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Priscila Santos Carvalho

Systematics and Biogeography of the Hydropsini tribe (Serpentes: Xenodontinae)

São José do Rio Preto
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Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Biologia Animal, junto ao Programa de Pós-Graduação em Biodiversidade, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

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Às mulheres.

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Ano passado eu morri, mas esse ano eu não morro.
(Sujeito de sorte - Belchior)

Respeita a existência ou espere resistência.
(Noite inteira - Pitty)

Nada em biologia faz sentido exceto à luz da evolução.
(Theodosius Dobzhansky, 1973)

Resumo

A região Neotropical é caracterizada pela alta diversidade biológica e pela presença de grandes bacias hidrográficas, que exercem papel importante no padrão de distribuição e evolução da biota. Tal diversidade está também associada, dentre vários fatores, ao soerguimento final dos Andes a partir do Mioceno. Apesar dos recentes avanços na sistemática de serpentes, a diversidade de várias linhagens neotropicais permanece pouco compreendida. Aqui, abordamos a sistemática e biogeografia de Hydropsini (*Pseudoeryx*, *Hydrops* e *Helicops*), e apresentamos uma análise filogenética com base na maior amostragem molecular feita até o momento, cerca de 83% da diversidade da tribo (22 das 26 espécies), incluindo 1.080 sequencias de dois genes mitocondriais (16S e Cytb) e quatro nucleares (Cmos, NT3, BDNF e R35). Dividimos a tese em três capítulos. No primeiro capítulo inferimos a filogenia de Hydropsini, cujos resultados mostraram que os três gêneros são válidos e recuperamos a monofiletismo da tribo. A inclusão de mais espécies resultou em relacionamentos contrastantes quando comparados com inferências filogenéticas anteriores. No segundo capítulo, com base na filogenia datada do capítulo 1, conduzimos uma estimativa de área ancestral, a fim de investigar a história biogeográfica dessas serpentes aquáticas. Revelamos que o cenário ancestral mais provável para a diversificação de Hydropsini foi a região amazônica, por volta de 21 milhões de anos, no início do Mioceno. Discutimos como o dinamismo da paisagem durante o Mioceno na região amazônica teve um grande impacto na diversificação de Hydropsini, influenciado principalmente pelo sistema Pebas. Por fim, no terceiro capítulo, nos baseamos nos achados sobre o complexo de *Helicops leopardinus* (*He. leopardinus*, *He. modestus*, *He. infrataeniatus* e *He. tapajonicus*) do primeiro capítulo e estudamos a filogeografia deste complexo para compreendermos sua diversidade e estruturação genética em níveis mais recentes de diversificação. O complexo de *Helicops leopardinus* se originou durante o Pleistoceno (~1,2 Mya), e mostramos que este complexo pode representar uma única espécie com cinco agrupamentos geneticamente estruturados com fluxo gênico de forma desigual entre eles. A diferenciação genética do complexo de *He. leopardinus* é explicada principalmente pela interação da distância geográfica, variação climática e bacias hidrográficas. Fornecemos novas propostas sobre padrões e processos de diversificação para um complexo de espécies de cobras aquáticas amplamente distribuído ao longo da América do Sul.

Palavras-chave: América do Sul. Dipsadidae. Distribuição geográfica. Filogenia molecular. Filogeografia. Sistemática. Xenodontinae.

Abstract

The Neotropical region is characterized by high biological diversity and the presence of large hydrographic basins, which play an important role in the distribution pattern and evolution of the biota. Such diversity is also associated, among several factors, with the final uplift of the Andes during the Miocene. Despite recent advances in snake systematics, the diversity of several Neotropical lineages remains poorly understood. Here, we address the systematics and biogeography of Hydropsini (*Pseudoeryx*, *Hydrops* e *Helicops*) and present a phylogenetic analysis based on the largest molecular sampling to date, about 83% of the tribe's diversity (22 of 26 species), including 1,080 sequences of two mitochondrial genes (16S and Cytb) and four nuclear (Cmos, NT3, BDNF, and R35). We organized the thesis into three chapters. In the first chapter, we inferred the phylogeny of Hydropsini, and our results showed that the three genera are valid and we recovered the monophyly of the tribe. The inclusion of more species resulted in contrasting relationships when compared to previous phylogenetic inferences. In the second chapter, based on the dated phylogeny from chapter 1, we conducted an ancestral area estimate in order to investigate the biogeographic history of these aquatic snakes. We reveal that the most likely ancestral scenario for Hydropsini diversification was the Amazon region, around 21 million years ago, in the early Miocene. We discuss how the landscape dynamism during the Miocene in the Amazon region had a great impact on the diversification of Hydropsini, mainly influenced by the Pebas system. Finally, in the third chapter, based on the findings on the *Helicops leopardinus* complex (*He. leopardinus*, *He. modestus*, *He. infrataeniatus* and *He. tapajonicus*) from the first chapter, we carried out a phylogeography for these snakes to understand their diversity and genetic structure. The *Helicops leopardinus* complex originated during the Pleistocene (~1.2 Mya), and we show that this complex may represent a single species with five genetically structured clusters with uneven gene flow between them. The genetic differentiation of the *He. leopardinus* complex is mainly explained by the interaction of geographic distance, climatic variation, and watersheds. We provide new proposals on diversification patterns and processes for a complex of aquatic snake species widely distributed across South America.
Keywords: South America. Dipsadidae. Geographic distribution. Molecular phylogeny. Phylogeography. Systematics. Xenodontinae.

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1. General introduction

Hydropsini: A brief systematic history

With over 3,900 species encompassing a diversity of morphologies, ecologies, and lifestyles (arboreal, fossorial, terrestrial, aquatic) (Vitt & Caldwell 2013), snakes represent about 35% of squamate diversity (Uetz 2022) and approximately 30% of global snake diversity occurs in the Neotropical region (Guedes et al. 2018). Within the extensive Neotropical diversity of snakes, the family Dipsadidae is one of the largest radiations of Caenophidia ("Advanced Snakes") (>750 species; *sensu* Grazziotin et al., 2012). Dipsadids were considered restricted to the New World, but recent placement of *Stichophanes* and *Thermophis* as sister to Dipsadidae expanded their distribution into the Old World (e.g., Figueroa et al. 2016). Dipsadidae is morphologically supported by two putative hemipenial synapomorphies (body calyces on the asulcate surface of the lobes and lateral enlarged spines disposed on the sides of the hemipenial body) and molecularly (Zaher et al. 2009).

Among dipsadids, the tribe Hydropsini belongs to Xenodontinae Bonaparte, 1845, and is composed by three genera: *Helicops* Wagler, 1828 (21 species); *Hydrops* Wagler, 1830 (3 spp); and *Pseudoeryx* Fitzinger, 1826 (2 spp). This tribe is endemic to South America, occur from Trinidad to Uruguay, and their species are strongly associated and adapted to the aquatic environment (Moraes-da-Silva et al. 2019). Among the variety of lifestyles in snakes, about 5% have aquatic habitats (Pauwels et al. 2008) (e.g., members of Acrochordidae, Boidae, Colubridae, Dipsadidae, Elapidae, Homalopsidae, Natricidae and Viperidae). Thereby, snakes have independently invaded the aquatic environment many times (Pauwels et al. 2008). Interestingly, the Oriental and Neotropical regions have a great hydrological richness and are the two largest regions of aquatic or semiaquatic freshwater snake diversity (Pauwels et al. 2008). Some morphological specializations in aquatic/semiaquatic snakes include position of nostrils on the snout top, valvular nostrils, dorsolaterally oriented eyes (Pauwels et al. 2008; Segall et al. 2016). This morphology adaptations are observed, irrespective of the phylogenetic relationships among species of snakes from different families, suggesting that the aquatic environment does indeed drive the evolution of morphology in snakes, thus driving the evolutionary trajectory of this group of animals (Segall et al. 2016).

Among the current taxonomy of Hydropsini, *Pseudoeryx* has two valid species: *P. plicatilis* and *P. relictualis* with allopatric distributions. Though, *P. plicatilis* presents two subspecies *P. p. plicatilis* and *P. p. mimeticus* both with cis Andean distribution. Meanwhile, *P. relictualis* is a trans Andean species, occurring in Lake Maracaibo, Venezuela (Schargel et al. 2007). *Hydrops* contains three species: *Hy. triangularis*, *Hy. martii* and *Hy. caesurus*.

