses used a concatenated dataset of 41 individuals for which both cyt *b* and *ND2* sequences were available (*c*. 2025 bp).

Polymerase chain reaction (PCR) amplifications were performed with 50 μL reaction volumes containing 1 × PCR buffer (Bioron, Ludwigshafen, Germany), 100 μM dNTPs, 0.2 units of Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 200 ng of DNA, and 5 pmol of primers. Optimal annealing temperature was determined by gradient PCR in a Tgradient thermocycler (Biometra). Thermal cycling involved: (1) an initial denaturing step at 94°C for 5 min; (2) 35 cycles: 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; and (3) a final 5-min extension at 72°C. PCR products were precipitated with 4M NH_4Ac and ethanol (1:1:6) and centrifuged for 15 min (13,000 rpm). Sequencing was carried out on an ABI 3730 automated capillary sequencer (Applied Biosystems, USA) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 by STARSEQ GmbH (Mainz, Germany). To confirm observed mutations, both strands of each sample were sequenced.

Phylogenetic analyses

Phylogenetic analyses were performed using Bayesian inference (BI) implemented in MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) and maximum likelihood (ML) via RAxML GUI vs. 0.93 (Silvestro and Michalak 2011). The evolutionary models were selected with JMODELTEST using the BIC (Bayesian information criterion; (Posada 2008). Four different MRBAYES analyses were conducted, each with two parallel simultaneous runs, for a total of 5×10^6 generations each, and trees were sampled every 500 generations. The mitochondrial genes (cyt *b* and *ND2*) were analyzed independently and concatenated in a single data matrix. In the combined dataset, a mixed model Bayesian analysis was employed using the GTR + Propinv model for *ND2* and the HKY + G model for cyt *b*.

To visualize genealogical relationships among individuals, haplotype networks were constructed with maximum parsimony in the program TCS 1.18 (Clement et al. 2000). Mean pairwise (*p*)-distances (Nei 1987) within and among lineages were calculated using MEGA 4.0 (Tamura et al. 2007).

Molecular dating

Estimates of divergence time of main lineages of *M. hemimelaena* were conducted using two data partitions (cyt *b* and *ND2*), each with individual models of molecular evolution chosen by JMODELTEST 0.1.1 (Posada 2008). The widely used cyt *b* mutational rate of 2.1% sequence

divergence per million years (0.0105 substitutions/site/lineage/million years) was applied (Weir and Schluter 2008; Weir et al. 2009). Because a clock-like evolution was rejected using a likelihood ratio test in PAUP*4.0b10 (SWOFFORD 2002) and by the estimation of the parameter ucld.stdev in BEAST, it was applied an uncorrelated lognormal relaxed-clock. Two independent simultaneous runs of 2×10^7 generations were performed, sampling one every 1,000 trees in BEAST 1.6.1 (Drummond and Rambaut 2007). Posterior probabilities of the nodes were computed for all Bayesian analyses across the sampled trees after burn-in. The number of generations required to reach stationarity of the posterior distribution was determined by examining marginal probabilities plotted as a time series in TRACER 1.5 (Rambaut and Drummond 2007).

Population genetics and historical demography

For population genetic analyses, groups were defined according to the major clades of haplotypes recovered in the phylogenetic analyses. Calculations of the following population parameters based only on cyt *b* sequences were calculated in ARLEQUIN 3.1 (Excoffier et al. 2005): nucleotide diversity (π), haplotype diversity (*h*), Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) statistic. Significance was determined based on 1000 coalescent simulations. To confirm and expand on the results obtained from the phylogenies, it was performed *a posteriori* analysis of population genetic structure using AMOVA (Excoffier et al. 1992). The defined groups were also the major clades recovered in the phylogenies.

The historical population size dynamics was reconstructed using GMRF skyride plots (Minin et al. 2008), implemented in BEAST 1.6.1 (Drummond and Rambaut 2007). The GMRF skyride plot is a nonparametric analysis that uses the waiting time between coalescent events in a gene tree to estimate changes in effective population size over time. It differs from the related Bayesian skyline plot (Drummond et al. 2005) by not requiring the imposition of a user-defined prior on the number of population size changes in the history of the sample (Ho and Shapiro 2011). The defined groups were the clades recovered in the phylogenies for which more than five individuals were available. The GMRF skyride plots were constructed using both mtDNA genes using the cyt *b* mutational rate of 2.1% sequence divergence per million years (0.0105 substitutions/site/lineage/million years), time-aware smoothing and strict molecular clock priors. All other parameters were identical to the molecular dating described above.

3.2.4 Results

Phylogenetic analyses

Phylogeographic analyses recovered a sister relationship and the reciprocal monophyly between *M. castanea* and *M. hemimelaena*, which are separated by a genetic distance of 6.2% (uncorrected *p*-values within *M. hemimelaena*). Three clades of *M. hemimelaena* were identified on the basis of mtDNA data. They were found to correspond to vocally and morphologically distinct lineages (Figs. 24, 25, and 26; Isler et al. 2002; Zimmer and Isler 2003): (1) *M. h. hemimelaena* (west of the Madeira River), (2) *M. h. pallens* (east of the Madeira River ranging eastward to the Xingu River), and (3) *M. h. pallens* (endemic to the Jiparaná/Aripuanã interfluvium). In addition to being monophyletic in mtDNA and separated by the highest average uncorrected genetic distance within *M. hemimelaena* (3.1%), populations of *M. h. pallens* from inside and outside the Jiparaná/Aripuanã interfluvium are also vocally and morphologically distinct (Isler et al. 2002; Zimmer and Isler 2003), thus indicating that these populations are best

treated as different evolutionary units. While the intention in this study does not extend to a formal taxonomic revision, for convenience hereafter we refer to populations found within the Jiparaná/Aripuanã interfluvium (Fig. 24) as ***M. taxon novum***.

Within *M. h. hemimelaena*, significant phylogeographic structuring also exists (Figs. 25 and 26), but in this case genetic distances are much smaller (0.8–1.4%), and the lack of congruent mtDNA, morphological, and vocal diagnoses precludes their recognition as different taxa (see also Zimmer 1932; Isler et al. 2002). Posterior probability values supporting the monophyly of these three main clades were high, as was the major split separating *M. h. hemimelaena* from *M. h. pallens/M. taxon novum*; on the other hand, support for the sister relationship between *M. h. pallens* and *M. taxon novum* was weak (Figs. 25 and 26).

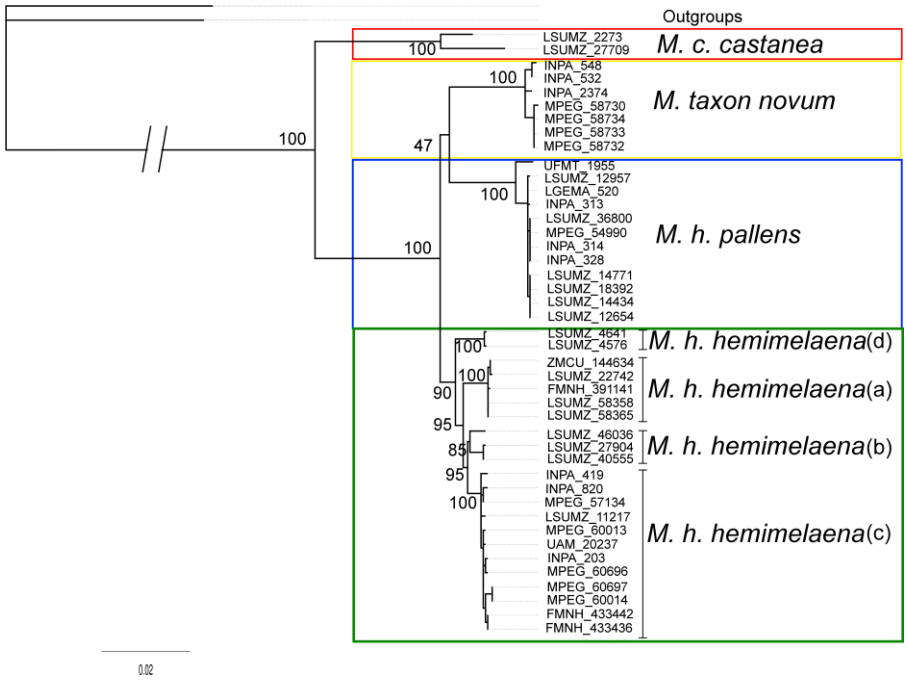


Figure 25 – Maximum likelihood phylogeny based on *ND2* and cyt *b* sequences (*n* = 41) showing the relationships among different phylogroups of the *Myrmeciza hemimelaena* complex in Amazonia. Numbers beside the nodes denote bootstrap values (based on 1000 replicates) from maximum likelihood (–ln L = 5805.788528) analysis.

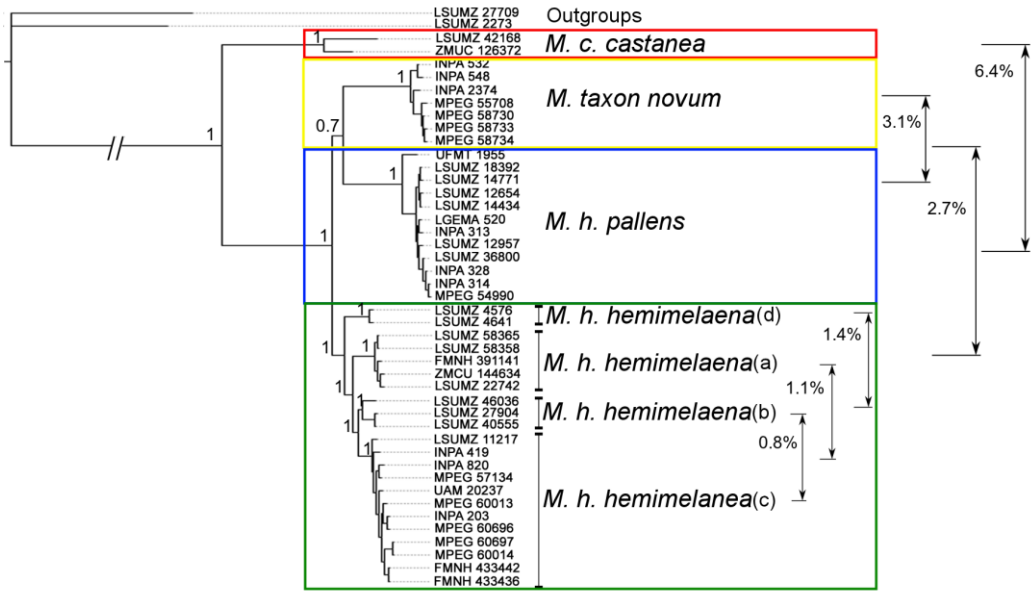


Figure 26 – Bayesian phylogeny based on *ND2* and cyt *b* sequences (*n* = 41) showing the relationships among different phylogroups of the *Myrmeciza hemimelaena* complex in Amazonia. Numbers next to the nodes correspond to posterior probability values; numbers in % are the uncorrected pairwise (*p*)-distances; a, b, c and d are clades of *M. h. hemimelaena* as shown in Fig. 24.

Maximum parsimony haplotype networks (95% connection limit) mirrored the Bayesian phylogenetic tree in recovering the same three major clades corresponding to the three genetically and phenotypically diagnostic forms of *M. hemimelaena* (*M. h. hemimelaena*, *M. h. pallens* and *M. taxon novum*). These clades display non-overlapping geographic distributions and are separated by larger than average numbers of mutational steps (Fig. 27).

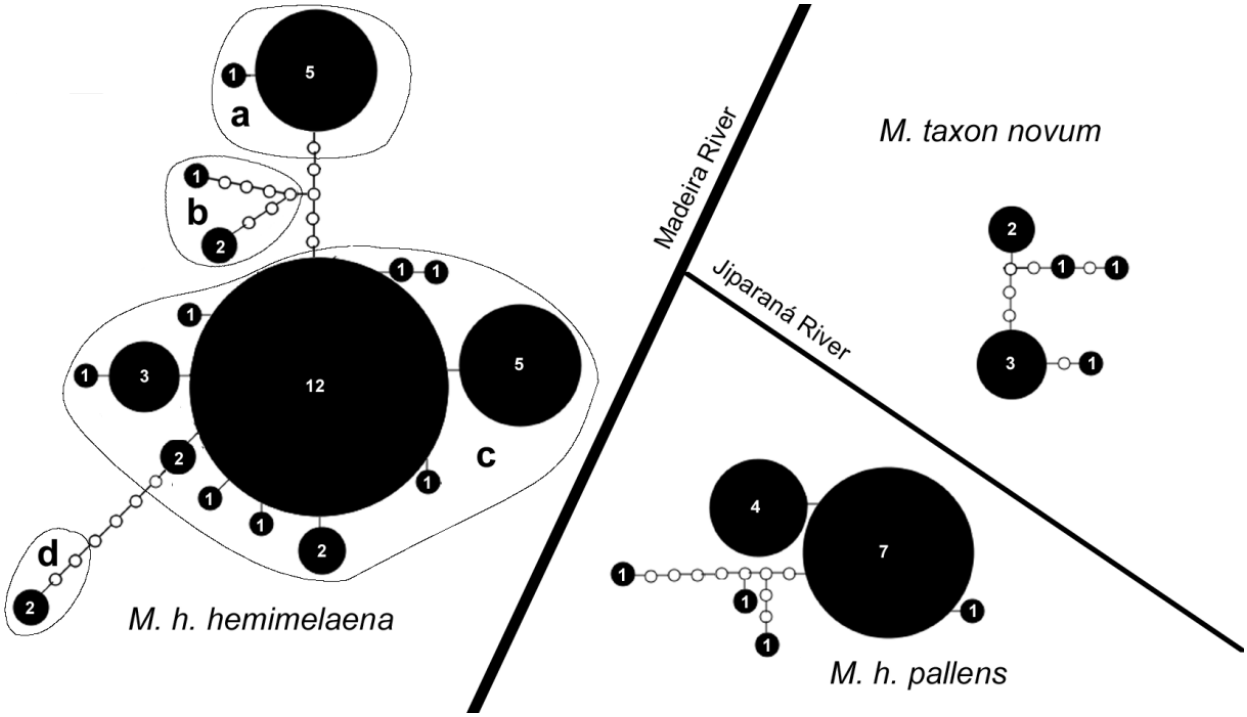


Figure 27 – Haplotype networks (95% connection limit) based on cyt *b* sequences estimated for 66 individuals of the *Myrmeciza hemimelaena* three main vocal/morphological lineages separated by the Madeira and Jiparaná rivers in Amazonia. Each *black circle* represents a haplotype whose size corresponds to its total frequency in the respective lineage. *Numbers* inside black circles refer to the number of sampled individuals possessing that particular haplotype. *Solid lines* connecting haplotype clades represent single mutational steps. *Blank circles* correspond to unsampled haplotypes. Note that each of the three main clades (which were also recovered by the Bayesian phylogeny estimate; Fig. 24) is supported by a higher level distance in TCS, and hence are not connected;
a, b, c, and d are clades of *M. h. hemimelaena* as shown in Fig. 24.

Molecular dating

According to the relaxed-clock model, diversification times of *Cercomacra* and the *M. hemimelaena* complex took place from the Late Miocene through the entire Pliocene between 2.7 and 8.8 Mya (mean 5.7 Mya; Fig. 28). The earliest split in the *M. hemimelaena* complex occurred during the Pliocene–early Pleistocene (1.8–4 Mya; mean 2.9 Mya) and involved *M. castanea* and all taxa grouped under *M. hemimelaena* (Fig. 4). Subsequent splits within *M. hemimelaena* were all clustered during the mid–early Pleistocene (1.8–0.3 Mya; Fig. 28).

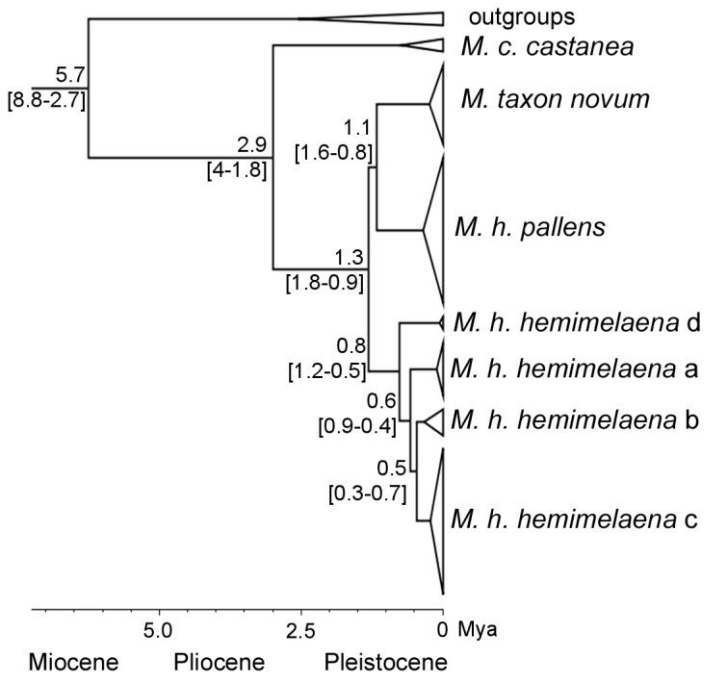


Figure 28 – Bayesian divergence times of *Myrmeciza hemimelaena* lineages in Amazonia (in million years ago, Mya) estimated with a relaxed-clock model based on *ND2* and cyt *b* sequences (n = 41). Numbers within *brackets* indicate 95% posterior age intervals.