Hydrops martii and *Hy. triangularis* have widely overlapping geographic distributions, and are often found in syntopy, especially along the central part of the Amazon basin. Meanwhile, *Hy. Caesurus* is endemic in the La Plata basin (Argentina, Paraguay and Brazil) (von May et al. 2019). *Hydrops* differs from *Helicops* and *Pseudoeryx* in its smooth dorsal scales (keeled in *Helicops*) and in its maxillary diastema and color pattern with transverse bands (no diastema and longitudinal lines or dots in *Pseudoeryx*) (Roze 1957a, b). Lastly, *Helicops* is the most diverse genus of Hydropsini with 21 known species, diagnosed by the presence of nude flounces on the lobes of the hemipenis (Zaher 1999), and differs from *Hydrops* and *Pseudoeryx* by the presence of keels on the dorsal scales, absent in both latter taxa (Roze 1957a, b; Moraes-da-Silva et al. 2019). *Helicops* has species with a wide and restricted distribution throughout South America (Nogueira et al. 2019) and most occur in the Amazon region (e.g., *He. petersi*, *He. pastazae*, *He. yacu*), while *He. leopardinus* and *He. angulatus* are widely distributed. In this genus, there are two species with restricted trans Andean distribution (Moraes-da-Silva et al. 2019; 2021; Citeli et al. 2022).

Many species within this tribe have undefined type localities, which makes taxonomic decisions difficult. Some species are only known from a few specimens or their type specimens, as many specimens were collected when tissue collection for molecular analysis was not yet standard practice (e.g., *He. yacu*, *He. hogei*). Currently, only 22 specimens have been sequenced representing 12 species out of the 26 recognized. There have been several taxonomic rearrangements in recent decades, mainly due to morphological differences in hemipenis between the three genera (see below) (Roze 1957b; Rossman 1973). Since the three genera share a single internasal scale, eyes and nostrils located in a dorsal position, Roze (1957a, b) indicated a phylogenetic affinity between them, and considered such traits as adaptations of these genera to aquatic habits. The author also suggested that *Hydrops* and *Helicops* would be more closely related due to dentition characteristics, qualitative (color pattern) and quantitative (dorsal scales) features, and that *Pseudoeryx* would be an sister clade of this clade. However, Rossman (1973) based sole on hemipenial evidence considered that such characteristics would be convergent adaptations of the aquatic habit and rejected the hypothesis of Roze (1957a, b). Although he did not place the genera into any family, the author suggested that new evidence beyond the hemipenis might alter his conclusion. Within Xenodontinae, *Pseudoeryx* and *Hydrops* are the only genera that do not have hemipenial characteristics that would diagnose them as belonging to this subfamily (Zaher 1999). Despite this, the relationship of the three genera was supported by sharing the presence of a highly

developed adductor mandible externus superficialis muscle at their point of origin (Zaher 1999).

The taxonomic history of *Pseudoeryx* and *Hydrops* is confusing and complex, mainly because the species and subspecies have been described from qualitative and quantitative variations of a limited number of specimens (Roze 1957b; Mertens 1965; Albuquerque 2000; Albuquerque & Lema 2008). Furthermore, in *Pseudoeryx* the lack of information on type localities makes it difficult to solve taxonomic problems. The two currently subspecies of *P. plicatilis* (*P. p. plicatilis* and *P. p. mimeticus*) show great overlap in qualitative and quantitative characters and occur in sympatry at some areas (Dixon & Soini 1986; Cunha & Nascimento 1993; Schargel et al. 2007). Such overlaps seems to indicate that the subspecies of *P. plicatilis* do not represent distinct evolutionary lineages and merely depict artificial clusters of geographic/individual variation (Schargel et al. 2007), although, so far this issue has not been resolved. Variations in hemipenial morphology in three specimens of *P. plicatilis* were observed and there is a possibility that some populations may represent different species (Zaher 1999). Roze (1957b) performed a taxonomic review of *Hydrops* and described two subspecies for *Hy. martii* (*Hy. m. martii* and *Hy. m. callostictus*) and six for *Hy. triangularis* (*Hy. t. triangularis*, *Hy. t. fasciatus*, *Hy. t. venezuelensis*, *Hy. t. neglectus*, *Hy. t. bassleri*, *Hy. t. bolivianus*), based on morphological characters. Almost 50 years later, Albuquerque (2000) and Albuquerque & Lema (2008) reviewed the genus with a larger sample and found that there was a great overlap between the subspecies of both *Hy. triangularis* and *Hy. martii*, and placed the subspecies of each taxon in synonymy with the nominal species.

As it presents the greatest diversity within the tribe, *Helicops* presents the greatest taxonomic issues. There is polymorphism of several quantitative and qualitative data, and most variables overlap between several species, both in taxa with restricted and wide distributions (Kawashita-Ribeiro et al. 2013; Moraes-da-Silva et al. 2019, 2021; Costa et al. 2016). Briefly, Rossman (1976) coined the *pastazae* complex (*He. pastazae* and *He. petersi*) and the "*polylepis* section" (*He. pastazae*, *He. petersi*, *He. polylepis* and *He. yacu*). Soon after, Rossman and Abe (1979) discussed the possible synonymy of *He. yacu* with *He. pastazae*, since two additional specimens of *He. yacu* were collected from the upper Amazon region that resembled *He. pastazae*. Rossman (2002) reported that he "believes" that *He. hogei* would be a junior synonym of *He. scalaris* based on morphology. From the 2000s until now, there have been five descriptions of new species of *Helicops* (Kawashita-Ribeiro et al. 2013; Costa et al. 2016; Moraes-da-Silva et al. 2019, 2021, 2022). However, several widespread taxa, such as *He. angulatus*, *He. leopardinus*, *He. hagmanni* and *He. polylepis*

may represent species complexes and have never had their taxonomic status tested. Therefore, the relationships between *Helicops* taxa need strong investigations considering integrative approaches, and with larger sampling data.

Our understanding of snake phylogenetic relationships has significantly improved in recent years due to the advancement and development of molecular genetics (Zaher et al. 2009; Pyron et al. 2013; Figueroa et al. 2016; Zaher et al. 2019). Molecular phylogenies confirmed the monophyly of the tribe, establishing its validity, although the intergeneric and interspecific relationships remain unresolved, given that taxonomic and geographic sampling remains limited (e.g., Moraes-da-Silva et al., 2019, 2021, Murphy et al. 2020). With the recent discoveries of new species of *Helicops*, Costa et al. (2016) and subsequent works began to use a molecular phylogeny approach to validate such species and verify their phylogenetic positioning. From these phylogenies with low taxonomic and geographic sampling, a phylogenetic perspective on the tribe began to be elucidated.

Summarily, in the description of *He. nentur*, Costa et al. (2016) revealed phylogenetic affinity of this taxa with *He. carinicaudus*, and which was corroborated by subsequent studies (Moraes-da-Silva et al., 2019, 2021, Murphy et al. 2020). Moraes-da-Silva et al., 2019, 2021 described the species *He. boitata* and *He. phantasma*, respectively. Such studies recovered the first as a sister rate of *He. carinicaudus* and *He. nentur*, while *He. phantasma* was closely related to *He. leopardinus*. Moraes-da-Silva et al. (2019) presented for the first time the close relationship of *He. leopardinus* with *He. infrataeniatus* and *He. modestus*. They still revealed *He. polylepis* as sister taxa of the clade formed by *He. gomesi* and *He. angulatus*. Moraes-da-Silva et al. (2021) also included *He. hagmanni* in this new study that was recovered as a sister taxon of the clade formed by *He. polylepis*, *He. gomesi* and *He. angulatus* with low support value. Murphy et al. (2020) pointed out that *He. angulatus* is paraphyletic with respect to *He. gomesi* and revalidated *Helicops cyclops* Cope, 1868 to solve the problem. However, the study presents some taxonomic inconsistencies that need to be reviewed. Despite the panorama presented here, molecular phylogenetic studies so far have used a limited sample of the diversity of the tribe, in addition to using only species and samples occurring in Brazil.