Population genetics and historical demography

High values of haplotype diversity (h) and low values of nucleotide diversity (π) were obtained for the three major clades of *M. hemimelaena* identified in the maximum parsimony networks. Neutrality tests showed signs of population fluctuations for all three clades. Values of Fu's F_S statistic were significantly negative for all lineages, whereas Tajima's D was significantly negative only for *M. h. hemimelaena* (Table 11). AMOVA confirms that most of genetic variation in *M. hemimelaena* is partitioned geographically among the three main lineages identified in both the Bayesian phylogeny and haplotype networks (86%, P = 0.0000, F_{ST} = 0.86).

Table 11 – Genetic diversity and demographic population parameters for different clades of the *Myrmeciza hemimelaena* complex in Amazonia based on cyt *b* sequences.

Taxon	***N***	***h***	***Π***	***D***	***F*$_S$**
M. h. hemimelaena	42	1.00	0.003	**–1.40**	**–25.70**
M. h. pallens	15	1.00	0.004	–1.12	**–15.75**
M. taxon novum	8	1.00	0.003	0.34	**–4.50**

Values given in bold are significant at $P < 0.05$; ***n***, number of sequences; ***h***, haplotypic diversity; ***Π***, nucleotide diversity; ***D***, Tajima's D (1989); ***Fs***, Fu (1997).

Most of the Bayesian skyride plots (BSPs) estimated for the three *M. hemimelaena* clades agree with the neutrality tests in inferring histories of demographic fluctuations during the mid–late Pleistocene and Holocene. *Myrmeciza h. pallens* presents a clear pattern of population decline during the last 0.3 million years (Mya) in eastern Amazonia (Fig. 28). A slight signal of gradual population expansion was also recovered for *M. h. hemimelaena* (population a) in the Andean

foothills south of the Amazon/Marañón River during the last 0.15 Mya (Fig. 29). The BSP for *M. h. hemimelaena* (population c) in western Amazonia shows an abrupt decline between 0.3 and 0.25 Mya, followed by an expansion *c*. 0.2 Mya, then population stability between 0.2 and 0.1 Mya, and another expansion during the last 0.1 Mya. Finally, *M. taxon novum* appears to have maintained a relatively stable size during the last 0.4 Mya (Fig. 29).

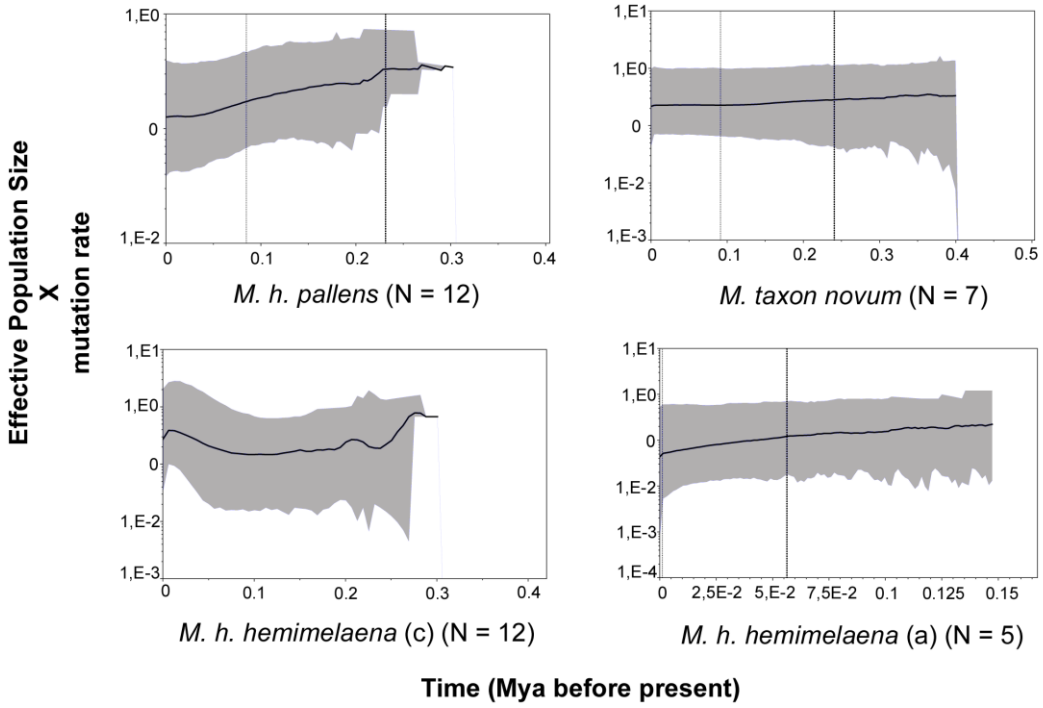


Figure 29 – Demographic history of *Myrmeciza hemimelaena* lineages in Amazonia determined by Bayesian skyride plots based on *ND2* and cyt *b* sequences. The *black line* represents the median values, the *shaded area* denotes 95% Bayesian credible intervals; a and c are clades of *M. h. hemimelaena* as shown in Fig. 24.

3.2.5 Discussion

Temporal and spatial patterns of diversification and historical demography

Overall, the temporal diversification pattern estimated herein for the *M. hemimelaena* complex is at odds with the notion that Avian diversity in Amazonia was generated mostly during the Pliocene and Pleistocene. Most Amazonian avian lineages for which dated molecular phylogenies are available have shown the same pattern of ongoing diversification during those epochs (Aleixo and Rossetti 2007; Patané et al. 2009; Patel et al. 2011; Ribas et al. 2012). Therefore, as more studies with extensive taxonomic sampling and multiple character evidence become available, I predict that chronograms estimated for Amazonian lineages should point towards diversification scenarios concentrated in both the Pliocene and Pleistocene. The relative importance of each of those epochs to the current patterns of diversity and endemism in Amazonia will become clearer with additional studies.

Rivers of different sizes conspicuously delimit the distribution of most species of the *M. hemimelaena* complex. The only exceptions are *M. castanea* and *M. hemimelaena*, which share a narrow and barrier-free contact zone south of the upper Marañón River in central Peru (Isler et al. 2002). However, as shown in the Bayesian phylogeny (Fig. 26), the population of *M. hemimelaena* of this contact zone (population b) is sister to the *M. hemimelaena* population c, which is distributed throughout most of western Amazonia south of the Amazon and east of the Ucayali River (Fig. 24). Hence, the contact zone between *M. castanea* and *M. hemimelaena* can

be interpreted as a secondary contact between non-sister lineages that diversified elsewhere rather than an area of primary differentiation. This interpretation challenges the role of the upper Marañón River in promoting cladogenesis in the *M. hemimelaena* complex. In contrast, even though the phylogenetic relationships among *M. h. hemimelaena*, *M. h pallens* and *M. taxon novum* could not be resolved with confidence because of the low (0.7) nodal support for sister relationship between *M. h. pallens* and *M. taxon novum*, any combination involving this triplet implies that at least parts of the Madeira River could have acted as a barrier involved in the primary diversification of lineages of the *M. hemimelaena* complex (Fig. 24). In the most likely scenario recovered by the Bayesian tree, the differentiation between *Myrmeciza h. hemimelaena* and *M. h. pallens*/*M. taxon novum* took place at *c*. 1.3 Mya across the entire course of the Madeira River (Fig. 28). Alternative tree topologies would imply that either only the upper (in the case of a sister relationship between *M. h. hemimelaena* and *M. taxon novum*) or middle (in the case of a sister relationship between *M. h. hemimelaena* and *M. h. pallens*) courses of the Madeira River might have acted as barriers of primary diversification (Fig. 24). Interestingly, the distributions of both *M. h. hemimelaena* and *M. h. pallens* extend farther to the south into the headwaters of the Madeira River in eastern Bolivia, where the few available records suggest that populations are likely to be separated by the Mamoré River, the largest tributary forming the Madeira River (Isler et al. 2002; Zimmer and Isler 2003; Fig. 24). However, as this is a fairly small river, they could as well come into contact somewhere else, as also observed for the warbling antbird (*Hypocnemis cantator*) complex (Isler et al. 2007).

Together, the Bayesian phylogeny estimate and the current distribution patterns of *M. h. hemimelaena* and *M. h. pallens* allow the interpretation that the Madeira River and possibly its headwaters have been functioning as a primary diversification barrier in the *M. hemimelaena* complex. The same can be said about the much smaller Jiparaná and Aripuanã rivers, which separate *M. taxon novum* from its most likely sister taxon (*M. h. pallens*; Fig. 24). However, large tributaries bisecting the range of *M. h. hemimelaena* population c in western Amazonia (e.g., Juruá and Purus rivers), do not separate sister clades on their opposite banks, and thus do not appear to be directly involved with primary population differentiation in this species (Fig. 24).

Bayesian skyride plots (BSPs; Fig. 29) and population genetics neutrality tests (Table 8) both point towards ubiquitous demographic fluctuations throughout the Pleistocene for all subspecies in the *M. hemimelaena* complex, except maybe *M. taxon novum*, for which only Fu's F_S statistic showed a significant departure from neutrality. Even though the power of those tests and analyses can be severely compromised by our limited sampling and reliance on mtDNA, the emerging pattern seems to be one of demographic instability throughout the Pleistocene, as predicted by the refuge hypothesis (Zink 1997; Hewitt 1999; Aleixo 2004; Cheviron et al. 2005). The historical demographic pattern shown by *M. h. hemimelaena* in western Amazonia and the foothills of the Andes (for which there is the most complete sampling, i.e. 42 individuals) matches models of climatic oscillation proposed worldwide for the mid–early Pleistocene (e.g., Head and Gibbard 2005) as follows: (1) the abrupt population decrease after *c*. 0.3 Mya followed the onset of a glacial period; (2) the slight population increase before *c*. 0.2 Mya is consistent with the establishment of an interglacial warmer period; (3) the new population decrease starting after 0.2 Mya followed another glacial period lasting until 0.1 Mya, after which a new population rebound is apparent; and (4) the most recent demographic sink started after *c*. 0.02 Mya and can be tentatively linked to the LGM; apparently, the effective population size of *M. h. hemimelaena* has not yet recovered to pre-LGM levels (Fig. 29). If the Bayesian skyride plot produced for *M. h. pallens* is interpreted at face value, a steady population decline after *c*. 0.3 Mya is apparent

as also found in *M. h. hemimelaena*, but unlike in the latter species, no episodic population rebounds are visible (Fig. 29). However, both *M. h. hemimelaena* and *M. h. pallens* populations share the same overall mid–early Pleistocene decline. The interpretation of our historical estimates of effective population sizes for *M. taxon novum* is particularly complicated by our limited sampling of this taxon (Fig. 29).

The drivers of cladogenesis

The spatial and temporal patterns of diversification and historical demography presented and discussed herein for the *M. hemimelaena* complex allowed to discuss the relative importance of two main hypotheses accounting for the differentiation of the Amazonian biota: the *refuge* and *riverine barrier hypotheses* (see Haffer 1997b; Antonelli et al. 2010 for a synopsis of these and other hypotheses of Amazonian diversification).

In the light of the riverine barrier hypothesis, rivers would always be associated with areas of primary differentiation in the *M. hemimelaena* complex. However, as shown previously, western Amazonian rivers flowing through the Solimões sedimentary basin (e.g., Marañon, Juruá, and Purus) have less stable courses that experience frequent lateral channel migration rarely observed in rivers located on the Brazilian and Guianan shields such as Madeira, Jiparaná and Aripuanã (Aleixo 2004; Bates et al. 2004). Thus, to this day, discrepancies with the riverine barrier hypothesis involving *terra-firme* forest species, such as those grouped in the *M. hemimelaena* complex, appear to be restricted to a small subset of wide rivers flowing through the relatively young Solimões sedimentary basin in western Amazonia (Patton et al. 1994; Lougheed et al. 1999; Aleixo 2004; Wanderley-Filho et al. 2010). Indeed, when molecular phylogenies of Amazonian birds associated with *terra-firme* forests are viewed together, the Madeira, Jiparaná, and Aripuanã rivers have all been shown also to delimit sister and monophyletic groups of many other lineages, thus demonstrating the generality of their role as likely vicariant agents; river width and water load alone are not necessarily correlated with a strong barrier effect (Aleixo 2004; Nyári 2005; Sardelli 2005; Fernandes 2007; Patel et al. 2011). Varying degrees of barrier effects in the Amazon seem to be determined primarily by an interaction between geology and river width and discharge (Antonelli et al. 2010). Again, the Bayesian phylogeny estimate obtained for the *M. hemimelaena* complex also supports this broad conclusion because it fits a general pattern shown by several other avian lineages, whereby populations from the Brazilian shield are monophyletic, but those from the Solimões sedimentary basin are paraphyletic (Aleixo and Rossetti 2007).

The timing and mode of the diversification of the *M. hemimelaena* complex is consistent with the most recent models based on geological and palaeontological evidence proposed for the historical development of the Amazon drainage (Espurt et al. 2010; Latrubesse et al. 2010; Mora et al. 2010). According to these models, the continuous subduction of the Nazca Ridge under the South American Plate during the Miocene caused the uplift of the Fitzcarrald Arch located in the Acre basin distributed in Brazil and Peru during Pliocene times (Espurt et al. 2010). This, in turn, prompted the Amazonian foreland basins located on the eastern Andean foothills in Bolivia, Brazil, Peru, Ecuador and Colombia, to evolve between the late Pliocene–early Pleistocene from a depositional to a predominantly erosional state, draining sediments eastward and hence creating the modern trans-continental Amazon drainage flowing towards the Atlantic (Espurt et al. 2010; Latrubesse et al. 2010; Mora et al. 2010; see also Campbell et al. 2006). This major continental-wide drainage reorganization was responsible for the modern configuration of most Amazonian

rivers, including those located far away from the sub-Andean foreland basins such as the Tapajós, which also drains in an eastward direction as do those major tributaries whose sources are located well into the foreland basins (e.g. Rio Negro, Amazon/Marañon/Solimões and Madeira rivers). Thus, the first split in the *M. hemimelaena* complex involving *M. castanea* and *M. h. hemimelaena*, *M. h. pallens* and *M. taxon novum* and dated as of late Pliocene in age (Fig. 28) is consistent with this model of drainage formation. The fact that the Marañon River appears not to have acted as a primary barrier of diversification between *M. castanea* and the remaining species in the *M. hemimelaena* complex should not be interpreted as evidence undermining the riverine barrier hypothesis because populations of those two lineages co-occurring nowadays south of the Marañon are not sisters and either one or both could have dispersed more recently into this area across the river after a long period of differentiation in isolation (see above; Fig. 24). Currently, the entire Amazon and most of the Marañon rivers delimit the ranges of *M. castanea* and *M. hemimelaena* in northern Peru (Isler et al. 2002; Zimmer and Isler 2003), so their split could still be associated with the formation of a proto-Amazon river following the separation of the Amazonian foreland basins into a northern and a southern Amazonian foreland basin following the uplift of the Fitzcarrald Arch during the Pliocene (Espurt et al. 2010).

Similarly, the splits involving the remaining species in the *M. hemimelaena* complex seem to be associated with the formation of the modern Madeira River basin following a major continental-wide drainage reorganization of the Amazon Basin following the establishment of the Fitzcarrald Arch. Exact dates for the establishment of the modern Madeira River basin are still uncertain but have not been thought of as older than the Late Pliocene (Westaway 2006), thus theoretically preceding the major splits across the Madeira River between *M. h. hemimelaena* and *M. h. pallens*/*M. taxon novum* by *c*. 1 Mya (Fig. 28). Clearly, the absence of more detailed data-rich models accounting for the temporal development of the Madeira drainage prevents a more accurate assessment of the congruence between the ages of the estimated splits in the *M. hemimelaena* complex and the formation of the modern Madeira River basin. However, the documentation of two separate active large mega-fans of suggested late Pleistocene age involving both the Jiparaná and the Aripuanã drainages (Latrubesse 2002), is indicative of much wider and complex palaeo-drainages in this interfluvium than those found today. This history may explain patterns of primary diversification in this sector of Amazonia involving the *M. hemimelaena* complex as well as other avian and even primate lineages (van Roosmalen et al. 1998; Sardelli 2005; Fernandes 2007). A second non-exclusive and somewhat related explanation could also involve tectonically mediated mega-drainage capture as shown for the Rio Negro River (Almeida-Filho and Miranda 2007), whereby either the Jiparaná or Aripuanã once captured a significant part of the Madeira or even the neighbouring Tapajós drainages or both, but later became mere tributaries following the major continental-wide drainage reorganization of the Amazon Basin during the late Pliocene (see also Willis 1969). When applied to the Madeira drainage, future studies with a similar perspective as that by Almeida-Filho and Miranda (2007) should clarify the causes of the seemingly disproportional riverine barrier effect verified for the *M. hemimelaena* complex and other vertebrate lineages across the Aripuanã and Jiparaná rivers.