Evolutionary approach

The molecular approach, combined with advances in bioinformatics, have helped to understand the evolution of biota, aiding in the development and knowledge of systematics, taxonomy and biogeography (Posada & Crandall 2001; Hickerson et al. 2010; Burbrink & Gehara 2018). Phylogeographic studies focus on the patterns and processes that determine the geographic distribution of genealogical lineages, trying to correlate, for example, geoclimatic

events, geographic distance, topography (historical and contemporary events) with the evolutionary history of lineages (Avise 2012; Knowles 2009). Such studies can help in the delimitation of species, in the recognition of new and cryptic species, as well as in the understanding of events such as extinction, dispersal, vicariance, and the demographic history of a species or population (e.g., Ledo et al. 2017; Dal Vechio et al. 2019; Carvalho et al. 2020; Damasceno et al. 2021). Phylogeographic studies of aquatic snakes from other regions such as Asia and North America have revealed that the evolutionary trajectory of the species does not depend only on geomorphological and climatic processes, but also intrinsic traits of each species. Therefore, species phylogenetically and ecologically related show different phylogeographic patterns (e.g., Guiher & Burbrink 2008; Brandley et al. 2010; Lukoschek et al. 2011; McCartney-Melstad et al. 2012; Carvalho et al. *in press*). Besides, comparative observations are determined by shared ancestry, and testing evolutionary hypotheses requires focusing on closely related taxa (Pyron 2015). Methods based on estimates of reconstruction of ancestral areas demand dated trees to connect the spatial and temporal dimensions (e.g., Ronquist 1997; Matzke 2014).

The history of the uplift of the Andes includes several elevation phases that triggered a long and complex history of the landscape and river systems linked to the evolutionary history of the South American biota (e.g., Wesselingh & Salo 2006; Hoorn et al., 2010a, b). Therefore, geological events and landscape remodeling on a continental scale have a strong influence on dispersal and vicariance processes, which reflects on the distribution patterns of organisms (Lundberg et al. 1998). Among the most important of these events are marine introgressions, the formation of the Pebas and Acre systems, changes in drainage connectivity and fusion, and the formation of the current transcontinental Amazon River drainage (Wesselingh & Salo 2006; Hoorn et al., 2010a, b; Rosa et al. 2003).

The dispersal of freshwater species requires corridors of aquatic habitat connecting basins, and the range limits of most of these species coincide with the limits of watersheds, although many others have distributions that extend beyond the limits of watersheds between basins (Albert & Reis 2011). However, for species that are not restricted to the aquatic environment, as in the case of aquatic or semiaquatic snakes, which can also forage on the terrestrial environment, hydrographic limits are not necessarily barriers (Carvalho et al. 2020; Carvalho et al. *in press*). Furthermore, species with a wide distribution are less likely to show phylogeographic breaks caused by geographic barriers (e.g., Avise 2012) and may still show greater genetic differentiation due to local adaptation (Kremer et al. 2012; Berthouly-Salazar et al. 2013).

Despite the panorama presented, the molecular phylogenetic studies presented so far for Hydropsini used a limited sample of the diversity of the tribe, using only species and samples that occur in Brazil. Furthermore, given its widely and narrowly distributed taxa and aquatic habitat, Hydropsini is an excellent model to study the spatial distribution of aquatic snake diversity in the South American region. Therefore, here we address the systematics and biogeography of Hydropsini, presenting for the first time a robust multi loci (two mtDNA, four nuDNA) molecular phylogenetic analysis including 1080 sequences from 22 of the 26 species. We also discuss the origin and diversification of the tribe based on a dated tree and geological events.

Main results

We have divided the thesis into three chapters. In the first chapter we inferred the phylogenetic relations of Hydropsini, which our results showed that the three genera are valid and we recover the monophyly of the tribe. In addition, we have redefined new groupings based on phylogenetic evidence. We recovered paraphyletic lineages in *He. carinicaudus*, *He. leopardinus* complex, *He. pastazae* and *He. angulatus* and discussed new relationships and taxonomic issues. Within *Helicops*, the inclusion of the majority of species had an impact on previous proposed phylogenetic positions. In the second chapter, based on the phylogeny of chapter 1, we performed an ancestral area estimation, in order to investigate the biogeographic history of these aquatic snakes. We reveal that the most likely ancestral scenario for Hydropsini diversification was the Amazon region, around 21 Mya in the early Miocene. The mountain ranges of Colombia and Venezuela acted as a vicariant barrier separating the cis and trans Andean taxa. Furthermore, colonization to adjacent watersheds further to east was also caused by distinct dispersal events. We discuss how landscape dynamism during the Miocene in the Amazon region had a great impact on the diversification of Hydropsini, mainly influenced by the Pebas system. Lastly, in the third chapter we based on the findings for *Helicops leopardinus* complex (*He. leopardinus*, *He. modestus*, *He. infrataeniatus* and *He. tapajonicus*) in the first chapter, we performed a phylogeography of this species complex in order to better understand its diversity and genetic structure. *Helicops leopardinus* complex originated during the Pleistocene (~1.2 Mya). We found that this complex may represent a single species with five genetically structured clusters, which show gene flow unevenly shared among them. The genetic differentiation of the *He. leopardinus* complex is mainly explained by the interaction of geographic distance (IBD), climatic variation (IBE), and

hydrographic basins. We provided new insights about diversification patterns and processes for a species complex of a broadly distributed group of watersnakes along South America.

2 CHAPTER 1:

Phylogeny and systematic of Hydropsini (Serpentes: Dipsadidae: Xenodontinae)

Abstract

The Hydropsini tribe is the most diverse group of water snakes in South America with three genera (*Pseudoeryx*, *Hydrops* and *Helicops*) and 26 valid species widely distributed in the continent. However, the relationships between genera and species are not well established. Therefore, we provide the most comprehensive phylogenetic analysis among *Pseudoeryx*, *Hydrops* and *Helicops*. We have two goals in this study: (1) present the relationship of the genera and species within Hydropsini; and (2) evaluate the diversity of lineages in Hydropsini and test whether the current taxonomy reflects the phylogeny of the tribe. We incorporate new data for seven previously unsampled taxa in the Hydropsini, using the highest levels of taxon and geographic sampling, a total of 22 species (85% of known diversity), which allowed us to improve the phylogenetic relationships among genera and species of the tribe. The dataset included 260 samples with two mitochondrial (16S, Cytb) and four nuclear genes (Cmos, NT3, BDNF and R35). Our phylogenetic analyses show that the three genera are valid and corroborated the monophyly of Hydropsini, and recover *Pseudoeryx relictualis* as a *Hydrops*. Within *Helicops*, the inclusion of almost all species of the genus had impact on previous notions of its relationship. We recovered paraphyletic lineages in *He. carinicaudus*, *He. leopardinus* complex, *He. pastazae* and *He. angulatus*. We recovered *He. scalaris* within the *He. angulatus* group. While *Helicops danieli* and *He. scalaris* are not closely related. We present the first comprehensive phylogeny of Hydropsini and provide new relationships that will assist in insights into the evolution in reproductive mode, aquatic habit and, a valuable resource for future comparative (phenotypic or ecological) evolutionary processes and taxonomic studies.

Keywords: Bayesian Inference, Diversification, Evolution, Hydrobasin, Multi-loci, South America, Taxonomy, Watersnake.

2.1 Introduction

Phylogenies are reconstructions of evolutionary history, and provide an understanding of relationships between species, as well as providing the historical basis for test ecological inferences and evolutionary processes (de Queiroz & Gauthier 1990; Edwards 2009). The understanding of phylogenetic relationships among snakes has progressed significantly in recent years, mainly due to the use of molecular data (e.g., Pyron et al. 2013; Zaher et al. 2019). Considering all extant snake species (~3.971 species), about 14% of them have an aquatic or semiaquatic habit, and inhabit freshwater ecosystems and the greatest diversity of freshwater snakes is located in the Oriental and Neotropical regions (Pauwels et al. 2008; Murphy 2012).

About half of all aquatic Neotropical species belongs to the Hydropsini Dowling 1975 tribe, a clade currently composed by 26 snakes allocated in three genera (*Pseudoeryx*, *Hydrops* and *Helicops*) distributed throughout South America with cis-andine (23 species) and trans-andine (three spp) species (Moraes-da-Silva et al. 2019; 2021; 2022; Citeli et al. 2022). *Helicops* Wagler (1828) represents the most diverse and widely distributed genus within the tribe with 21 species. *Hydrops* Wagler (1830) and *Pseudoeryx* Fitzinger (1826) contain three and two species, respectively, and occur mainly in the western region of the continent. Species within this tribe have highly morphological specializations that include position of nostrils on the snout top and dorsolaterally oriented eyes (Roze 1957a; b). Within the tribe, *Helicops* differs from *Hydrops* and *Pseudoeryx*, among other characteristics, by the presence of keels on the dorsal scales, absent in both last taxa, while diastema is present in *Helicops* and *Hydrops* and absent in *Pseudoeryx* (Roze 1957 a; b). Therefore, the systematics of the tribe has been historically based on phenotypic traits, which were later shown to lack phylogenetic support (Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021). Furthermore, the evolution of the reproductive mode within Hydropsini is intriguing, given that there are oviparous and viviparous species, and even *Helicops angulatus* (Linnaeus, 1758) presents populations with different reproductive modes, that is, it is a species with reproductive bimodality with allopatric distribution, oviparous populations are distributed from north to east-central and northeast, while viviparous populations occur from northwest to west-central South America (Braz et al. 2016; 2018). Ancestral state reconstructions suggest that oviparity is plesiomorphic in Hydropsini, while *Hydrops* and *Pseudoeryx* are exclusively oviparous (Braz et al. 2016). Nonetheless, the reproductive mode of some species is still unknown (i.e., *He. boitata* and *He. petersi*) (Braz et al. 2016; 2018; Moraes-da-Silva e al. 2022).

The knowledge of the diversity in Hydropsini has grown remarkably in recent years, with an increase of species descriptions (e.g., Scrocchi et al. 2005; Schargel et al. 2007; Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021). *Pseudoeryx* possess two valid species, which *P. plicatilis* has wide distribution in the Amazon region, Pantanal and also in the Chaco of northern Argentina and Paraguay. *Pseudoeryx* remained for a long time a monospecific genus, when Schargel et al. (2007) described *P. relictualis*, endemic to Lake Maracaibo, Venezuela, which is geographically isolated from other plains by the Cordillera de Mérida to the east and south and the Sierra de Perijá to the west. Roze (1957a) highlighted the need for a detailed review of *Pseudoeryx*, considering the possibility of *P. plicatilis* harboring two taxa, which *P. mimeticus* Cope, 1885 could represent a valid taxon, nonetheless it has not been done so far. However, Hoge and Nina (1960–1962), based mainly on ventral scales and coloration pattern, superficially allocated *P. mimeticus* as a subspecies of *P. plicatilis* (Linnaeus, 1758). Later, Mertens (1965) described *P. p. ecuadorensis* (SMF 60435, female) from a single specimen to Ecuador without a specific type locality. However, Dixon and Soini (1986) synonymized it with *P. p. mimeticus* given that it fits the morphological variation of the latter. Other studies have pointed out several intermediate characters and overlap between *P. p. plicatilis* and *P. p. mimeticus* and highlighted that both subspecies lack a taxonomic and geographic resolution (Dixon & Soini 1986; Cunha & Nascimento 1993; Schargel et al. 2007).

On *Hydrops*, Roze (1957b) revised *Hy. martii* (Wagler, 1824) and *Hy. triangularis* (Wagler, 1824), and separated both species into two and six subspecies, respectively, based on scale counts, color pattern and geographic distribution. Years later, Albuquerque (2000) and Albuquerque and Lema (2008), supported by a larger sampling, synonymized all subspecies of the genus due to the large overlap of qualitative (i.e., color pattern) and quantitative (i.e., scale count) characters, and raised the subspecies to nominal species. Furthermore, Scrocchi et al. (2005) described *Hy. caesurus*, an endemic species from the La Plata basin (Argentina, Paraguay and Brazil), while both *Hy. triangularis* and *Hy. martii* occur in North South America, predominantly in the Amazon basin.

Lastly, *Helicops* is the most diverse, widespread genus within the Hydropsini tribe, and still, has more species with wide distribution, arising questions on species taxonomy (Rossman 1976; Rossman & Abe 1979; Kawashita-Ribeiro et al. 2013; Moraes-da-Silva et al. 2019; 2021; 2022; Murphy et al. 2020). The knowledge of the diversity in *Helicops* has grown with an increase of news species description, since species that were described from chance encounters to species that were already suspected of their taxonomic status (e.g., Kawashita-Ribeiro et al. 2013; Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021; 2022). In addition, some taxa have

had their taxonomic status contested, for example, Rossman and Abe (1979) assumed that *He. yacu* could be a synonym of *Helicops pastazae* Shreve, 1934 because they share several morphological characters, but he refrains to do it, and preferred to wait to analyze a larger number of specimens of the two species. In addition to the taxonomic issues on *Helicops*, is always recovered a polytomy among *He. leopardinus*, *He. infrataeniatus* and *He. modestus* (Moraes-da-Silva et al. 2021). Furthermore, recent molecular phylogenies have also found *He. angulatus* paraphyletic (Vidal et al. 2010; Murphy et al. 2020). *Helicops cyclops* Cope, 1868 was removed from the synonymy of *He. angulatus* (Murphy et al. 2020). Possibly, may have caused more confusion to the history of this species, given that the authors did not carry out a significant taxonomic revision of this species that shows a continental occurrence. Commonly, species with a wide distribution may present a complex and/or cryptic species (e.g., Geurgas & Rodrigues 2010; Nunes et al. 2012; Ruane et al. 2014). *Helicops angulatus*, *He. leopardinus* (Schlegel, 1837), *He. polylepis* Günther, 1861 and *He. hagmanni* Roux, 1910 had their taxonomic status questioned because they present wide occurrence and are difficult to delimit based on certain morphological characters, making the systematics of these taxa problematic (Kawashita-Ribeiro et al. 2013; Moraes-da-Silva et al. 2019). Therefore, within *Helicops*, taxonomic problems range from species complexes to multiple polymorphic or polychromatic species that may represent a single taxon (e.g., Rossman 1976; Rossman & Abe 1979; Rossman 2002a). Consequently, in order to know more precisely the phylogenetic relationships of the species, it is important to understand the real diversity, and to sample the entire distribution of their taxa in a phylogeny. Moreover, we can provide answers about their taxonomic scenario, which is essential to elucidate their phylogenetic relationships.

Phylogenetic relationship hypotheses were conflicting within the tribe even before molecular data was available (e. g., Roze 1957a; b; Rossman 1973). Roze (1957a; b) was the first to recognize that *Pseudoeryx*, *Hydrops* and *Helicops* had a close relationship, pointing out that these genera shared, as he mentioned, morphological synapomorphies such as: nostrils located in a dorsal position and a single internasal scale, therefore, considered it to be an adaptation to aquatic habit. However, he did not perform any explicit phylogenetic analysis. Furthermore, Roze (1957a; b) proposed *Pseudoeryx* as the outgroup among the three genera due to dentition without diastema, no-banded color pattern, and lack of dorsal scale reduction (15-15-15). While the presence of keels and the greater number of rows of dorsal scales in *Helicops* are characterized as the most derived genus among the three hydropsins.

Based on hemipenial characters, Rossman (1973) disagreed that these genera could have some type of phylogenetic proximity, and even concluded that the nostrils and eyes in the dorsal

position would be an evolutionary convergence. The hemipenial morphology effectively caused confusion in the systematics of *Helicops*, *Hydrops*, and *Pseudoeryx* (Roze 1957a; Rossman 1973). In fact, the hemipenis of *Hydrops* and *Pseudoeryx* have notable morphological differences from any other xenodontines, including *Helicops*, which made it difficult to establish phylogenetic relationships and, therefore, they were often allocated to separate groups (Roze 1957a; Rossman 1973). In the study on hemipenis of Xenodontinae, Zaher (1999) considered the tribe monophyletic because *Pseudoeryx* and *Hydrops* share with *Helicops* (a clear member of the Xenodontinae) the adductor muscle (*externus superficialis*) of the mandible well developed at its point of origin. Furthermore, he attributed these hemipenial differences to a secondary loss in *Pseudoeryx* and *Hydrops*. Most molecular phylogenies focused on understanding suprageneric relationships have always recovered Hydropsini as monophyletic (e.g., Vidal et al. 2000; Zaher et al. 2009; Grazziotin et al. 2012; Pyron et al. 2013; Figueroa et al. 2016). In some topologies, *Pseudoeryx* and *Hydrops* form a clade, which is the sister group of *Helicops* (Vidal et al. 2000; Pyron et al. 2013; Figueroa et al. 2016; Zaher et al. 2019); moreover, it was observed *Pseudoeryx* and *Hydrops* representing two successive outgroups for *Helicops* (Zaher et al. 2009; Grazziotin et al. 2012), or *Hydrops* more closely related to *Helicops*, and *Pseudoeryx* more distantly (Pyron et al. 2011).