Unlike the riverine barrier hypothesis, the refuge hypothesis makes no spatial prediction about the location of splitting events involving lineages isolated in different climatic refuges (Patton and da Silva 1998), but populations under this model of evolution are expected to show signs of demographic fluctuations during the Pleistocene (Zink 1997; Hewitt 1999; Aleixo 2004; Cheviron et al. 2005) and even before that (Haffer 1997a), such that the temporal predictions of this hypothesis are very vague. As discussed above, when neutrality tests and Bayesian skyride

plots are interpreted together, at least two lineages on the *M. hemimelaena* complex showed clear signs of demographic changes during the Pleistocene. Furthermore, it was possible to find a temporal match between the historical demographic dynamics of the most thoroughly sampled subspecies (*M. h. hemimelaena*) and glacial and interglacial cycles (Head and Gibbard 2005). However, all these intense demographic fluctuations detected for *M. h. hemimelaena* and *M. h. pallens* during the Pleistocene post-dated their differentiation as separate species and appeared not to have contributed to most splitting events in the *M. hemimelaena* complex. Two possible exceptions, whose earliest 95% posterior age intervals fall into the timeframe of the reconstructed historical population dynamics by the Bayesian skyride plots, could be the splits among three separate populations of *M. h. hemimelaena* (a, b and c) inhabiting different parts of western Amazonia between the Mamoré/Madeira and Amazon/Marañon rivers (Figs. 24 and 28). However, mean time estimates for those splits are older than the Bayesian skyride plots time frames (Fig. 29), and their wide 95% posterior age intervals prevent a more detailed correlation with specific glacial/interglacial times. Thus, as also verified for two independent lineages of Amazonian birds, it appears that historical demographic dynamics detected in the *M. hemimelaena* complex during the mid–early Pleistocene were not accompanied by intense cladogenesis, as predicted by the refuge hypothesis (Aleixo 2004; Ribas et al. 2012).

As discussed above, the spatial and temporal nature of splitting events leading to major lineage differentiation in the *M. hemimelaena* complex are more easily explained by the formation of the modern Amazon River drainage during the late Pliocene. The data and interpretations presented herein provide evidence that tectonic activity leading to major drainage reorganization during the late Pliocene–early Pleistocene may have been an important driver of speciation in Amazonia.

Species limits and taxonomy

The molecular phylogeny supports the *M. hemimelaena* complex as a monophyletic group and confirms that *M. castanea* and *M. hemimelaena* are distinct sister species. Except for a very small area of overlap in northern Peru (Isler et al. 2002; see also below), they replace each other across the upper Amazon River. This divergence dates back to ca. 3 Mya, i.e. the Late Pliocene. Three well-supported monophyletic groups were identified in *M. hemimelaena*, and they are separated by comparatively large genetic distances (see also Bates 2000, 2002); according to molecular clock estimates they originated during the early–mid Pleistocene. These genetically divergent groups are also distinguished by unique morphological and vocal characters as verified in this and other studies (Isler et al. 2002; Zimmer and Isler 2003).

The existence of mtDNA reciprocal monophyly among the three major *M. hemimelaena* lineages, along with allozymic (i.e. nuclear) genetic structuring across the upper Madeira River (Bates 2000), additionally supported by vocal/morphological diagnoses (Isler et al. 2002; Zimmer and Isler 2003) encourage their recognition as separate species under different species criteria, including the biological (Mayr 1963), phylogenetic (Cracraft 1983) and several evolutionary species concepts proposed so far (de Queiroz 1998).

Although *M. h. pallens* and *M. taxon novum* are morphologically very similar and diverged in presumed isolation, no evidence of intergradation (i.e. widespread mtDNA haplotype mixing) was observed, thus suggesting that they appear to behave as separate species even while in contact in the headwaters of the Jiparaná/Aripuanã rivers. The same

situation is found for *M. castanea* and *M. hemimelaena* in the upper Marañon. Those two species, long considered conspecific, occur side-by-side without apparent signs of interbreeding in northern Peru, demonstrating that they appear to consist reproductively isolated species as well. Unequivocal vocal diagnoses (to be presented elsewhere for *M. taxon novum*; see also (Isler et al. 2002) among those three main clades match results of a previous experimental study in which genetically and vocally divergent populations of *M. h. pallens* isolated in natural forest fragments responded more strongly to local rather than non-autoctonous song variants, suggesting that even minor differences in vocal characters are associated with assortative mating and some degree of reproductive isolation in this group (Seddon and Tobias 2007).

Conservation implications

The phylogeography of the *Myrmeciza hemimelaeana* complex corroborates the existence of a new species restricted to the mini-interfluvium Jiparaná/Aripuanã. This agrees with previous studies suggesting that smaller rivers can also delimit the distribution of birds and other vertebrates in Amazonia and that this seems to be a common pattern rather than an exception. At least two other apparently new avian taxa have their distributions bounded by the Jiparaná and Aripuanã rivers. The warbling antbird complex (*Hypocnemis* spp., Thamnophilidae; Isler et al. 2007; Tobias et al. 2008) has vocally, morphologically and genetically distinct populations also separated by these two rivers, and an undescribed taxon of the genus *Herpsilochmus* (Thamnophilidae) is also restricted to this interfluvium (pers. obs., M. Cohn-Haft, pers. comm.). The bar-breasted piculet (*Picumnus aurifrons*) shows distinct plumage colouration on opposite banks of the Aripuanã River (Cohn-Haft et al. 2007). Genetic differentiation among morphologically indistinguishable populations bounded by the Jiparaná and Aripuanã rivers has also been described for Snethlage's tody-tyrant (*Hemitriccus minor*; Sardelli 2005) and the wedge-billed woodcreeper *Glyphorynchus spirurus* (Fernandes 2007). These two rivers also delimit the ranges of several taxa of primates (van Roosmalen et al. 1998) and butterflies (Hall and Harvey, 2002). Therefore, the Jiparaná/Aripuanã interfluvium harbours a cryptically endemic fauna that is being accurately described only now with modern tools such as genetic and bioacoustical analyses unavailable a few decades ago. This has important conservation implications because this region is currently suffering high rates of deforestation (Fearnside 2002, 2006). As discussed by Aleixo (2009) and implied by some previous studies (Cohn-Haft et al. 2007; Fernandes 2007), conservation policies should take taxonomic uncertainties into account in delimiting proper conservation targets. Disregarding the significance of entire cryptically diverse sectors of Amazonia under the current high anthropogenic development pressure may lead to the irretrievable loss of valuable unrecognized species.

3.3 Phylogeography and Speciation of the Spot-backed Antbird (*Hylophylax naevius*) in Lowland Amazonia

3.3.1 Abstract

Molecular phylogeography of species distributed across Amazonian barriers such as rivers and mountains may help to understand speciation in the Neotropical region. The molecular phylogeography of the Spot-backed Antbird (*Hylophylax naevius*) (Aves, Thamnophilidae) was investigated throughout the Amazon basin. The sampling of a total of 80 individuals encompassed opposite banks of the major Amazonian rivers, both sides of the Andes, and an ancient paleogeographic barrier in western Amazonia (having originated during the Andes uplift). Nucleotide sequences from two mtDNA genes (1015 bp of cyt *b* and 1023 bp of ND2), and one nuclear gene (539 bp of BF5) were obtained. Allopatric/parapatric well-supported lineages were determined within *H. naevius* with high levels of genetic differentiation, on opposite sides of the rivers (0.6–7.1%), ridge (4.4%) and the Andes (6.9%). The striking result was that *H. naevioides* (its purported sister species) clusters together with *H. naevius*, indicating that *H. naevius* is paraphyletic and this species ought to be separated into distinct species. A second instance of paraphyly was observed in the subspecies *H. n. theresae* demonstrating the existence of a so far undescribed **new taxa** which can be distinguish also by morphological and vocal characters. Molecular dating and population demography indicating speciation at different periods and associated with distinct vicariant events suggest that biodiversity in the Amazon was modeled by different historical processes.

3.3.2 Introduction

The Amazonian lowland rainforest harbors the highest biodiversity on Earth (Mittermeier et al. 2003) having fascinated scientists for centuries who attempted to understand the processes that have generated this high degree of speciation (Wallace 1852; Wesselingh et al. 2010). Among the hypotheses proposed to explain the current patterns of species distribution in Amazonia are notably those that support divergence in allopatry (Wallace 1852; Haffer 1969; Vanzolini and Willians 1970; Nores 1999; Patton et al. 2000).

Wallace noted that range boundaries for a number of animal species in Amazonia seemed to coincide with the river barriers (Wallace 1852) which suggests that the Amazon's main rivers functioned as natural barriers to gene flow between populations. The **river barrier hypothesis** have been corroborated by molecular phylogenetic studies (Ribas et al. 2012; Fernandes et al. in press). These studies have shown splits coincident with the origin of river formation. Most other hypotheses attempting to explain allopatric diversification in Amazonia are difficult to test mainly because they are related to geographical barriers that are no longer present in the Amazon basin, such as Pliocene wetlands, ancient ridges, and Pleistocene forest refuges.

Aquatic conditions seem to have persisted in western–central Amazonia up to 7 Mya, when the modern Amazon system was established (Hoorn et al. 2010). Stratigraphic data suggest that this fluvial system extended westward up to the Purus arch, an ancient ridge of western Amazonia that separated the Solimões and Amazon basins and restricted the Amazonian mega-wetland to the west and the palaeo-Amazon River to the east (Shephard et al. 2010). The Purus arch is one of several ancient arches formed during uplift of the northern Andes and that subdivided the Amazon Basin (Graham 2009). A deep phylogeographic split was found in both echimyid

rodents and frogs, apparently unrelated to the presence of the rivers that delimit their ranges. For each of these two groups, two major clades were identified along the Juruá River (a white-water tributary of the Amazon; 1500 km long; ca. 500 m wide), corresponding to the river's upper and lower course (possibly across a supposed arch), rather than to opposing river banks (Patton et al. 1994, 2000; Lougheed et al. 1999).

The hypothesis that climatic and vegetational alteration during the Pleistocene fragmented the previously continuous ranges of many species into isolated refuges was first proposed by Edward Forbes in 1846 to account for disjunctions in the distributions of plants in Europe (see Darwin Correspondence Database, http://www.darwinproject.ac.uk/entry956). Haffer (1969) and Vanzolini and Willians (1970) proposed that this same phenomenon could have caused allopatric speciation in Amazonian vertebrates. The main criticism to apply this idea in Amazonia is that it is not known whether the Amazon rainforest was fragmented or reduced in its original size. Moreover, several authors argue that speciation in the Neotropics occurred in periods prior to the Pleistocene (for review see Antonelli et al. 2010).Under a population genetics framework, population bottlenecks are expected during periods of forest contraction (after the onset of dry climatic conditions), whereas population expansion is predicted during periods of forest expansion, following humid climatic conditions (Aleixo 2004). Although some studies have shown evidence of population fluctuations for some avian taxa in Amazonia (Ribas et al. 2012), there is no indication that forest fragmentation has caused cladogenesis. Molecular studies have shown that different geological processes promoted differentiation in Amazonia (e.g., marine incursions, orogeny, and river formation) and there is a consensus that diversification in this region may not be explained by a single vicariant event (Bush 1994; Costa 2003; Cheviron et al. 2005; Antonelli et al. 2010).

Divergent timing and patterns of species accumulation are prominent questions in Neotropical ornithology. Some authors have argued that avian speciation in Amazonia predates the Pleistocene (Weir and Schluter 2007; Antonelli et al. 2010; Hoorn et al. 2010; Weir and Price 2011b). Weir and Schluter (2007) analyzed diversification timing of several sister species of mammals and birds in a latitudinal gradient and concluded that rates of speciation in the Neotropical region are slower than in the higher latitudes and that Neotropical birds predate the Pleistocene. Tobias et al. (2008) argue that the conclusions of Weir and Schluter (2007) could be attributed to taxonomic uncertainty of the Neotropical birds and that their published evolutionary ages are thus overestimated. This debate illustrates that spatial and temporal patterns of diversification of Neotropical birds are still poorly understood. More refined data based on molecular phylogenies and systematic revisions must be obtained in order to accurately understand Neotropical bird evolution and the diversification in this region.

Small passerine birds, especially those restricted to the understory of *terra-firme* forests, such as several antbirds (Thamnophilidae), are good models for testing biogeographic hypotheses because they are more prone to present populations delimited by geographic barriers such as rivers and mountains (Capparella 1988; Fernandes 2007; Burney and Brumfield 2009). Hence this group of birds has been subject of historical biogeographic studies in Amazonia (Marks et al. 2002; Aleixo 2004; Cheviron et al. 2005; Fernandes 2007; Tobias et al. 2008). Molecular phylogenetic data available for antbirds reveals several incongruences regarding current subspecies classification (Fernandes 2007; Tobias et al. 2008; Fernandes et al. in press). Several gaps in the taxonomy of Thamnophilidae remain. Traditional phenotype-based subspecies taxonomy may not accurately reflect phylogenetic relationships among antbirds. Thus, improving

the molecular dataset of antbirds is critical to delimiting species and understanding the evolutionary mechanisms that have shaped their diversification.

The Amazonian endemic Spot-backed Antbird (*Hylophylax naevius*) (Aves, Thamnophilidae) is an adequate species for testing biogeographic hypotheses in the Neotropics. It occurs in the entire Amazon basin on opposite river banks of different sizes. Its purported sister species *Hylophylax naevioides* (Gómez et al. 2010) is restricted to the western Andes (Colombia, eastern Ecuador to Costa Rica, Panama, and Nicaragua) (Zimmer and Isler 2003). Both are typical understory birds found mainly in lowland t*erra-firme* forest. These ecological aspects of distribution and habitat allow to test the relevance of rivers and mountains for speciation in the Neotropical region.

Specimens of *H. naevius* from the Museu Paraense Emílio Goedi (MPEG) and the ornithological collection of the University of São Paulo (MZUSP) apparently represent a new morphological form (details of which to be communicated in a seminal paper). Populations of the new bird appear to be delimited by the Madeira River and its right bank tributary, the Jiparaná. Although the Jiparaná is a comparatively small river for Amazonia and it is not fully recognized to delimit the geographical distribution of Amazonian species (da Silva et al. 2005), it seems to be an important geographic barrier for vertebrates (van Roosmalen et al. 1998; Sardelli 2005; Cohn-Haft et al. 2007; Fernandes 2007; Isler et al. 2007). A comprehensive account on patterns of primate distribution in the Madeira-Tapajós interfluvium (van Roosmalen et al. 1998) and studies on the bioacoustics (Isler et al. 2007) and molecular phylogeny (Sardelli 2005; Fernandes 2007; Tobias et al. 2008) have indicated the importance of smaller tributaries of the Madeira as geographic barriers and even primary drivers of speciation (Fernandes et al. in press)

Here, using nuclear genes and mtDNA and large sampling covering the entire Amazon basin, the molecular phylogeography of the Spot-backed Antbird (*Hylophylax naevius*) has been investigated. This study aims to address the following questions:
1) Does the phylogeographical structure found for *H. naevius* have consequences on the current taxonomy of this Neotropical bird?
2) Do the speciation hypotheses available for explaining the Amazonian biodiversity predict the historical relationships found for *H. naevius*?
3) Are the *H. naevius* lineages of recent or ancient origin?

3.3.3 Materials and Methods

Taxon sampling

A total of 80 individuals from 45 localities covering all Amazonian areas of endemism and five subspecies of *Hylophylax naevius* were analyzed (Table 12 and Fig. 30). All tissues were either collected directly in the field or obtained from the following institutions: Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil (INPA), Museu Paraense Emílio Goeldi, Belém, Brazil (MPEG) and Universidade Federal de Mato Grosso, Cuiabá, Brazil (UFMT); tissue samples were subsequently vouchered together with research specimens (mostly skins). Sequences of *Hylophylax naevioides* and *Hylophylax punctulata* were used as outgroups, since the most complete phylogeny estimated so far for the Thamnophilidae recovered those two *Hylophylax* species as the sister clade to *H. naevius* (Brumfield et al. 2007; Gómez et al. 2010).