Rossman (1976), using morphological data (qualitative and quantitative), reviewed the "*pastazae* complex" (*He. pastazae*, *He. petersi*), described *He. petersi*, and even suggested a group called "*polylepis* section" composed of: *He. pastazae*, *He. petersi*, *He. polylepis* and *He. yacu*, which he claims to be closely related. The affinities of *He. hagmanni* were considered uncertain even with non-bright scales and a large number of rows with the spotted pattern, as evidence that it could be closely related to the *polylepis* section, but characteristics such as the maxillary dentition, relatively short tail and number of extremely low subcaudals were sufficient for Rossman (1976) to consider not including the section.

The most complete phylogenetic approach of the tribe until now included 13 Hydropsini taxa (Moraes-da-Silva et al. 2021), which comprises species occurring mainly in Brazil. The phylogenetic relationships between the genera and species of Hydropsini remain unclear, and a more complete sample, including more taxa, might help to clarify the phylogenetic relationship of the tribe. A phylogeny with large-scale taxon sampling is invaluable for comparative evolutionary studies and for approaching its taxonomy (e.g., Torres-Carvajal et al. 2017; Arredondo et al. 2020; Bernstein et al. 2021; Melo-Sampaio et al. 2021). Despite progress in understanding the evolutionary history of snakes, species with aquatic habits do not receive much scientific attention, and studies in several fields are still extremely scarce (Carvalho et al.

2020). Is widely justified the need for large-scale taxon sampling molecular phylogeny to better understand the relationships among species within Hydropsini. In this study we have two main goals: (1) infer and propose hypotheses on the phylogenetic relationships of the genera and species within Hydropsini based on updated geographic, molecular and taxonomic sampling; and (2) evaluate the diversity of lineages in Hydropsini and explore whether relationships between species represent their current taxonomy. Furthermore, we aim to build a phylogenetic relation of the tribe through molecular approach, and present the first large-scale phylogenetic estimate for Hydropsini.

2.2 Material and Methods

Taxon sampling and data acquisition

We sampled 13 individuals of *Pseudoeryx*, 12 of *Hydrops* and 215 of *Helicops* from 210 localities throughout South America (Fig. 1; Table S1). We generated 1012 novel DNA sequences from 221 specimens representing 22 species of Hydropsini. We also obtained sequences of Hydropsini from GenBank, as well as outgroup species (Table S1) and we used the species *Coluber constrictor* for rooted the tree. Our final dataset has 260 terminals, including 20 outgroup species (Table S2), from all currently recognized tribes of the Xenodontinae, besides some species of Dipsadidae and Colubridae (*sensu* Grazziotin et al. 2012). We included 22 species of the 26 recognized and for the first time, the species *Pseudoeryx relictualis*, *Hydrops caesurus*, *Helicops danieli*, *He. pastazae*, *He. scalaris*, *He. trivittatus* and *He. yacu* have their phylogenetic relationships evaluated. It was not possible to sample *He. apiaka*, *He. petersi* and *He. acangussu*. We do not consider *He. cyclops* given taxonomic issues (see Discussion for more information).

Molecular data

We extracted the DNA from muscle, liver or scales, and we used the phenol-chloroform extraction protocol (Sambrook et al. 1989). We amplified the partial sequences of the two mitochondrial genes (mtDNA): 16S ribosomal (16S) and Cytochrome b (Cytb); as well as four nuclear gene (nuDNA): Oocyte maturation factor Mos (Cmos), Neurotrophin-3 (NT3), Brain-Derived Neurotrophic Factor Precursor (BDNF) and RNA fingerprint protein 35 (R35) using the standard Polymerase Chain Reaction (PCR) technique. PCR amplification protocols and primers are presented in supplementary material 1. We aligned each locus using the Muscle algorithm (Edgar, 2004) in Geneious v9.1.8 (Geneious 2022; https://www.geneious.com).

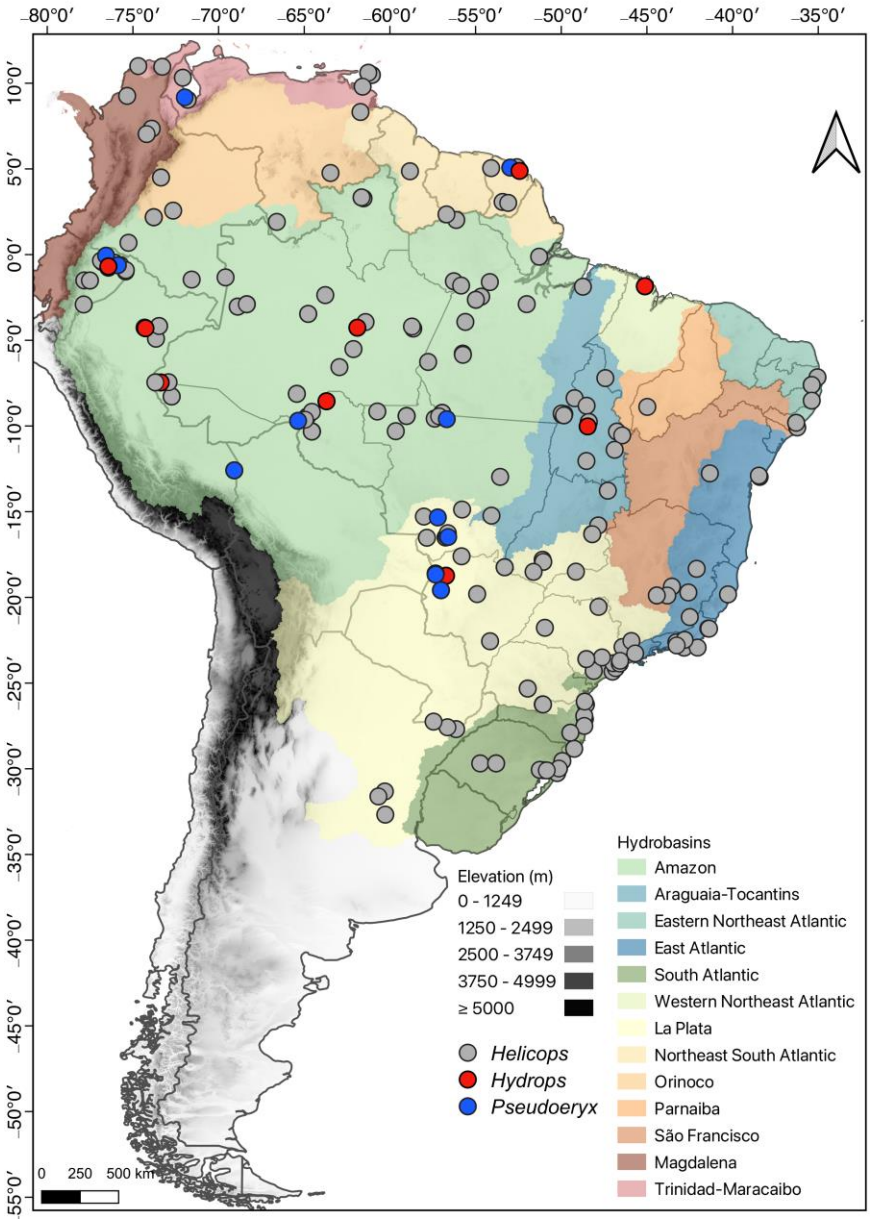


Figure 1. Sampling coverage used in this study for *Pseudoeryx*, *Hydrops*, and *Helicops* throughout South America.

Inferring phylogenetic relationships

We used PartitionFinder 2 to identify partitioning schemes and the most appropriate nucleotide substitution models (Lanfear et al. 2017). According to our concatenated alignment, we found eight partitions evaluated by BIC (Table S3). To test phylogenetic relationships among species of Hydropsini, we used Bayesian Inference (BI) and Maximum Likelihood (ML) methods. We performed a BI analysis using BEAST v.2.6.3 (Bouckaert et al. 2019) with a Yule process tree prior, a strict clock model, and we used rate of 1.3% substitutions per million years in Cytb that has been previously shown in closely related Dipsadidae snakes (Daza et al. 2009).