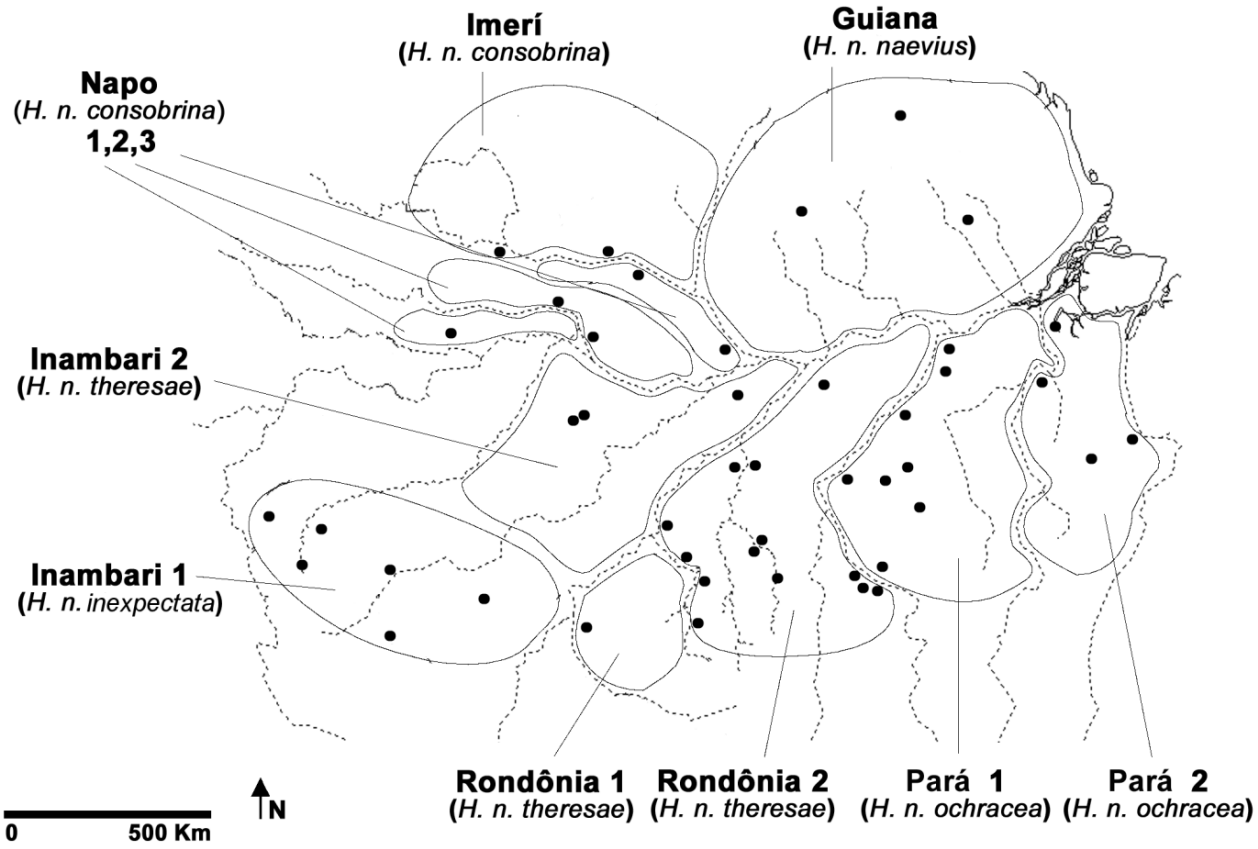


Figure 30 – Geographic distribution of samples (●) for Spot-backed Antbird (*Hylophylax naevius*) and Neotropical areas of avian endemism based on Cracraft (1985).

Table 12 – Collection locality, voucher number, institutions of origin for *H. naevius* samples used in this study.

Taxon	Locality	Voucher	Inst.	Cyt *b*	ND2	Fib.
H. n. consobrina	Brazil: AM, right bank Rio Negro, 3 km SW São Gabriel da Cachoeira. 0.13778°S, 67.0944°W	1134	INPA		X	
H. n. consobrina	Brazil: AM, right bank Rio Negro, 3 km SW São Gabriel da Cachoeira. 0.13778°S, 67.0944°W	1144	INPA	X	X	X
H. n. consobrina	Brazil: AM, RDS Amanã, Comunidade Nova Canaã.	217	INPA	X	X	X
H. n. consobrina	Brazil: AM, RDS Amanã; "volta grande"; ca. 100 km N Tefé. 2.48333°S, 64.63333°W	1301	INPA	X	X	X
H. n. consobrina	Brazil: AM, 110 km ENE Santa Isabel do Rio Negro. 0.06528°S, 64.0981°W	1613	INPA	X	X	X
H. n. consobrina	Brazil: AM, Novo Airão, Igarapé-Açu. 2.85361°S, 60.8514°W	59586	MPEG	X	X	
H. n. consobrina	Brazil: AM, Novo Airão, Igarapé-Açu. 2.85361°S, 60.8514°W	AMZ162	MPEG		X	
H. n. consobrina	Brazil: AM, Maraã, Lago Cumapi. 1.73333°S, 65.8793°W	62893	MPEG		X	
H. n. consobrina	Brazil: AM, Barcelos, left bank Rio Cuiuni. 0.78333°S, 63.2817°W	59584	MPEG	X	X	X
H. n. consobrina	Brazil: AM, Maraã, Lago Cumapi. 1.73333°S, 65.8793°W	62890	MPEG		X	
H. n. consobrina	Brazil: AM, Maraã, Lago Cumapi. 1.73333°S, 65.8793°W	62892	MPEG		X	
H. n. consobrina	Brazil: AM, Maraã, Lago Cumapi. 1.73333°S, 65.8793°W	62889	MPEG	X	X	
H. n. consobrina	Brazil: AM, ESEC Juami-Japurá; right bank Rio Japurá; ca 166 km W Japurá. 2.31667°S, 68.4167°W	675	INPA	X	X	X
H. n. naevius	Brazil: PA, Almeirim, REBIO Maicuru. 0.81667°N,53.91667°W	66222	MPEG		X	
H. n. naevius	Brazil: PA, Almeirim, REBIO Maicuru. 0.81667°N, 53.91667°W	66221	MPEG		X	

Taxon	Locality	Voucher	Inst.	Cyt *b*	ND2	Fib.
H. n. naevius	Brazil: Almeirim, REBIO Maicuru. 0.81667°N, 53.91667°W	66220	MPEG	X	X	X
H. n. naevius	Brazil: PA, Oriximiná, ESEC Grão Pará. 1.28333°N, 58.68333°W	65818	MPEG		X	
H. n. naevius	Brazil: PA, Oriximiná, ESEC Grão Pará. 1.28333°N, 58.68333°W	65817	MPEG		X	
H. n. naevius[1]	Suriname: Distrikt Sipaliwini	B55298	LSUMZ	X	X	X
H. n. naevius	Brazil: PA, Oriximiná, ESEC Grão Pará. 1.28333°N, 58.68333°W	65816	MPEG		X	
H. n. ochracea	Brazil: PA, Jacareacanga, FLONA do Crepori, Rio das Tropas, Cotovelo. 6.51895°S, 57.44456°W	65211	MPEG		X	
H. n. ochracea	Brazil: PA, Belterra, Flona do Tapajós, Br 163 km 083.	66132	MPEG	X	X	
H. n. ochracea	Brazil: PA, Serra dos Carajás. 5.77111°S, 50.55264°W	70631	MPEG		X	
H. n. ochracea	Brazil: PA, Marabá, Flona Tapirapé-Aquiri. 5.36667°S, 49.11667°W	67710	MPEG	X	X	X
H. n. ochracea	Brazil: PA, Itaituba, 7 km NW Moraes de Almeida. 6.20222°S, 55.68822°W	59186	MPEG		X	
H. n. ochracea	Brazil: PA, Itaituba, km 85 Transgarimpeira. 6.59503°S, 56.10151°W	69555	MPEG		X	
H. n. ochracea	Brazil: PA, Portel, FLONA do Caxiuanã, Plot PPBIO.1.95000°S, 51.60000°W	61818	MPEG		X	
H. n. ochracea	Brazil: PA, Portel, FLONA do Caxiuanã, Plot PPBIO. 1.95000°S, 51.60000°W	61815	MPEG		X	
H. n. ochracea	Brazil: Portel, FLONA do Caxiuanã, Plot PPBIO. 1.95000°S, 51.60000°W	61817	MPEG	X	X	X
H. n. ochracea	Brazil: PA, Senador José Porfírio, right bank Rio Xingu. 3.52986°S, 51.73650°W	55674	MPEG		X	
H. n. ochracea	Brazil: PA, Trairão, FLONA Trairão	69584	MPEG		X	
H. n. ochracea	Brazil:PA, Trairão, FLONA Trairão, Ramal do Areias.	69587	MPEG		X	
H. n. theresae	Brazil: AM, Maués, Flona do Pau Rosa, 3.91444°S, 58.29472°W	67064	MPEG		X	
H. n. theresae	Brazil: AM, Maués, Flona do Pau Rosa, Comunidade Sta. Teresa. 3.91444°S, 8.29472°W	67065	MPEG		X	
H. n. ochracea	Brazil: PA, Marabá, Flona Tapirapé-Aquiri. 5.36667°S, 49.11667°W	67711	MPEG		X	
H. n. ochracea	Brazil: PA, Belterra, Fazenda Treviso, Igarapé Moju. 3.47294°S, 54.56546°W	67630	MPEG		X	
H. n. ochracea	Brazil: PA, Novo Progresso, left bank Rio Jamanxim	66028	MPEG	X	X	X
H. n. theresae	Brazil: RO, Guajará-Mirim, Reserva Biológica Ouro Preto. 10.8333°S, 64.75°W	55024	MPEG		X	
H. n. theresae	Brazil: RO, Guajará-Mirim, Reserva Biológica Ouro Preto. 10.8333°S, 64.75°W	55019	MPEG	X	X	
H. n. theresae	Brazil: RO, Guajará-Mirim, Reserva Biológica Ouro Preto. 10.8333°S, 64.75°W	55021	MPEG	X	X	X
H. n. theresae	Brazil: AC, ESEC Rio Acre, ca. 78 km W Assis Brasil. 11.0514°S, 70.2714°W	59853	MPEG		X	
H. n. theresae	Brazil: AC, left bank Rio Purus, Santa Rosa, foz do Chandless. 9.11594°S, 69.8316°W	63362	MPEG		X	
H. n. theresae	Brazil: AM, left bank Rio Aripuanã, 130 km S de Novo Aripuanã. 6.3°S, 60.4°W	473	INPA	X	X	X
H. n. theresae	Brazil: RO, Reserva Biológica JARU, right bank Rio Jiparaná. 9.45°S, 61,6667°W	2421	INPA	X	X	
H. n. theresae	Brazil: AM, right bank Rio Aripuanã, Igarapé Extremo, 135 km S Novo Aripuanã. 6.3°S, 60,3333°W	512	INPA	X	X	X
H. n. theresae	Brazil: AM, right bank Rio Aripuanã, 135 km S Novo Aripuanã. 6.3°S, 60.3333°W	513	INPA		X	
H. n. theresae	Brazil: AM, left bank Rio Aripuanã, 127 km S Novo Aripuanã. 6.25°S, 60.45°W	541	INPA		X	

Taxon	Locality	Voucher	Inst.	Cyt *b*	ND2	Fib.
H. n. theresae	Brazil: AM, Humaitá, T. Indígena Parintintin, Aldeia Pupunha, 7.46667°S, 62.8167°W	58749	MPEG	X	X	
H. n. theresae	Brazil: MT, Paranaíta, Fazenda do Apiacás.	JB 439	UFMT	X	X	
H. n. theresae	Brazil: AC, Tarauacá, left bank Rio Liberdade. 7.81667°S, 72.0269°W	60688	MPEG	X	X	X
H. n. theresae	Brazil: AM, Coari, Rio Urucu. 4.93472°S, 65.2914°W	62334	MPEG	X	X	
H. n. theresae	Brazil: MT, Aripuanã.	JB652	UFMT		X	
H. n. theresae	Brazil: AC, ESEC Rio Acre. 11. 01483°S, 70. 21742°W	58900	MPEG		X	
H. n. theresae	Brazil: AC, Porto Acre, Reserva Humaitá. 9.75669°S, 67.6706°W	59998	MPEG	X	X	X
H. n. theresae	Brazil: AC, ESEC Rio Acre. 11.0514°S, 70.2164°W	58899	MPEG		X	
H. n. theresae	Brazil: ESEC Rio Acre, ca. 78 km W Assis Brasil. 11.0568°S, 70.2713°W	59854	MPEG		X	
H. n. theresae	Brazil: MT, Paranaíta, Fazenda do Apiacás.	JB528	UFMT		X	
H. n. theresae	Brazil: AM, Humaitá, T. Indígena Parintintin, Aldeia Pupunha, 7.46667°S, 62.8167°W	58750	MPEG		X	
H. n. theresae	Brazil: MT, Aripuanã.	JB658	UFMT		X	
H. n. theresae	Brazil: AM, RDS Aripuanã; left bank Rio Aripuanã; 8.42847°S, 59.7822°W	1280	INPA		X	
H. n. theresae	Brazil: AM, RDS Aripuanã; left bank Rio Aripuanã; 8.42847°S, 59.7822°W	1281	INPA		X	
H. n. theresae	Brazil: AM, RDS Aripuanã; left bank Rio Aripuanã; 8.42847°S, 59.7822°W	1289	INPA		X	
H. n. theresae	Brazil: AM, RDS Aripuanã, right bank Rio Aripuanã. 8.36667°S, 59.8417°W	1277	INPA	X	X	
H. n. theresae	Brazil: MT, Paranaíta, Rio Teles Pires, left bank. 9.47500°S, 56.47667°W.	69311	MPEG		X	
H. n. theresae	Brazil: MT, Paranaíta, right bank Rio Paranaíta, Fazenda Paranaíta. 9.58306°S, 56.71556°W	69314	MPEG		X	
H. n. theresae	Brazil: AM, Paranaíta, right bank Rio Teles Pires, Sete Quedas. 9.30436°S, 56.75864°W	67480	MPEG		X	
H. n. theresae	Brazil: AC, Parque Nacional Serra do Divisor, Tabocal. 8.74522°S, 72.83469°W	58366	MPEG		X	
H. n. theresae	Brazil: AM, Careiro, Br 319 km 158, Tupana Lodge. 4,08333°S, 60.66050°W	68901	MPEG	X	X	
H. n. theresae	Brazil: RO, Ji-Paraná, Igarapé Lurdes, Aldeia Gaviões. 10.43333°S, 61.65000°W	58214	MPEG		X	
H. n. theresae	Brazil: RO, Ji-Paraná, Igarapé Lurdes, Aldeia Gaviões. 10.43333°S, 61.65000°W	58213	MPEG		X	
H. n. theresae	Brazil: AM, Tefé, Base Petrobras/Urucu, Papagaio. 4.85000°S, 65.06667°W	57121	MPEG		X	
H. n. theresae	Brazil: AM, Tefé, Base Petrobras/Urucu, Papagaio. 4.85000°S, 65.06667°W	57120	MPEG		X	
H. n. theresae	Brazil: AM, Tefé, Base Petrobras/Urucu, Igarapé Lontra. 4.86667°S, 65.11667°W	57118	MPEG	X	X	
H. n. theresae	Brazil: AM, Tefé, Base Petrobras/Urucu, Igarapé Lontra. 4.86667°S, 65.11667°W	57119	MPEG		X	
H. n. theresae	Brazil: AC, Mâncio Lima, right bank Alto Rio Moa. Igarapé Ramon. 7.45000°S,73.76667°W	52771	MPEG		X	
H. n. theresae	Brazil: RO, Machadinho D'Oeste, right bank Rio Jiparaná. 8.90914°S, 62.00001°W	71108	MPEG		X	

Institution acronyms:
INPA – Instituto Nacional de Pesquisas da Amazônia;
MPEG – Museu Paraense Emílio Goeldi;
LSUMZ – Louisiana State University Museum of Natural Science;
UFMT – Universidade Federal de Mato Grosso. [1] Sequence downloaded from GenBank.

DNA extraction, amplification, and sequencing

DNA was extracted from breast muscle (0.2 g, approximately) using a standard phenol/chloroform protocol (Sambrook et al. 1989). The mitochondrial NADH dehydrogenase subunit 2 (ND2, 1023 bp) gene for 75 individuals and cytochrome *b* gene (cyt *b*, 1015 bp) for 26 individuals were sequenced using the published mtDNA primers (*i*) ND2: Brumfield et al. (2007) and Fernandes et al. (in press) and (*ii*) cyt *b*: (Sorenson et al. 1999) and (Dietzen et al. 2003). In order to confirm the structure found in the mitochondrial genes, the nuclear intron 5 of the gene for β-fibrinogen (BF5, 539 bp) was sequenced for a subset of 15 individuals of *Hylophylax naevius* using published primers (Brumfield et al. 2007). The sequence of one individual collected in Suriname (Appendix A) and four individuals used as outgroups were downloaded from GenBank.

PCR amplifications were performed with 50 μl reaction volumes containing 1 × PCR buffer (Bioron, Ludwigshafen, Germany), 100 μM dNTPs, 0.2 units of Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 200 ng of DNA, and 5 pmol of primers. Optimal annealing temperature was determined by gradient PCR in a Tgradient thermocycler (Biometra). Thermal cycling was performed under the following conditions: (1) an initial denaturing step at 94°C for 5 min; (2) 35 cycles: 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C; and (3) a final 5-min extension at 72°C. PCR products were precipitated with 4 M NH_4Ac and ethanol (1:1:6) and centrifuged for 15 min (13,000 rpm). Sequencing was carried out on an ABI 3730 automated capillary sequencer (Applied Biosystems) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 by STARSEQ GmbH (Mainz, Germany). To confirm observed mutations, both strands of each sample were sequenced.

Phylogenetic analyses

Phylogenetic analyses were performed using Bayesian inference (BI) implemented in MrBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) and maximum likelihood (ML) via RAxML GUI vs. 0.93 (Silvestro and Michalak 2011). The evolutionary models were selected with JMODELTEST using the BIC (Bayesian Information Criterion) (Posada 2008). Two independent runs of 5^6 generations each were conducted and trees were sampled every 500 generations. The first 500 samples were discarded as burn-in. The mtDNA genes (cyt *b* and ND2) were analyzed independently and concatenated in a single data matrix.

Using sequences of the three genes it was estimated a species trees using the method proposed by (Heled and Drummond (2010) in *BEAST 1.5.4 (Bayesian Evolutionary Analysis Sampling Trees). The haplotypes of the three genes were grouped in different trait sets, defined by the phylogroups found in the mtDNA analyses. The models were estimated in JMODELTEST (Posada 2008) was used for each partition *a priori*. The model parameters were estimated together with the tree topologies in the same analysis. In addition to the substitution model, the tree topologies were estimated independently for each gene except for the mtDNA genes. Due to a lack of recombination among mitochondrial genes in many organisms including birds, mtDNA genes should be considered as linked in this kind of analyses (Heled and Drummond 2010).

In the combined dataset, a mixed model Bayesian analysis was employed using the GTR + G model for ND2 and the HKY + G model for cyt *b* and BF5. The Bayesian haplotype reconstruction for the nuclear loci was performed using PHASE 2.1 (Stephens et al. 2001).

To visualize genealogical relationships among individuals, haplotype networks were constructed using ND2 sequences with maximum parsimony in the program TCS 1.18 (Clement et al. 2000). Mean pairwise *p*-distances (Nei 1987) within and among lineages were calculated using MEGA 4.0 (Tamura et al. 2007).

Molecular dating

Estimates of divergence times of main lineages of *H. naevius* were conducted using two data partitions (cyt *b* and ND2), each with individual models of molecular evolution chosen by JMODELTEST 0.1.1 (Posada 2008). The widely used cyt *b* mutational rate of 2.1% sequence divergence per million years (0.0105 substitutions/site/lineage/million years) was applied (Weir and Schluter 2008; Weir et al. 2009). Two runs were performed setting the clock model as relaxed, uncorrelated lognormal, followed by two independent simultaneous runs of 10^6 generations, sampling one of every 1,000 trees in BEAST v1.6.1 (Drummond and Rambaut 2007). Posterior probabilities of the nodes were computed for all Bayesian analyses across the sampled trees after burn-in. The number of generations required to reach stationarity of the posterior distribution was determined by examining marginal probabilities plotted as a time series in TRACER 1.5 (Rambaut and Drummond 2007).

Population genetics and historical demography

For population genetic analyses, groups were defined according to the major clades of haplotypes recovered in the phylogenetic analyses. Calculations of the following population parameters were based only on ND2 sequences: nucleotide diversity (π), haplotype diversity (h), Tajima's D, and Fu's F_s statistic were calculated in ARLEQUIN 3.1 (Excoffier et al. 2005). Significance was determined based on 1,000 coalescent simulations. To confirm and expand on the results obtained from the phylogenies, *a posteriori* analysis of population genetic structure was performed using AMOVA (Excoffier et al. 1992). The defined groups were also the major clades recovered in the phylogenies.

The historical population size dynamics was reconstructed using the GMRF Skyride Plot (Minin et al. 2008), implemented in BEAST v1.6.1 (Drummond and Rambaut 2007). The GMRF Skyride Plot is a nonparametric analysis that uses the waiting time between coalescent events in a gene tree to estimate changes in effective population size over time. It differs from the related Bayesian Skyline Plot (Drummond et al. 2005) by not requiring the specification of a user-defined prior on the number of population size changes in the history of the sample (Ho and Shapiro 2011). The defined groups were the main clades recovered in the phylogenies.
The GMRF Skyride Plots were constructed using both mtDNA genes using the ND2 mutational rate of 2.5% sequence divergence per million years (0.0125 substitutions/site/lineage/million years) (Smith and Klicka 2010), time-aware smoothing, and strict molecular clock priors.
All other parameters were identical to the molecular dating described above.

3.3.4 Results

Phylogenetic analyses

The Bayesian inference and maximum likelihood from ND2, cyt *b*, and the concatenated mtDNA phylogeny provided the same tree topologies. Phylogeographic analyses recovered 11 main clades partially associated with the Amazonian areas of endemism. More restricted structures were found in the Rondônia area of endemism (2 clades apparently bounded by the Jiparaná river), Imeri, and Inambari (clades not related to rivers) (Figs. 30–34).

The current subspecies taxonomy does not entirely reflect the molecular phylogeny. The subspecies *H. n. theresae* is not monophyletic and it was subdivided into four phylogroups. *Hylophylax n. ochraecea* from Pará was subdivided into two and *H. n. consobrina* into four subgroups. One of the most striking results was that *Hylophylax naevioides*, its purported sister species (Gómez et al. 2010), clusters together with *Hylophylax naevius* showing that *H. naevius* is not monophyletic and may be split into different species (Figs. 30 and 31). These results were supported with high posterior probability and bootstrap values in the concatenation of the ND2 and cyt *b* genes (Fig. 33). The mtDNA structure was corrobared by the nuclear gene; BF5 recovered a similar topology. Phylogenetic analyses of BF5 recovered the northern and southern clades found in the mtDNA analysis with high support, but with no monophyly in southwestern Amazonia (Inambari region) (Fig. 33).The mean pairwise genetic *p*-distance between major clades ranged from 0.6–7.1% while the mean genetic *p*-distance measured within major clades was found to be 0.0–0.3% (Fig. 31).

The species tree estimated with *BEAST shows the same topology as those retrieved from the mtDNA gene analysis and the concatenation of all three genes (Fig. 6 and Table 12). As in the mtDNA dating analysis, the multilocus approach yields two main clades separated by the Amazon River. A sister relationship between the southern Amazonian clade and *H. naevioides* was determined (Fig. 35).

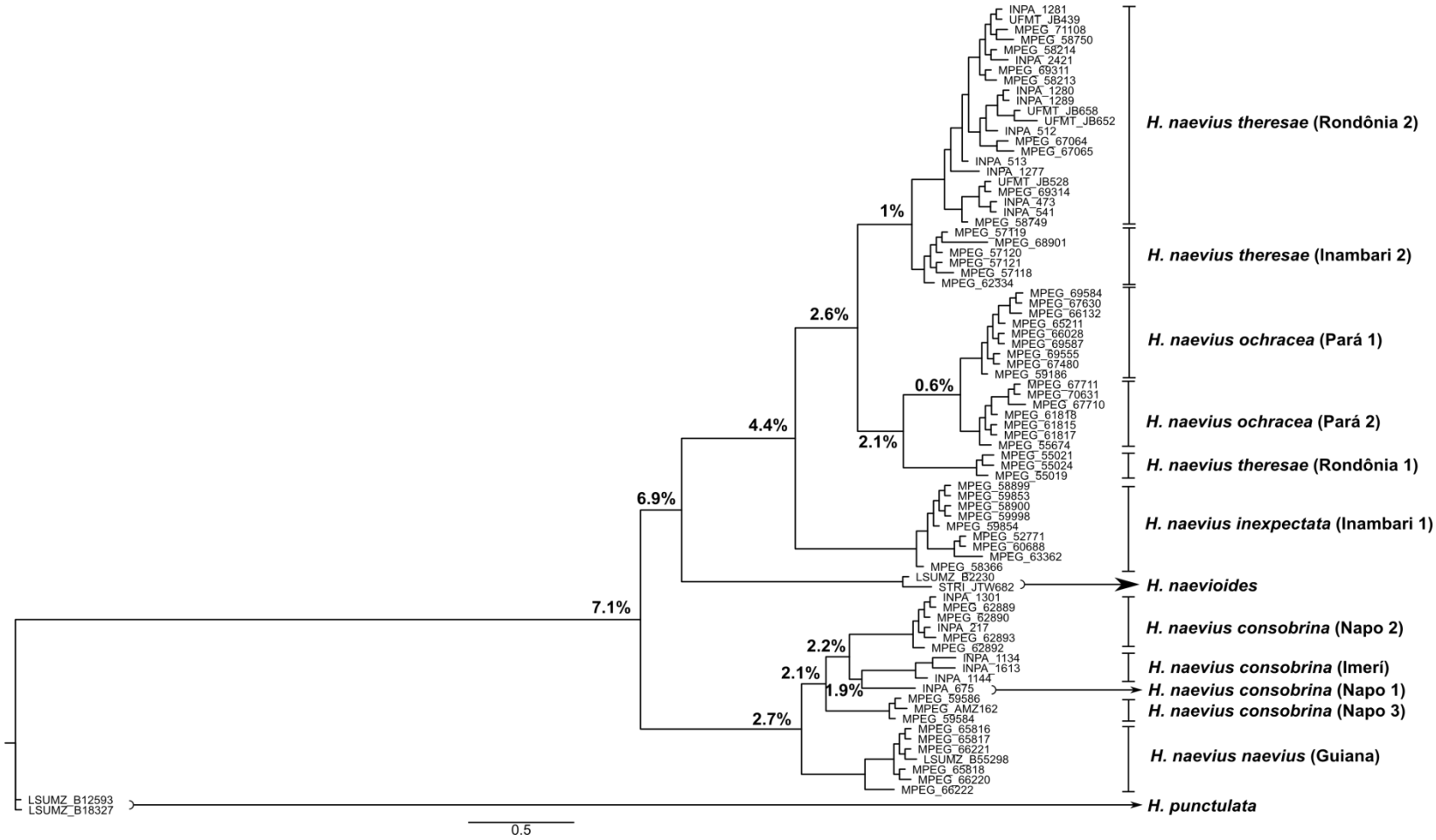


Figure 31 – Bayesian phylogeny based on ND2 sequences showing the relationships among different populations of *Hylophylax naevius* distributed throughout the Amazon region. Numbers next to nodes refer to uncorrected genetic *p*-distances (%)

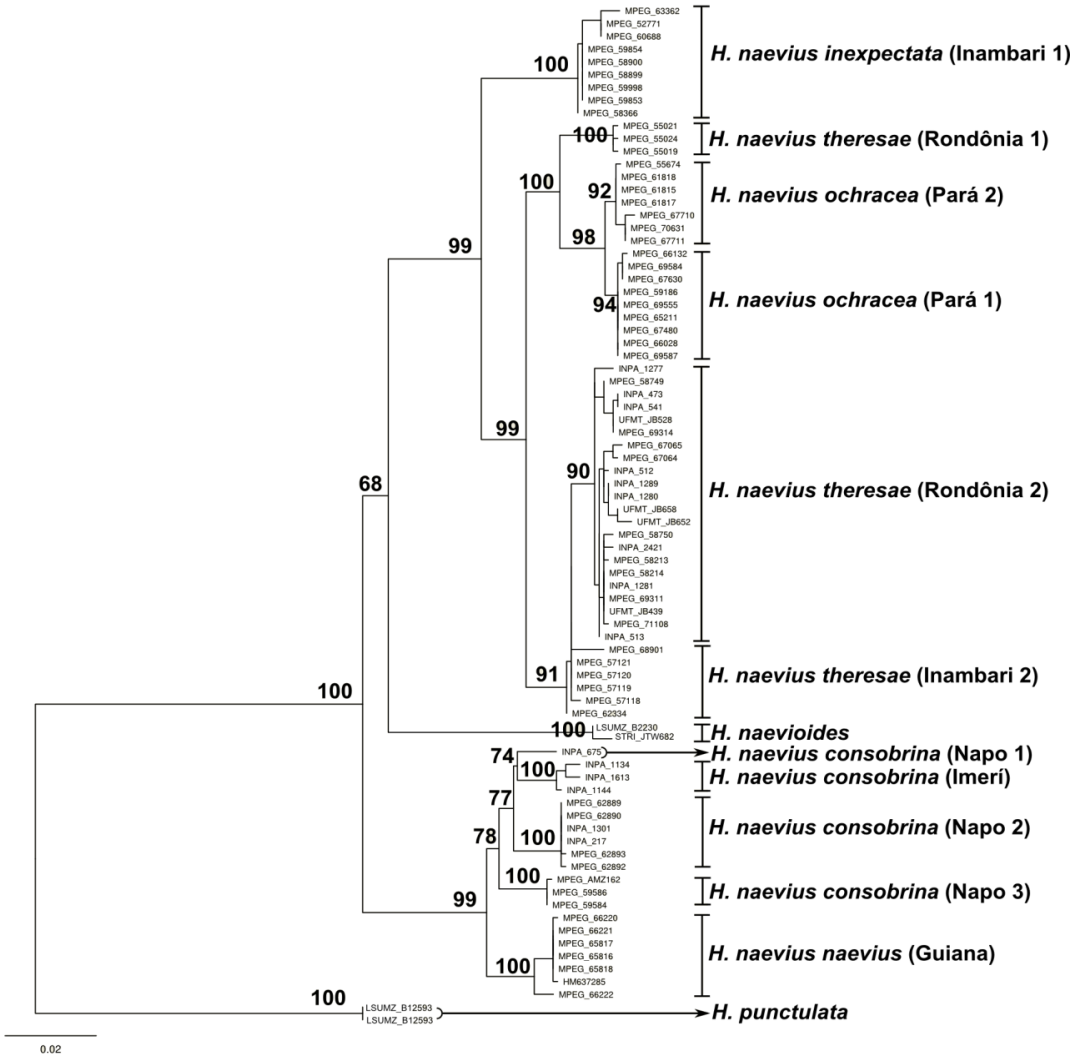


Figure 32 – Maximum likelihood (–ln L = 18.268264) phylogeny based on ND2 sequences showing the relationships among different populations of *Hylophylax naevius* distributed throughout the Amazon region. Numbers next to nodes refer to bootstrap values

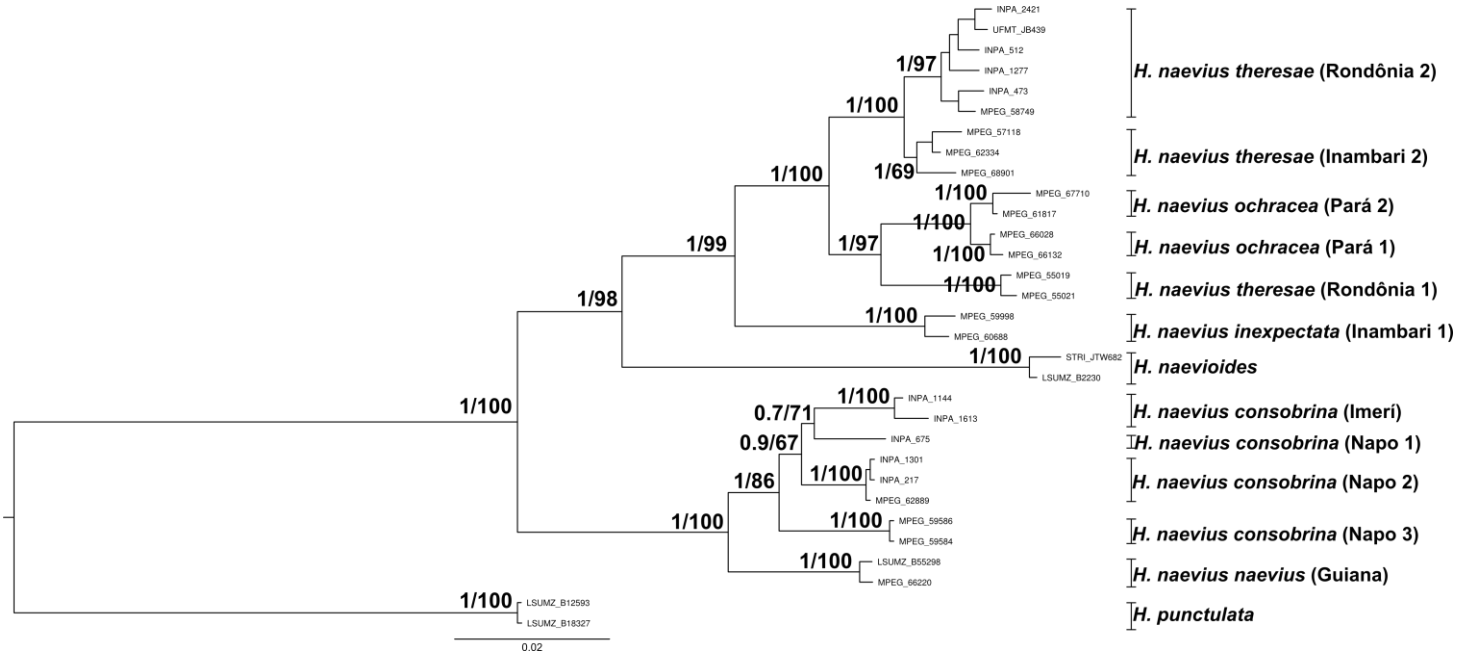


Figure 33 – Bayesian phylogeny based on ND2 and cyt *b* genes. The areas of avian endemism are indicated for each specimen. Numbers correspond to posterior probability values and the bootstrap values (based on 1000 replicates) from maximum likelihood (–ln L = 6127.155924) analysis