We ran two analyses for 100 million generations, sampling every 10,000 steps. We checked for stationarity by visually inspecting trace plots and ensuring that all ESS values were above 200 in Tracer v1.7.1 (Rambaut et al. 2018). We combined both runs in LogCombiner v2.6.3 (with a 10% burn-in) allowing us to summarize the results in a maximum clade credibility tree in TreeAnnotator v2.6.3. We implemented a Maximum Likelihood tree inferred in RAxML (Stamatakis 2014) via raxmlGUI 2.0 (Edler et al. 2021) with partitions restricted to GTR-based models and using GTRGAMMA, with rapid bootstrap method and 1000 bootstrap iterations.

2.3 Results

The final alignment of our sequences (including gaps) was 426pb for 16S, 851pb for Cytb, 541pb for Cmos, 584pb for NT3, 646pb for BDNF, 633pb for R35. The length of the concatenated alignment was 3581pb. Both BI and ML trees showed a similar topology, except by a clade (see below), with the relationships of most clades well supported, and nodes with high posterior probabilities (PP) > 0.95 and high bootstrap values (BS) > 70% (Fig. 2 and Fig. S1). Hydropsini was recovered as monophyletic with maximum support in both analyses. We recovered *Pseudoeryx* and *Hydrops* as sister taxa, with *Helicops* as sister clade of both. *Pseudoeryx* was recovered as paraphyletic, *Pseudoeryx relictualis* was recovered as sister to all other *Hydrops*. Besides, our analyzes recovered two well established lineages for *P. plicatilis* with maximum support. The monophyly of *Hydrops* was well supported in BI (PP=1), and we found two lineages within *Hy. triangularis*. Lastly, the diverse genus *Helicops* is monophyletic with robust support values (PP=1; BS=100%) (Fig. 2).

Pseudoeryx relictualis was recovered as sister taxa of the remaining *Hydrops* species in BI (PP=0.94), and as sister species to all other *Hydrops* species (Fig. 3; Fig S2). To better represent the monophyly of this clade, we therefore propose the relocation of *P. relictualis* within the genus *Hydrops*: *Hydrops relictualis* **comb. nov**. We emphasize that a taxonomic review is extremely important to understand how to diagnose this species within *Hydrops*. We recovered *Hy. caesurus* as sister taxa of *Hy. martii* and *Hy. triangularis* (Fig. 3; Fig S2). We recovered two lineages well supported for *Hydrops triangularis* (PP=1; BS=98%). The L1 from Ecuador, Peru and Brazil and the L2 from French Guiana and Brazil (Fig. 3; Fig S2).

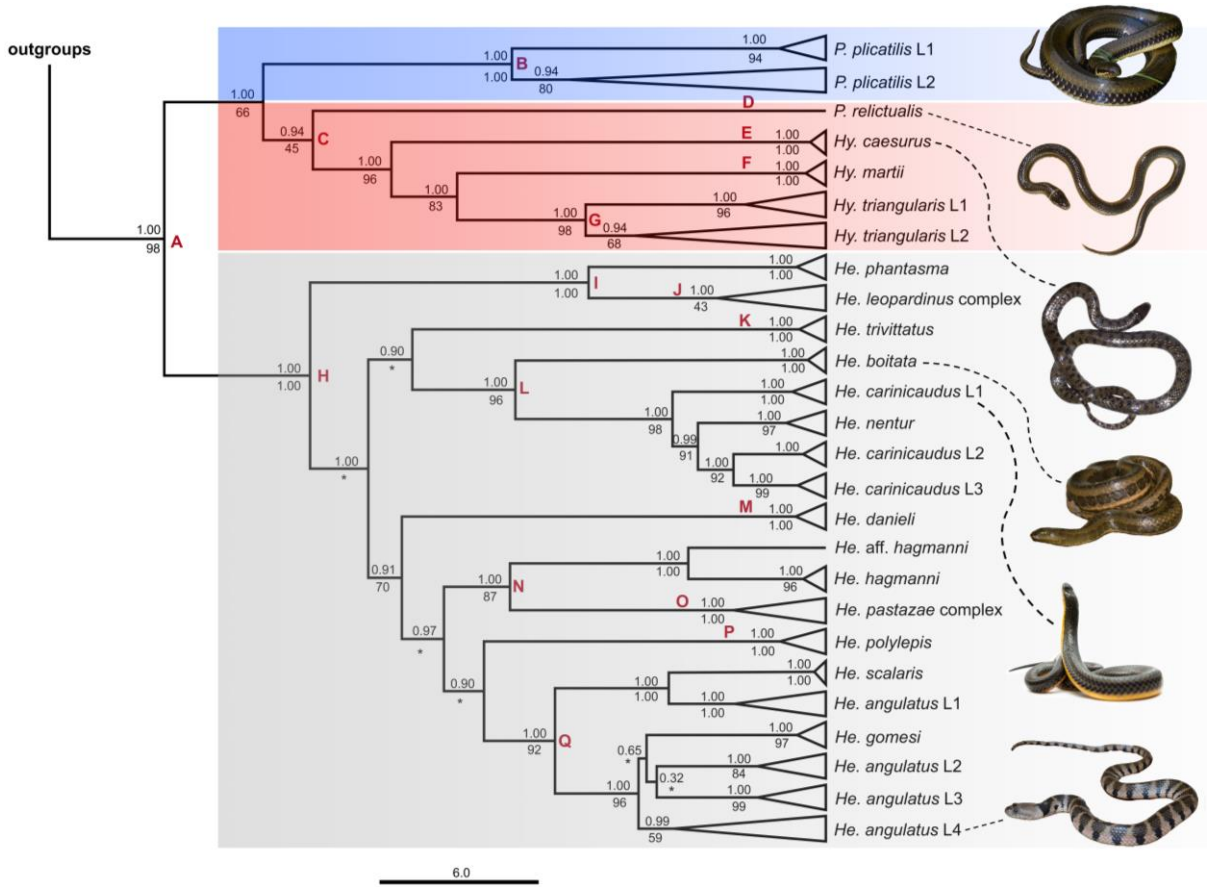


Figure 2. Bayesian phylogenetic inference of Hydropsini based on mtDNA (16S and Cytb) and nuDNA (Cmos, NT3, BDNF, and R35) genes using BEAST. Posterior probabilities (PP) and bootstrap (BS) values are ≥ 0.95 and ≥ 70%, above and below, respectively (we present only well-supported node values). Asterisks (*) indicate different phylogenetic relationships found in the ML tree. Clade A: Hydropsini tribe; Clade B: *Pseudoeryx*; Clade C: *Hydrops*; Clade D: *P. relictualis*; Clade E: *Hy. caesurus*; Clade F: *Hy. martii*; Clade G: *Hy. triangularis*; Clade H: *Helicops*; Clade I: *He. leopardinus* group; Clade J: *He. leopardinus* complex; Clade K: *He. trivittatus*; Clade L: *He. carinicaudus* group; Clade M: *He. danieli*; Clade N: *He. hagmanni* group; Clade O: *He. pastazae* complex; Clade P: *He. polylepis*; Clade Q: *He. angulatus* group. Photos: DJSantana (*P. plicatilis*, *He. angulatus*); FJMRojas-Runjac (*P. relictualis*); AACerqueira Junior (*Hy. casesurus*); Moraes-da-Silva et al. 2019 (*He. boitata*) and CHdeONogueira (*He. carinicaudus* L1).

Within *Helicops*, we recovered two clades in BI: we named the first clade as *He. leopardinus* group with *He. phantasma* as sister species from the clade formed by, *He. leopardinus*, *He. infrataeniatus*, *He. modestus* and *He. tapajonicus*, which were all paraphyletic. Within this later clade, we name it as *He. leopardinus* complex (*He. tapajonicus*, *He. modestus*, *He. infrataeniatus* and *He. leopardinus*), which all species are paraphyletic/polyphyletic (Fig. 4A; Fig S3). While the second clade by BI, we recovered *He. trivittatus* as a sister to *He. carinicaudus* group with moderate support value (PP=0.90). In ML,

excepted for *H. leopardinus* group, *He. trivittatus* was recovered as sister taxa of all other species with a well-supported node value (BS=87%) (Fig. 4B; Fig. S4).

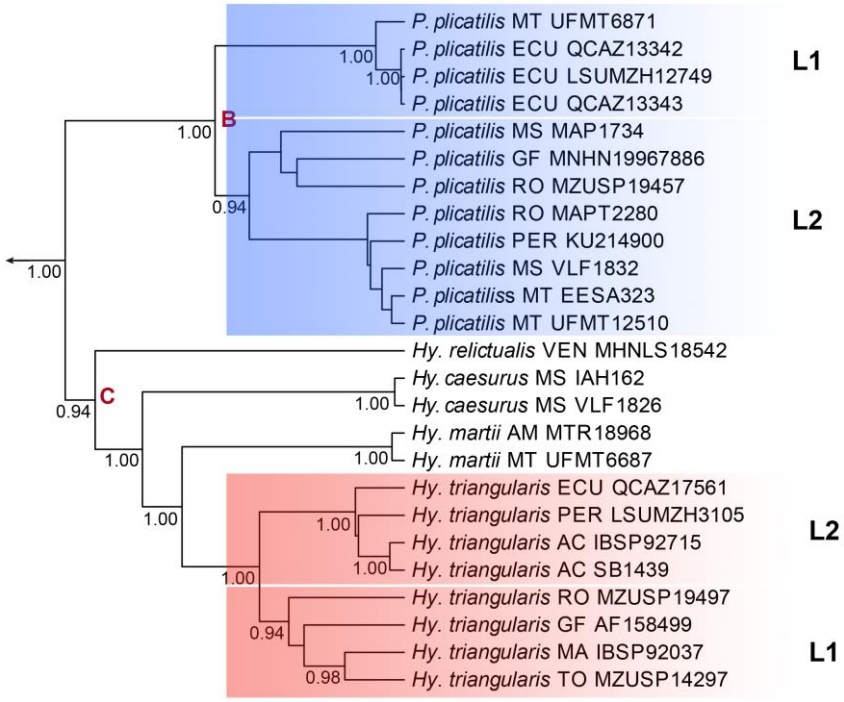


Figure 3. Bayesian phylogenetic inference of *Pseudoeryx* and *Hydrops* based on mtDNA (16S and Cytb) and nuDNA (Cmos, NT3, BDNF, and R35) genes using BEAST. Posterior probabilities (PP) values are ≥0.95 (we present only well-supported node values). Clade B: *Pseudoeryx*; Clade C: *Hydrops*; Clade D: *Hy. relictualis*; Clade E: *Hy. caesurus*; Clade F: *Hy. martii*; Clade G: *Hy. triangularis*. L: indicates distinct lineages.

The *He. carinicaudus* group, which is formed by *He. boitata*, as sister species of the clade conformed by, *He. carinicaudus* and *He. nentur*. In our topology, *He. carinicaudus* species is paraphyletic, with three lineages and the nominal species belongs to the Lineage 1 occurring in Espírito Santo and northern Rio de Janeiro (Fig. 4B; Fig. S4). *Helicops nentur* is the sister species of the other two lineages of *He. carinicaudus* (L2 and L3), which may represent distinct evolutionary lineages, then, we consider here as candidate species. All clades within this group have their nodes well supported (PP≥0.99; BS ≥91%).

He. danieli was recovered as a sister species of the *He. hagmanni* group, *He. angulatus* group and *He. polylepis* with moderate support values (PP=0.91; Fig. 2). The sole topological difference between the BI and ML trees was the placement of *He. polylepis*. In BI (Fig. 2 and 5), *He. polylepis* is the sister taxa of the *He. angulatus* group (PP=0.90), while in ML (Fig. S1) it is the sister taxa of *He. hagmanni* group (BS=87%). The recognized here *He. hagmanni* group, which was recovered with high support values in both analyses (PP=1.00; BS=91%; Fig.

2 and 4C), including *He. hagmanni*, *He. yacu*, *He. pastazae*, with *He. hagmanni* as sister taxon of the remaining species in the group. Besides, we considered the sample of *He. hagmanni* (IAVH-CT21340) as a candidate species. In addition, we recovered *He. yacu* closely related to *He.* pastazae and belongs to *He. pastazae* complex (Fig. 4C; Fig. S5).

The *Helicops angulatus* group is composed by two clades (Fig. 5; Fig. S6). The first clade with *He. scalaris* as sister species of the *He. angulatus* from northern South America (*He. angulatus* Lineage 1). The second clade within this group is formed by three remaining lineages of *He. angulatus* (L2, L3 and L4) and *He. gomesi*. However, the three *He. angulatus* lineages are paraphyletic related to *He. gomesi*.

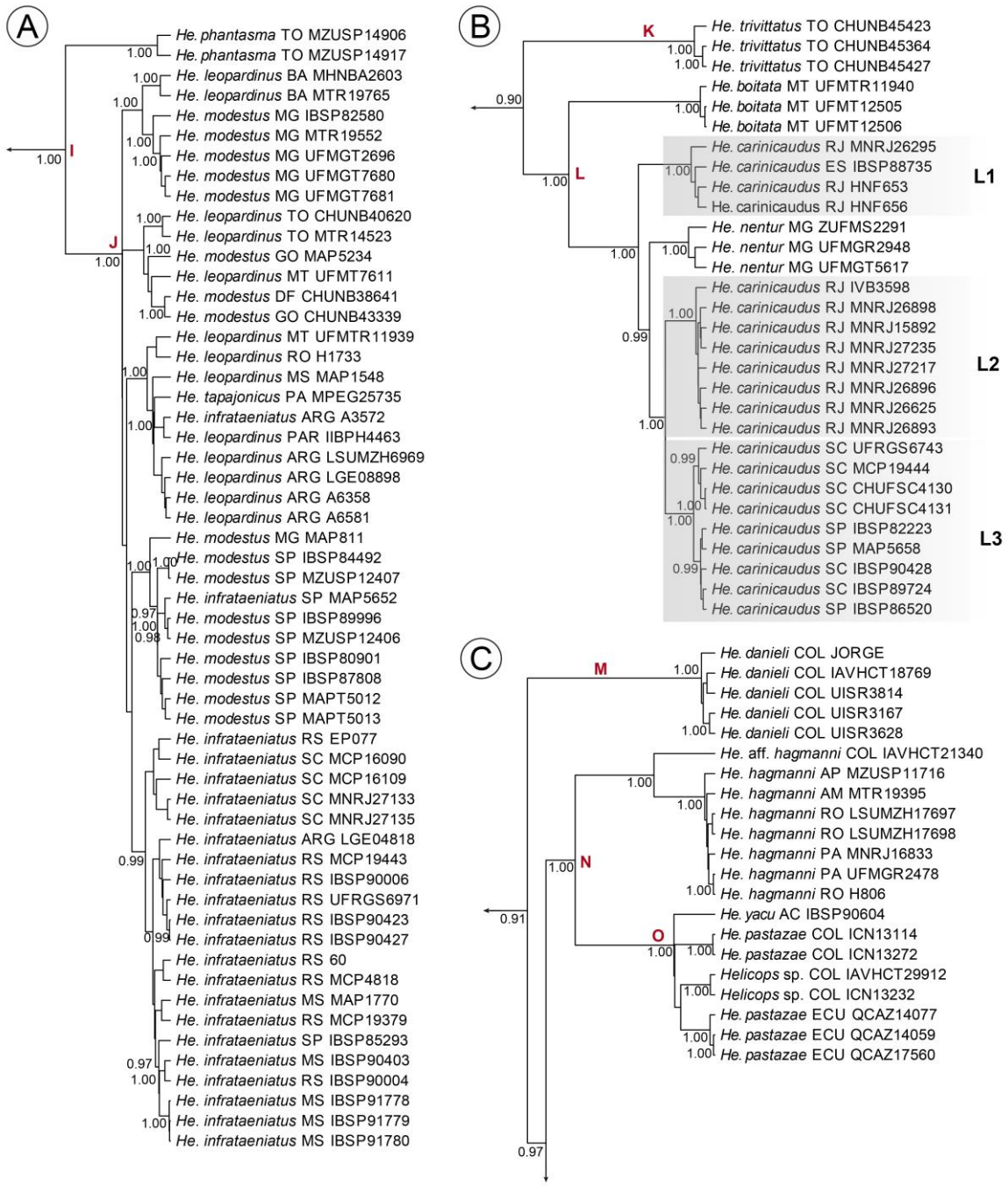


Figure 4. Bayesian phylogenetic inferences based on mtDNA (16S and Cytb) and nuDNA (Cmos, NT3, BDNF, and R35) genes using BEAST. Posterior probabilities (PP) values are ≥ 0.95 (we present only well-supported node values). (**A**) *Helicops leopardinus* group and *He. leopardinus* complex. Clade I: *He. leopardinus* group; Clade J: *He. leopardinus* complex. (**B**) *Helicops trivittatus* and *He. carinicaudus* group. Clade K: *He. trivittatus*; Clade L: *He. carinicaudus* group. (**C**) *Helicops danieli*, *He. hagmanni* group and *He. pastazae* complex. Clade M: *He. danieli*; Clade N: *He. hagmanni* group; Clade O: *He. pastazae* complex.