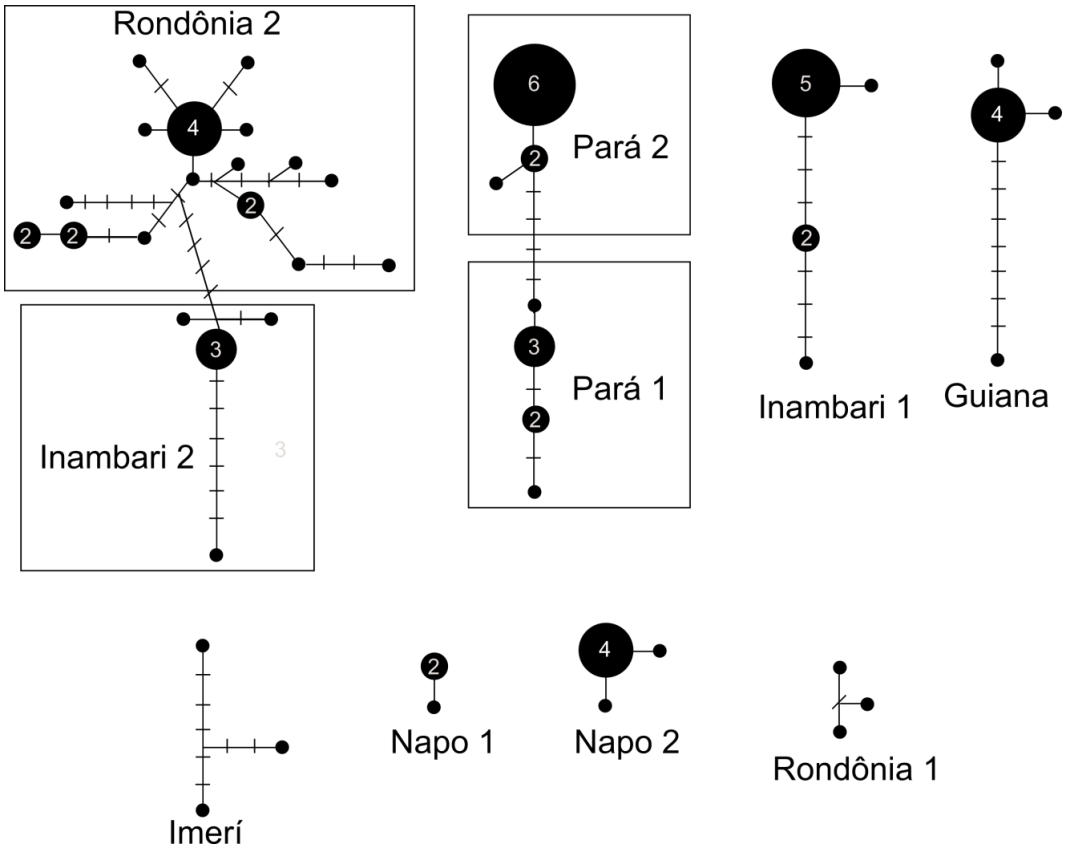


Figure 34 – Haplotype networks estimated for different clades within the ND2 phylogeny for *Hylophylax naevius* (see also Figs. 2 and 3). *Black circles* represent different haplotypes and their size is approximately proportional to haplotype frequency. *Numbers inside the black circles* refer to the number of sampled individuals possessing that particular haplotype. The mutational steps are indicated by short *solid lines* on each branch connecting haplotypes.

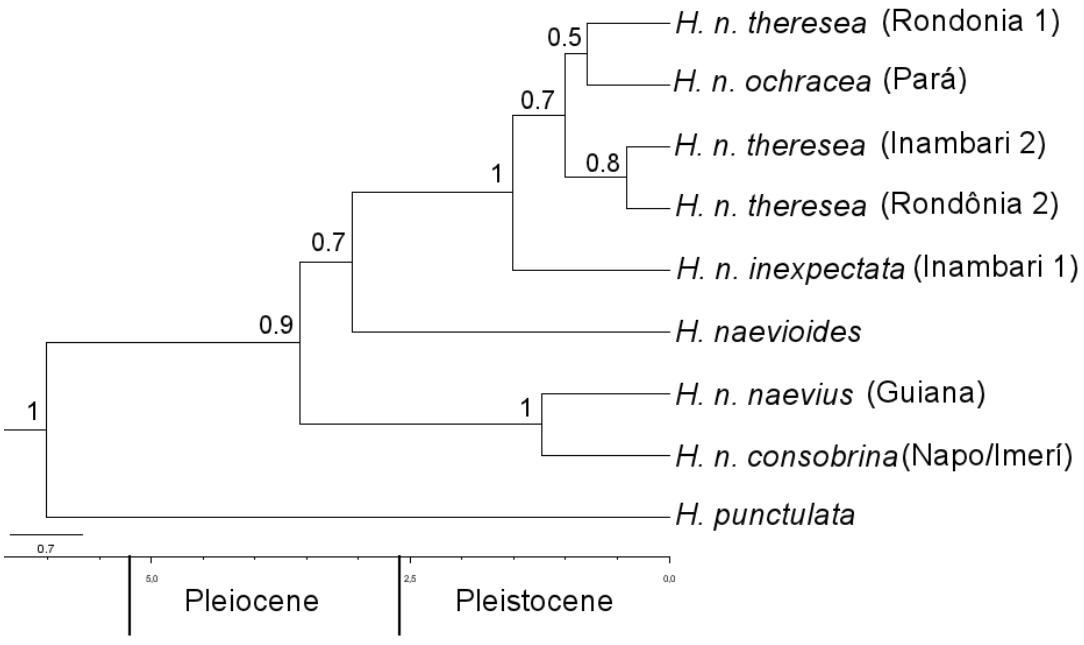


Figure 35 – Species tree based on ND2, cyt *b* and FB5 genes for *Hylophylax naevius* indicating the relationship among areas of avian endemism. *Numbers next to nodes* refer to posterior probability values.

Molecular dating

According to the relaxed-clock model the earliest split in *H. naevius*/*naevioides* (*H. n. consobrina*/*H. n. naevius* and *H. naevioides*/*H. theresae*/*H. ochracea*) took place in the Pliocene (3.2–4.9 Mya; mean 4.1 Mya) (Fig. 36). Subsequent splits between *H. n.theresae*/*ochracea* and *H. naevioides* occurred in the late Pliocene (2.6–4.1 Mya; Fig. 35). The latest diversification involved the subspecies from the north of the Amazon River (*H. n. consobrina* and *H. n. naevius*) and the subspecies from the Brazilian Shield (*H. theresae* and *H. ochracea*) during the Pleistocene (1.1–1.9 Mya; Fig. 36).

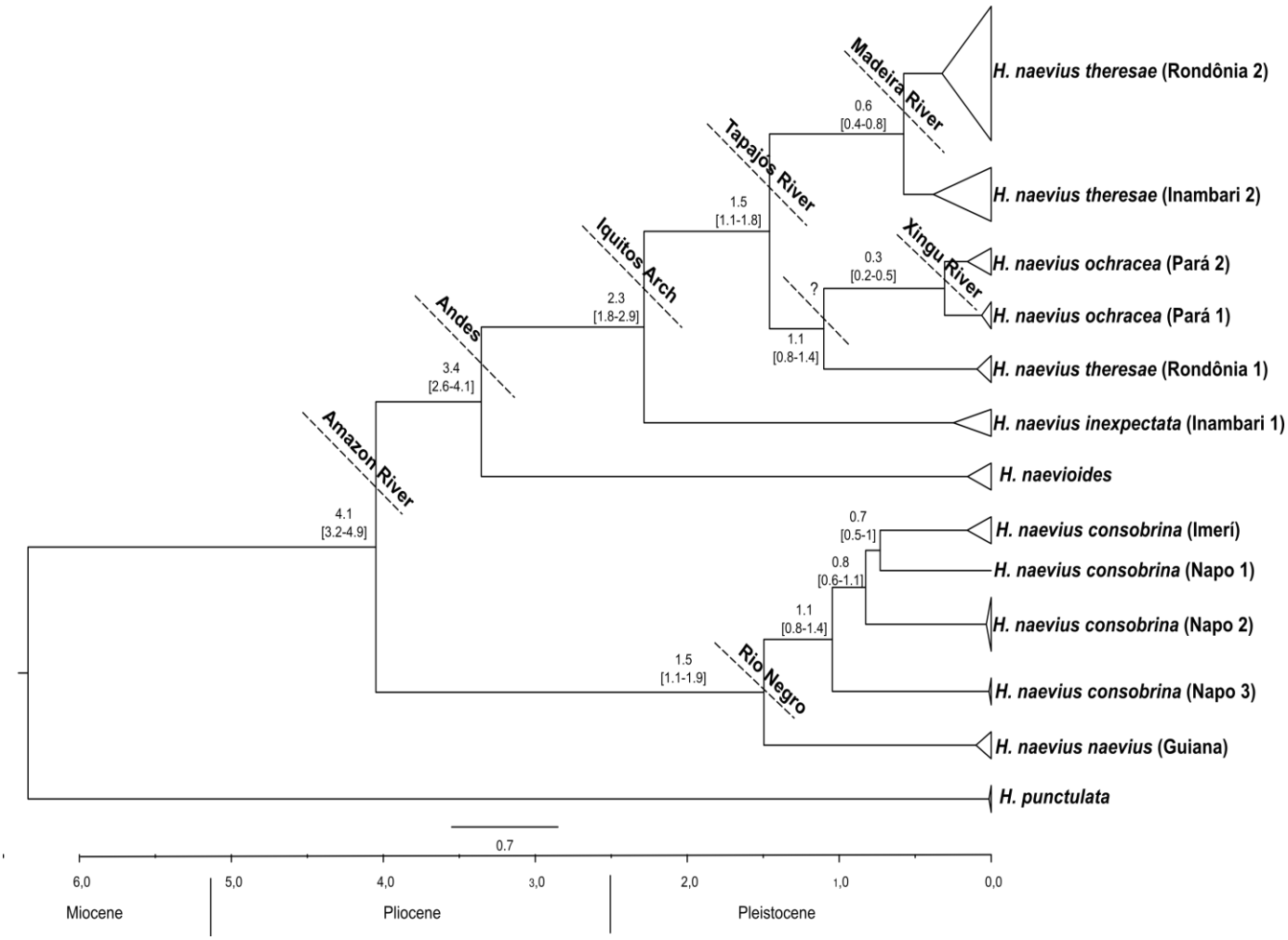


Figure 36 – Bayesian divergence times (in million years ago, Mya) estimated with a relaxed-clock model based on ND2 and cyt *b* sequences (*n* = 31). *Numbers* within *brackets* indicate 95% posterior age intervals. *Dashed lines* indicate the main geographic barriers.

Population genetics and historical demography

The analyses conducted to detect demographic change histories suggest population fluctuations for all phylogroups. Values of Tajima's *D* statistic were significantly negative for Inambari 2 and Guiana and Fu's F_s statistic were significantly negative for all lineages. Maximum values o f genetic diversity (*h*) and low nucleotide diversity (π) were found, which is expected for populations under demographic changes (Table 1). However, in the most part of the Amazonian basin *H. naevius* appears to have maintained a relatively stable size during the last 0.2 million years except in the Rondônia 2 that shows a signal of population expansion during the last 0.15 million years (Fig. 37).

Table 13 – Diversity and demographic population parameters

Region	*N*	*h*	*Π*	*D*	F_s
Inambari 1	9	1.0	0.0024	0.46802	–7.86778*
Inambari 2	6	1.0	0.0033	–1.43477*	–3.03187*
Para 1	9	1.0	0.0009	–0.06382	–14.87480*
Para 2	7	1.0	0.0020	–0.09908	–5.74939*
Rondônia 2	22	1.0	0.0053	–1.23945	–21.70056*
Napo/Imeri	13	1.0	0.0161	0.22804	–3.87477*
Guiana	7	1.0	0.0028	–1.60974*	–4.55660*

* Significant values ($P < 0.05$). *N* = number of individuals sampled; *h* = gene diversity; *Π* = nucleotide diversity; *D* = Tajima's *D* statistic; *F* = Fu's F_s statistic.

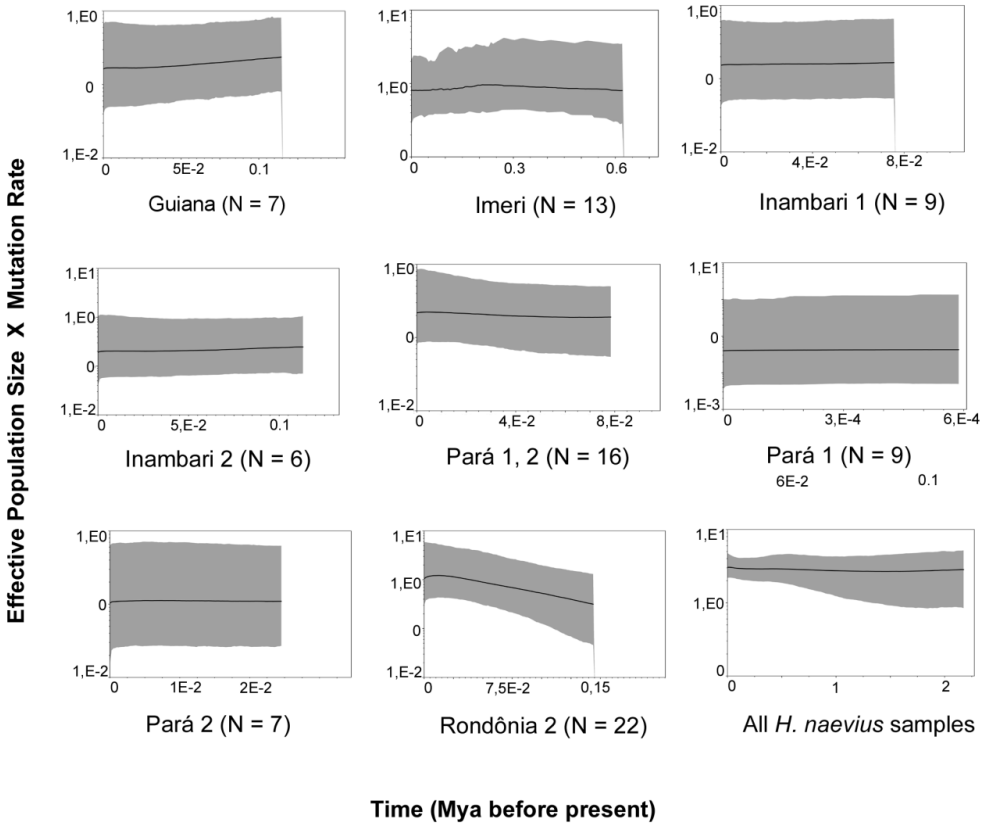


Figure 37 – Demographic history of *H. naevius* lineages determined by Bayesian Skyride Plots based on ND2 sequences. The *black line* represents median values, the *blue area* denotes 95% Bayesian credible intervals.

3.3.5 Discussion

Historical biogeography

The high degree of phylogeographic structure and splits dating from different periods suggest that distinct vicariant historical processes may have played a role in the speciation of *H. naevius*/*naevioides*. The phylogeographic pattern showing the earliest split occurring between the north and south Amazon during the Pliocene is evidence that populations of *H. naevius* may have become isolated by a proto-Amazon River. The temporal and spatial phylogenetic results presented here support this hypothesis. The phylogeographic patterns indicate sister lineages across the Amazon River with splits dating from the Pliocene, the epoch in which the Amazon is considered to have originated (Hoorn et al. 2010).

The second split involving the differentiation of *H. naevioides* and the remaining southern *H. naevius* subspecies is clearly associated with the Andean uplift since these two clades are sisters and allopatrically distributed on both sides of the Andes. The subsequent phylogeographic split is spatially associated with the formation of ancient ridges of western Amazonia. The uplift of the Andes during the late Miocene (Latrubesse et al. 2010) led to the subsequent uplift of ridges through the Amazon Basin, such as the Iquitos arch, which could have separated several Amazonian taxa (Lougheed et al. 1999; Patton et al. 2000). The concordance of the phylogeographic split within *Hylophylax naevius* from the upper Purús/Juruá (Inambari 1 region) and lineages from the lower Purús/Juruá rivers of western Amazonia during the late Pliocene with the localization of this ancient paleogeographic barrier suggests an association with this geological event. However, the ridges seems to be older (late Miocene in age) than the estimated time point of this split. So this hypothesis should be considered with caution.

Clearly, rivers delimit the distribution of lineages of the *H. naevius* species. Very conspicuous is the effect of the upper Tapajós River, mid Rio Negro, and the Amazon River where collection points were directly on opposite sides. At least the rivers from the Brazilian Shield seem associated with primary diversification after the establishment of the Fitzcarrald Arch which caused a drainage reorganization of the Amazon basin between the late Pliocene (Espurt et al. 2010; Latrubesse et al. 2010; Mora et al. 2010) and early Pleistocene (Espurt et al. 2010; see also Campbell et al. 2006). These periods correspond to the here presented temporal estimates of phylogeographic splits in this region (Fig. 36). The Xingú River may have had some effect on evolutionary differentiation, but a more complete sampling is still needed to confirm this assumption.

The pattern found in the Japurá/Rio Negro interfluvium is particularly striking. In northern Amazonia, between the Japurá and Rio Negro (Napo region), fine-scale phylogeographical structure is found despite the absence of present-day rivers. Evidence of a dramatic change in the course of the Rio Negro governed by neotectonics was shown by Almeida-Filho and Miranda (2007). They suggest that the lower course of the Rio Negro has suffered frequent changes in landscape throughout the Neogene-Quaternary. As a result of a regional tilting, the southward flow of the ancient course of the lower Rio Negro was redirected southeastward. This geological factor may have played a decisive role in the evolution of the current biodiversity. The data of *H. naevius* is in accordance with this paleogeographic reconstruction, indicating deep divergence among lineages across the locations of the former course of the Rio Negro. A second nonexclusive explanation is that the extensive areas of open vegetation (campinas and campinaranas) that predominate on the mid-western Rio Negro Basin may function as barriers

to dispersal and thereby reproductively have isolated populations of forest birds as has been suggested for other species (Naka 2011; Ribas et al. 2012). Large areas of open vegetation on white-sand soil (campinas and campinaranas) probably formed over ancient paleochannels and today may function as a secondary barrier. A secondary river barrier effect could be interpreted also for the Japurá River. This river does not separate sister clades on its opposite banks, and thus does not appear to be directly involved with primary population differentiation in these birds although it apparently delimits distinct bird assemblages as documented by da Silva et al. (2005).

Divergence of some *H. naevius* lineages estimated to have occurred in the Pleistocene corroborates the argument that speciation in the Neotropical region has also taken place in recent periods as discussed by Tobias et al. (2008). Another example of recent speciation in the Amazon is demonstrated by *Myrmeciza hemimelaena*, an Amazonian taxon, now shown to comprise three biological species with splits dating from the Pleistocene (see Sect. 3.2; Fernandes et al. in press). Although the refuge hypothesis is difficult to test since the location and the existence of the forest refuges are uncertain, one of its predictions is testable. According to this hypothesis, populations are expected to have expanded and contracted during the Pleistocene (Zink 1997; Hewitt 1999; Aleixo 2004; Cheviron et al. 2005). The results indicate negative values in the neutrality tests suggesting population fluctuations for all lineages. High values of haplotype diversity and low values of nucleotide diversity which characterize a population undergoing sudden demographic expansion after recent population bottleneck were also found. Recent population expansion was indicated by the Bayesian Skyride analysis (GMRF) in the Rondônia 2 region suggesting influence of the last glacial maximum (LGM) in the population dynamics from this area and maybe in the splits between the Rondônia 2 and Inambari 2 regions (within *H. n. theresae*). The molecular dating indicates that the phylogeographic split between these two lineages occurred approx. 0.6 Mya which corresponds to the LGM and not to the origin of the Madeira River in the late Pliocene–early Pleistocene. Thus, although LGM environmental changes do not explain entirely the differentiation of *H. naevius* subspecies – because all subspecies of *H. naevius* existed prior to 1.1 Mya when the temperature was warmer – the influence of the LGM as a generator of evolutionary diversity can not be eliminated. Absence of signs of population fluctuation in the Bayesian Skyride analysis for the other regions may not be used as an argument against the influence of climatic cycles in the population demography of *H. naevius*. Low nucleotide diversity with high haplotype diversity was determined for all lineages, which is an indication for recent population expansion. Thus, the absence of signs of population changes in the Bayesian analysis may be related to the low number of gene samplings. The GMRF is sensible to the number of loci and the GMRF estimates were based on only one mtDNA gene.

The data presented here show that distinct events of vicariance and speciation influenced the evolution of *H. naevius*. It clearly reveals a distinct history among different regions which reinforces that speciation was generated by more than one single vicariant mechanism. The origin of Amazonian birds may have occurred in different periods caused by distinct historical events. The Amazon River, the Andes, and the Iquitos Arch are associated with older divergence, whereas the Rio Negro, Tapajós, and Xingú rivers are related with more recent speciation. The last glacial maximum may have been important in generating lineages in unique evolutionary trajectories (i.e., “evolutionary significant units (ESU)” *sensu* Moritz 1994) and the Madeira River seems to be associated with a secondary barrier effect.

Phylogeographic structure and subspecific taxonomy

H. naevius has consisted of six subspecies described on the basis of phenotypic differentiation (Zimmer and Isler 2003). Five subspecies from the Amazon Basin covering most of the distribution of *H. naevius* were sampled. Phylogenetic analyses revealed ten deeply divergent clades (1–7.1% genetic distance) with allopatric or parapatric distribution, allowing for the correlation between geographic distribution and population structure. One of these five subspecies, *H. n. theresae* (distributed from southeastern Colombia and eastern Ecuador/Peru to the left bank of the Tapajos River in the Brazilian Amazon, Fig. 30) is not monophyletic as indicated by the molecular data, so that the current subspecies taxonomy is considered not to accurately reflect the molecular phylogeny.

The paraphyly of *H. naevius* as determined by mtDNA and nuclear genes points to the necessity of a taxonomic revision (see also Tobias et al. 2008). One approach could be to recognize three different species. The estimated coalescence of the youngest sisters is at 3.4 million years ago (Mya) during the mid-Pliocene: one species north of the Amazon River including two subspecies (*H. n. naevius* and *H. n. consobrina*), the second south of the Amazon River also comprising two subspecies (*H. n. theresae* and *H. n. ochracea*), and the third being *H. naevioides*.

On the other hand, the *H. naevius* subspecies are clearly ancient, as diagnosed by phenotype (Zimmer and Isler 2003) and several molecular synapomorphies, and thus likely represent biological species. This would imply that the subspecies of ***H. naevius*** **should be raised to full species level.** Due to the paraphyly of *H. n. theresae* a separate description including more detailed morphological or bioacoustical information must be performed in order to denominate a new taxon: a **new species from Rondônia 1** (Fig. 30).

Conservation implications

High levels of genetic divergence in relative absence of morphological differentiation seems to be a common and prevalent Amazonian pattern. Particularly for the uniqueness of this phenomenon it seems to represent an unprecedented diversification process and therefore must be preserved and better understood. If cryptic endemism is very prevalent in Amazonia, then it affects a huge proportion of global biodiversity. Thus future studies should focus on detected cryptic species expecially in the Madeira-Tapajós interfluvium that has been shown to be a region with great potential for the discovery of new species.

The geographic barrier of the Jiparaná and Aripuanã rivers for primates (van Roosmalen et al. 1998) and birds (Sardelli 2005; Cohn-Haft et al. 2007; Fernandes 2007; Isler et al. 2007) and the presence of genetically differentiated small populations in the Madeira-Tapajós interfluvium is increasingly evident. This study shows one more case of a so far undescribed new taxon with a distribution restricted to a small region delimited by the Jiparaná River. The data confirms the existence of areas of endemism smaller than that established by Cracraft (1985) indicating that new phylogeographical provinces may need to be delineated.These new endemic areas are situated in one of the most threatened regions of Amazonia. Thus the geographic barrier of the Jiparaná River should be recognized and this region must have priority in conservation programs.

4 Conclusions and General Discussion

Based on a combination of phylogeographic tools, molecular dating, and population genetic methods in this study was reconstructed the spatio-temporal scenario of diversification of different passerine bird species. Using deep sampling of three Amazonian understory birds it was possible to reveal unusual phylogeographic patterns. The results presented here highlight two major issues: unforeseen role of rivers of different sizes on ancient substrates and cryptic diversity in Amazonia. The comparison of the patterns described for these three birds allows the deduction of some general aspects about the evolution of birds in lowland Amazonia.

4.1 Area Cladograms and Historical Biogeography

Detection of congruent phylogeographic patterns among codistributed species can provide insight into the processes that may have shaped the distribution of regional biota (Avise 2000). By substituting the taxa on the molecular phylogenetic trees with their regions of geographic distribution (area cladograms), it is possible to hypothesize the historical relationship among geographic subregions (Fig. 38). Co-distributed taxa that indicate concordant area cladograms support a common evolutionary history.

The data presented on three widespread Amazonian birds in this study (Chap. 3) shows relative concordance in area cladograms, supporting a single shared evolutionary history. The three bird species show a strong hierarchical structure over the whole sampled geographical area. The topologies of the phylogenetic trees were quite similar in time and space. *M. hemimelaena, H. naevius*, and *G. spirurus* show a basal split between northern and southern Amazon River dating from the Pliocene (approx. 4 million years ago) (Fig. 38). In southern Amazonia, although there are some particularities for each one of the three species, the tree topologies show the next split corresponding to the Madeira River which separates the Brazilian Shield from the rest of Amazonia. In northern Amazonia – with the exception of *M. hemimelaena*, which does not occur in this region – the bird lineages are separated by the lower Rio Negro, although the sequence of divisions differ among *G. spirurus* and *H. naevius*. This difference may be attributed to the low nodal support and unresolved branch in the phylogenetic relationships among *G. spirurus* lineages from the Guiana, Napo, and Imeri areas.

The three species in general show congruent patterns of geographic history within the Brazilian Shield (Rondônia, Pará, and Belém regions). The Madeira River divides the lineages of the Inambari (western Amazonia) from those of the Brazilian Shield (eastern Amazonia).
East lineages subsequently split on opposite banks of the Tapajós River. Similar area cladograms were described for the *Hemitriccus minor* species complex (Tyrannidae) (Cohn-Haft 2000; Sardelli 2005) and *Hypocnemis cantator* species complex (Tobias et al. 2008).

Replicated patterns of phylogenetically deep molecular divergence were found within some areas of endemism suggesting fine-scale phylogeographical structure. In the Rondônia region the phylogeny shows clades delimited by two right-bank tributaries of the Madeira River, the Aripuanã and Jiparaná, for the three birds (Fig. 38). These results together with previous studies (Willis 1969; Sardelli 2005; Fernandes 2007; Isler et al. 2007; Tobias et al. 2008, see also Introduction) indicate that new phylogeographical provinces may need to be delineated. Bird populations delimited by the Aripuanã and Jiparaná rivers seem to be a common pattern and not an exception. This revised view of endemism should be incorporated into conservation and management planning of the Madeira-Tapajós interfluvium (Rondônia region), previously treated as a single, uniform area of endemism (Figs. 1 and 3) (Cracraft 1985; Dinerstein et al. 1995).

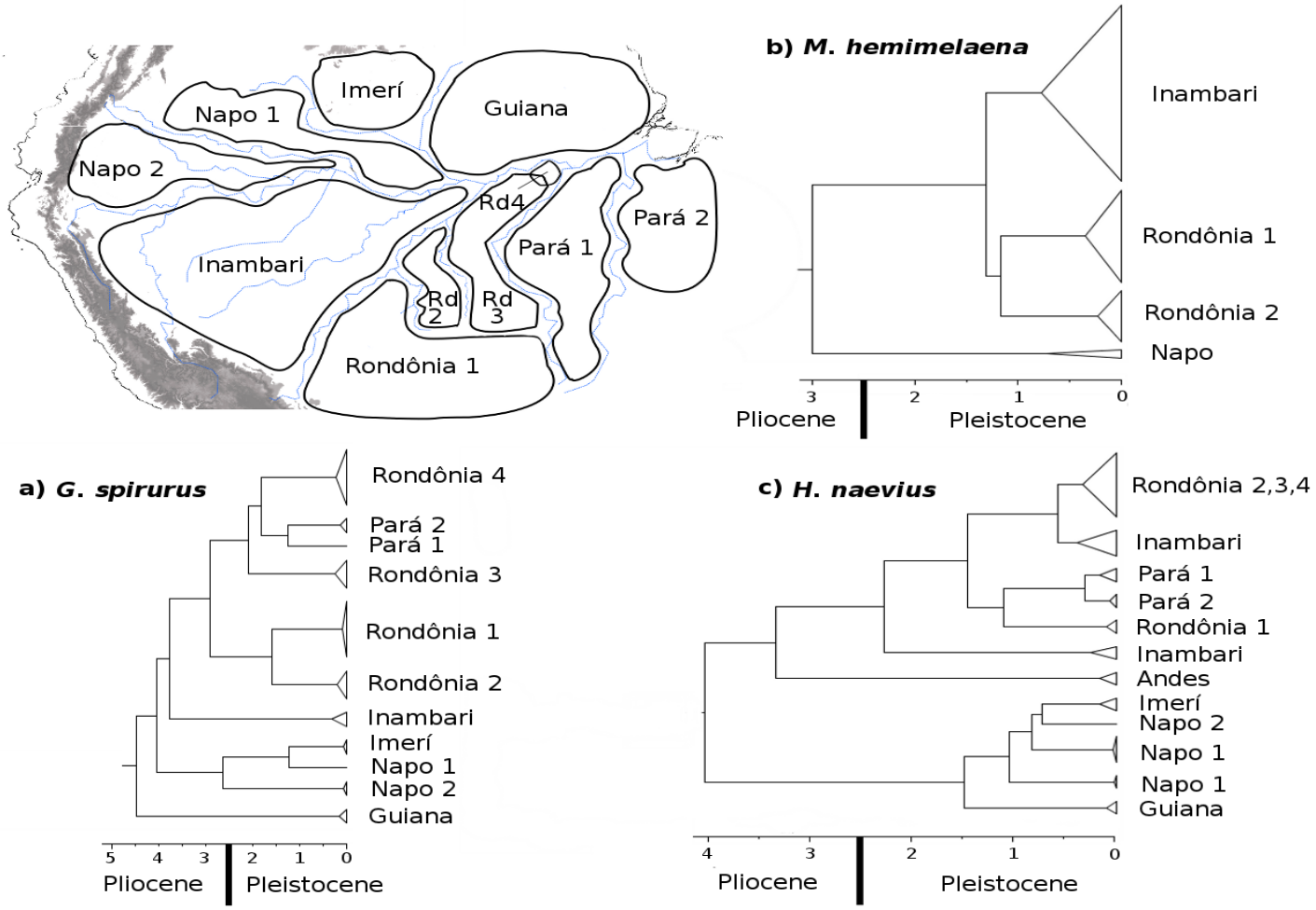


Figure 38 – Area cladograms based on phylogenetic and molecular dating analysis of this study. Map depicts major divisions of Amazonia following Cracraft (1985, see also Fig. 1). Note the subdivisons of Rondônia region delimited by the Jiparaná and Aripuanã rivers. Rd2 = Rondônia 2, Rd3 = Rondônia 3, Rd4 = Rondônia 4

The complexity of the Rondônia region is also reflected in the historical events of differentiation. Each of the three species here studied (*G. spirurus*, *H. naevius*, *M. hemimelaena)* and from previous studies (*H. minor* and *H. cantator*, two passerine birds which also show deep divergence among populations from this region) (Sardelli 2005) has a unique area cladogram between the Madeira-Tapajós interfluvium (Rondônia region) (Fig. 38). The two most similar patterns of geographic divergence within Rondônia are shared by *G. spirurus* and *H. minor*. In this case, both species show a basal split on opposite banks of the lower Jiparaná River, followed by the division of the current course of the Aripuanã River. However, the topology of *H. naevius* phylogeny shows a very different pattern of diversification between the Madeira and Tapajós rivers. The molecular phylogeny of *H. naevius* indicates that the Jiparaná River (a left-bank tributary of the Madeira River) has greater importance as a geographical barrier than the Madeira River itself. The division which corresponds to the course of the lower Jiparaná River has a more basal position in the phylogeny than the split across the mid-course of the Madeira. There are two explanations to this phenomenon (discussed also in Chap. 3). The documentation of two separate active large mega-fans allegedly of Late Pleistocene origin involving each the Jiparaná and the Aripuanã drainages (Latrubesse 2002), is indicative of much wider and complex paleo-drainages in this interfluvium than those found today, thus possibly explaining the complex diversification patterns in this region. The second somewhat related explanation was first addressed by Edwin

Willis in the 1960s (Willis 1969). Willis suggested that the complex pattern of geographic distribution of *Rhegmatorhina* spp. (an Ant-following Antbird) in the Madeira-Tapajós interfluvium could be attributed to changes in the course of the Tapajós River. Tectonically mediated mega-drainage capture was shown for the Rio Negro (Almeida-Filho and Miranda 2007). This may be applied to other regions in Amazonia. The Jiparaná or Aripuanã may once have captured a significant part of the Madeira or even the neighboring Tapajós drainages or both, but later became mere tributaries following the major continental-wide drainage reorganization of the Amazon basin during the Late Pliocene (see also Sects. 3.1 and 3.2).

In northern Amazonia, between the Japurá and Rio Negro (Napo region), fine-scale phylo-geographical structure was detected, but in absence of the current course of rivers (see Sects. 3.1 and 3.3). Evidence of a dramatic change in the course of the Rio Negro governed by neotectonics was show by Almeida-Filho and Miranda (2007). They suggest that the lower course of the Rio Negro has suffered frequent changes in landscape throughout the Neogene-Quaternary. As a result of a regional tilting, the southward flow of the ancient course of the lower Rio Negro was redirected southeastward. The data of *H. naevius* is in accordance with this paleogeographic reconstruction, indicating deep divergence among lineages across the locations of the former course of the Rio Negro. A second explanation is that the extensive areas of open vegetation (Campinas and Campinaranas) that predominate on the mid-western Rio Negro may function as barriers to dispersal and thereby reproductively isolated populations of forest birds as has been suggested for other species (Naka 2011; Ribas et al. 2012).

Phylogeographical divergence in absence of river barriers is also the pattern expected for southwestern Amazonia, in the Solimões-Madeira sedimentary basin. The excessively meandering rivers of this region formed by very high sediment influxes have induced frequent lateral channel migrations responsible for across-river transfer of large pieces of land. Studies of vertebrates (e.g., echimyid rodents and various frog species) show deep phylogeographical divisions that are unrelated to the presence of the river that delimit their ranges (Patton et al. 1994, 2000; Lougheed et al. 1999). These studies were focused on the Juruá River, a 1000-km-long, white-water tributary of the Amazon River (Fig. 1). For most echimyid rodents and frogs studied, two major clades, corresponding to the upper and lower portions of the river, were identified (Patton et al. 1994, 2000; Lougheed et al. 1999).These phylogeographical patterns were attributed to the presence of ancient mountain ridges that existed previous to the formation of the current lower Amazon River system. In the late Miocene, the Amazon Basin was split into two sub-basins separated by the Purus Arch. Once the Miocene Andean foreland basin was overfilled and the Purus Arch was overtopped, the modern Amazon River system was established during or after the late-Miocene (Hoorn et al. 2010). A second explanation for the absence of sister lineages across rivers in western Amazonia is commonly attributed to aquatic conditions with swamps, lakes, and internal deltas. However, the phylogeographic divisions found in western Amazonia for the three passerines here studied date from more recent periods (late Pliocene–early Pleistocene). Therefore, this seem not to be associated with mountains or wetland system present in the Miocene. The patterns from western Amazonia described in this study are more easily explained by the high sediment influx of the meandering rivers in this region.

Data presented in Chap. 3 show that populations of *G. spirurus*, *M. hemimelaena*, and *H. naevius* probably experienced periods of population bottlenecks and expansion. According to the refuge hypothesis, under a population genetics framework, population bottlenecks are expected during periods of forest contraction (after the onset of dry climatic conditions), whereas population

expansion is predicted during periods of forest expansion, following humid climatic conditions (Aleixo 2004). Expanding populations when encountered rivers are not not able to cross the widest stretches, but eventually the narrower parts. Once the populations that speciated in forest refuges come into secondary contact in the headwater regions they can not be established in the other river bank by competitive exclusion and not by the geographical barrier imposed by the river. Based on this model Haffer (1974) tried to explain patterns of geographic distribution of several taxa separated by the major Amazonian rivers, but that apparently seem to cross the river in the headwater regions. Another predictions of this model is that the phylogenetic divisions should date to the last glacial maxima (approximately 18,000 years ago).

In contrast to what one would expected from late Pleistocene differentiation, the genetic divergence among the main lineages inferred for *G. spirurus*, *M. hemimelaena* and *H. naevius* were very high (between 1–6%) suggesting that the differentiation were in periods prior to the last glacial maxima as discussed in Sects. 3.1, 3.2 and 3.3. The estimates for most recently phylogenetic divisions date from approximately 1 million years ago (Fig. 38). Thus, the last ice age appeared not to have contributed to the most splitting events, although the intense demographic fluctuations detected in populations of *G. spirurus*, *M. hemimelaena* and *H. naevius* suggest some influence of the glaciations in the population dynamics of these birds.

A complex history diversification were inferred for the three species and some differences in the sequence divergence (especially in the Madeira River Basin) were detected (see above). This suggest that the river hypothesis alone may not explain all diversification in Amazonia. Others processes may had importance as primary agents of divergence. Cohn-Haft (2000) suggest that lack of strict concordance among phylogenetic branching patterns and sequence divergence of codistributed lineages can be explain by current environmental conditions. Distribution over a large geographic area, low population densities, and limited dispersal might drive speciation under temporary isolation of some portion of a population. Having speciated, as long as two species remain ecologically identical they will maintain allopatry, not because a physical barrier such as rivers, but because of interspecific competition. This hypothesis predicts variable patterns of divergence as suggested by Cohn-Haft (2000).

4.2 The Role of Rivers in Amazonian Biogeography

In the mid-19th century, during the first Amazonian exploration, Alfred Russel Wallace postulated that the formation of large Amazonian rivers delimits the ranges of many vertebrates and fragments their formerly widespread populations into isolated units where differentiation occurred in allopatry (Wallace 1852). The simplicity and clearness of biogeographic patterns in Amazonia, where conspicuous animals such as birds and monkeys looked different across the rivers, make this hypothesis hardly contestable. For example, howler monkeys (genus *Alouatta*) to the south of the Amazon river bank are black and to the north bank are red. In birds, many pairs of presumed sister taxa are separated by Amazonian rivers (Haffer 1974).

This has led to the idea that the formation of the Amazonian rivers had a primary effect on diversification. Capparella (1988, 1991) provided evidence for alloenzyme differentiation between phenotypically similar and dissimilar taxa separated by the Amazon River, thus demonstrating the Amazon River’s potential effectiveness as an impediment to gene flow among bird taxa. However, this evidence corroborates that rivers are only current barriers and represent borders between the clades. The process leading to diversification is still very controversial.

Under the river barrier hypothesis it is expected that sister clades are separated by a river imposing restrictions of exchange of individuals within a center of origin of existing species (Patton et al. 2000). If the rivers were the primary agents of divergence between the clades it is also expected that the divergence times among co-distributed lineages are concordant and that the splits should date from the late Pliocene–early Pleistocene (Ribas et al. 2012). Another prediction is that the effectiveness of rivers as barriers to dispersal is greatest along the lower, wider portions of the river, where the river presents a greater obstacle than where it narrows upstream (Haffer 1974). The Amazonian rivers do not act as barriers for all species, and further, smaller rivers are less likely to be barriers than larger rivers, and any population delimited by a small river must also be divided by the larger one (Cohn-Haft 2000).

The data presented here support these predictions. For the three species it was found that, in general, the major rivers separate sister lineages or even morphologically differentiated sister forms. Assuming roughly equal rates of evolution, then *G. spirurus*, *M. hemimelaena*, and *H. naevius* populations may have differentiated across the Amazonian rivers at about the same time. This implies that these three species share a similar evolutionary history. The topologies of the phylogenetic trees were concordant and the splits dated approximately from the age of the rivers' formation. The data of the three species also suggest a hierarchical effect where larger rivers divide older clades, while smaller rivers are associated with more recent divergences. There is evidence of gene flow in the headwater regions where the rivers are narrower, strengthening the hypothesis of the hierarchical effect of the width of river on population structuring. This corroborates the effect of the rivers acting as geographic barriers and that the interfluvial areas harbor high levels of endemism. The Amazonian rivers may have functioned as primary agents of divergence, therefore they probably were an important agent in generating biodiversity.

While important rivers (such as the Juruá and Purus) in western Amazonia appear not to be a barrier to gene flow for bird populations, this study shows that a much smaller and apparently inconsequential river does form a relevant barrier. The reasons have been previous discussed and related to underlying substrate and its effects on river dynamics. The rivers from the west are well know to transfer extensive portions of soil and silt across their banks – frequently a meander loop is cut off or new river courses are created (Haffer 1997b). In this way, even the most sedentary animal may easily find itself located on the opposite river bank. This pattern seems to be consistent among different groups of vertebrates which suggests that the river width is not the only factor affecting divergence. The geology and origin of the rivers are also crucial factors to understand the origin and diversification of species in Amazonia.

4.3 Cryptic Diversity

As first noticed by Capparella (1988, 1991) the high degree of ancient structure among populations that show little phenotypic differentiation appears to be a common and predominantly Amazonian pattern. Deep divergence among populations of Amazonian birds corresponding to measures of intergeneric divergence among North American birds (up to 6% of genetic differentiation) in relative absence of morphological differentiation has been reported in several studies conducted in lowland Amazonia (Antonelli et al. 2010). This could be due to the high incidence of species complexes in this region. These complexes commonly feature absence or slight variation in morphological and behavioral characters, masking the occurrence of similar species that can be uncovered by genetic analyses. Particularly for the uniqueness of this phenomenon it seems to represent an unprecedented diversification process and therefore must be

preserved and better understood. If cryptic endemism is very prevalent in Amazonia, then it affects a huge proportion of global biodiversity. Thus it is important to conduct intensive and extensive surveys to detect more cases of cryptic speciation and to understand on what scale it works (Cohn-Haft 2000; Aleixo 2009).

The present study revealed previously unrecognized but similar morphological forms with high genetic distances across river banks. In two cases (*M. hemimelaena pallens* and *H. naevius theresea*) small morphological differences in plumage color of females, slight vocal differences, but considerable genetic divergence across the Jiparaná River has been detected. In the third case (*G. spirurus inornatus*), a pronounced genetic divergence was found across the Jiparaná and Aripuanã rivers, although no morphological or vocal differences could be detected (see Sect. 3.1). In the three cases, while the morphological differences were small or absent, the genetic distance among populations across rivers has been found to be equivalent to those found in other taxa that show great morphological distinction across the same rivers (Ribas et al. 2012). One possible explanations for this pattern, as suggested by Cohn-Haft (2000), is that strong stabilizing selection maintains phenotype and niche virtually unchanged over time. If environmental conditions are the same on opposite banks of the rivers, it is not expected that populations, even when isolated, exhibit large phenotypic differences. Thus, the high genetic divergence in absence of great phenotypic differentiation might be result of morphological stasis.

4.4 Conservation Implications

The presence of populations with high genetic divergence that show little or no morphological difference in various passerine species implies that the Amazon basin harbors a level of evolutionary diversity not taken into account in current taxonomy and is ignored in biodiversity conservation and management. The observation that even small rivers can separate genetically and morphological differentiated forms of small birds has important conservation implications, especially in regions suffering high rates of deforestation. Capparella (1991) recommends that the interriverine areas of the Amazon be object of special conservation efforts, and Mesquita et al. (2007) mentioned the importance of recognizing “mini-interfluvia” in the definition of Amazonian reserves. This would make a paramount difference in conceptualizing regional biodiversity conservation efforts. Moreover, the existence of contact zones between species and subspecies in the headwater regions means these regions are important natural laboratories for studying patterns and processes of speciation in addition to interspecific interactions in terrestrial Amazonian organisms. Based on the data presented here and on previous studies (Isler et al. 1998; Hall and Harvey 2002; Sardelli 2005; Fernandes 2007; Tobias et al. 2008) new phylogeographic provinces delimited by small rivers in addition to those described by Cracraft (1985) (Fig. 1) should be recognized in Amazonia – an area northeast of the Aripuanã, an area southwest of the Jiparaná, and an area between the Aripuanã and Jiparaná rivers (Fig. 38).

5 Acknowledgements

First of all I would like to thank my supervisor Prof. Dr. **Michael Wink** for supporting and encouraging my project and for kindly providing the opportunity to develop this study at Heidelberg University. My sincere appreciation goes to **Alexandre Aleixo** (Curator of Birds, Museu Paraense Emílio Goeldi, Belém) for his essential intellectual contribution during the entire period of my Ph.D.

This work has only been made possible by the tenacious effort of many specimen collectors, who throughout years of intense field work in the Amazon amassed the raw data necessary for the analyses contained in this dissertation. I thank the curators and curatorial assistants of the following collections for allowing me to study and sequence skins and tissues under their care: **John Bates**, **Dave Willard** (Field Museum of Natural History, Chicago); **Mario Cohn-Haft**, **Camila Ribas**, **Ingrid Macedo** (Instituto Nacional de Pesquisas da Amazônia (INPA, Manaus); **Cristina Miyaki** (Laboratório de Genética e Evolução Molecular de Aves, USP, São Paulo); **Robb Brumfield** and **Donna Dittmann** (Louisiana State University Museum of Natural Science); **Alexandre Aleixo** and **Fabíola Poletto** (Museu Paraense Emílio Goeldi, Belém); **João Pinho** (Universidade Federal de Mato Grosso); and **Jon Fjeldså** and **Jan Bolding Kristensen** (Natural History Museum of Denmark).

For four years I was generously supported by funding from the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (**CNPq**). Deutscher Akademischer Austauschdienst (**DAAD**) financed a six-month German course which was very important for my stay in Germany. The **Graduate Academy** of **Heidelberg University** supported me to attend the 25th International Ornithological Congress in Brazil. **Idea Wild** financed some necessary field equipment.

Fieldwork in Brazil was only possible because of the collaboration of several institutions, particularly the Instituto Nacional de Pesquisas da Amazônia (**INPA**) which provided essential logistic support for my research, and the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais (**IBAMA**) for providing collecting permits. I appreciate the logistic support of Reserva Biológica do Jaru (**REBIO JARU**) and of **SDS-APUÍ**. **Gisiane Lima** was essential for succeeding with my field work. Many thanks to **Isac Teobald** who gave logistic support during the field trip to the region of Apuí. I'm thankful for help by my friend **Claudeir Vargas** who gave me valuable assistance in Manaus and to **Sidnei Dantas** in Belém. Thanks to **Upiara** and **Carmen Fiuza** for much appreciated support in Heidelberg.

The technical assistants and colleagues from the Institute of Pharmacy and Molecular Biotechnology (**IPMB**), especially **Holger Schäfer**, **Hedwig Sauer-Gürth**, and **Heidi Staudter**, kindly helped and taught me the necessary techniques in the laboratory. **Javier Gonzalez** introduced me to valuable techniques in molecular biology and helped in interpreting the results. I also thank **Susanne Renner** for help with the data analyses. **Luciano Naka** and **Catherine Bechtoldt** gave useful and inspiring comments. To **Petra Fellhauer** I am immensely grateful for professional and logistic support.

I am indebted to **Theodor C. H. Cole** who not only proofread my manuscripts and improved my oral presentations, but also gave me support in difficult moments during my Ph.D.

My deep love and appreciation goes out to my **family**: to my **parents** and my two **sisters** who always supported me and provided essential encouragement – and to my wife, **Fernanda Antunes Carvalho**, who has been a reliable and brilliant companion providing intellectual support, an inspiring fellow adventurer accompanying me on difficult field trips to the Amazon, for being with me during my stay in Germany, and for encouraging me throughout.

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Affidavit for Dissertation

I hereby declare that this thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

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Alexandre Mendes Fernandes, Apr. 24, 2012