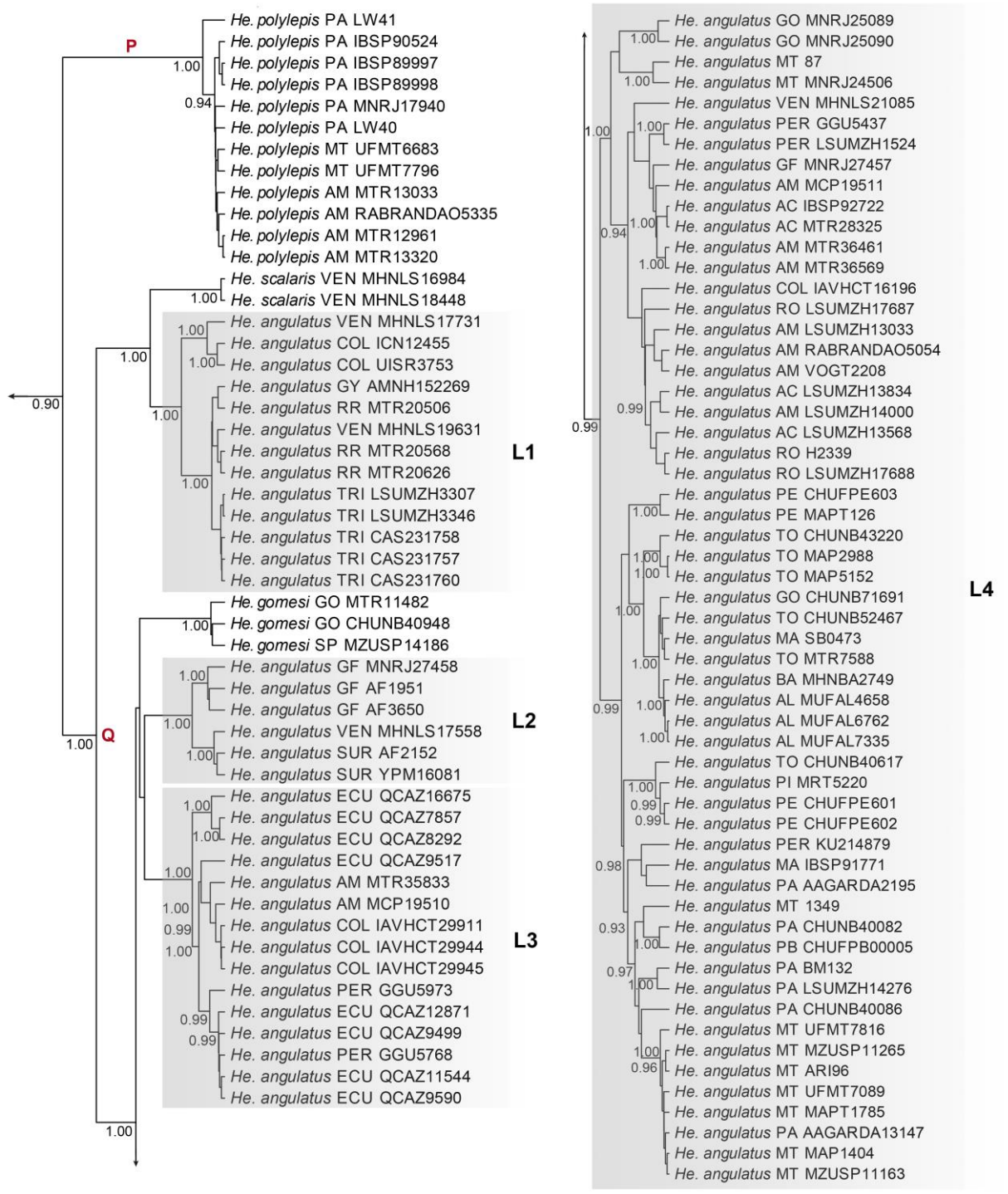


Figure 5. Bayesian phylogenetic inference of *He. polylepis*, *He. angulatus* group and *He. pastazae* complex based on mtDNA (16S and Cytb) and nuDNA (Cmos, NT3, BDNF, and R35) genes using BEAST. Posterior probabilities (PP) values are ≥0.95 (we present only well-supported node values). Clade P: *He. polylepis*; Clade Q: *He. angulatus* group. L: indicates distinct lineages.

2.4. Discussion

Tribe monophyly and the relationship among the genera

Hydropsini is robustly supported as monophyletic (PP=1.00; BS=98%) (Fig. 2 and Fig. S1). Here, we generated 1.012 novel DNA sequences from 221 specimens belonging to Hydropsini with a wide geographic coverage. We emphasize that this is the first approach that have tested the phylogenetic relationship of *Hydrops caesurus*, *Hy. relictualis*, *Helicops danieli*, *He. pastazae*, *He. scalaris*, *He. trivittatus* and *He. yacu*. We added for the first time exclusively trans-Andean lineages of *Helicops* species (*He. danieli* and *He. scalaris*). Our tree has 85% of the known diversity of the tribe (22 of the 26 recognized species). It was not possible to sample *He. acangussu*, *He. apiaka*, *He. hogei* and *He. petersi*, since DNA sample of these species were not available. We consider *He. cyclops* as synonym of *He. angulatus* (see Discussion below). Previous molecular studies focusing on supra-generic relationships have demonstrated the monophyly of the Hydropsini, confirming the hypothesis presented by Roze (1957b). However, such studies had a limited species sampling, which makes it difficult to taxonomically identify problematic species, cryptic species and phylogenetic relationships (e.g., Vidal et al. 2000; Zaher et al. 2009; Pyron et al. 2011; 2013; Figueroa et al. 2016). A recent study about the phylogeny of mud snakes (Homalopsidae) with a large taxon and geographic sampling have observed an undescribed diversity and, conversely, several indistinct species under different names (Bernstein et al. 2021). Even though, the Hydropsini monophyly was always unquestionable. Notwithstanding, the relationship between *Helicops*, *Hydrops* and *Pseudoeryx* have been uncertain. For example, Vidal et al. (2000) using mitochondrial DNA sequences recovered *Helicops* as sister to a clade *Hydrops* + *Pseudoeryx*. However, our inference suggests that *Pseudoeryx* and *Hydrops* sister taxa, which form a clade that are the sister group of *Helicops*, a relationship most commonly recovered in other phylogenies as Pyron et al 2013; Figueroa et al. 2016; Zaher et al. 2019; Murphy et al. 2020; Moraes-da-Silva et al. 2021. Zaher (1999) considers that *Hydrops* and *Pseudoeryx* do not have two derived hemipenial character states, which is present in all Xenodontinae and also in *Helicops*, confirming the close relationship between *Hydrops* and *Pseudoeryx*. On the other hand, Roze (1957b) proposed that characters such as the presence of a diastema, scales, and color pattern place *Hydrops* and *Helicops* more closely related. However, these characteristics pointed out by Roze (1957b) do not support the relationship of a clade formed by *Helicops* and *Hydrops*. Therefore, when comparing molecular and morphological inference results, the morphological data for this tribe

are incongruent due to the various polymorphic and/or homoplastic characteristics, as they possibly undergo a lot of selective pressure (e.g., Moraes-da-Silva et al. 2019; 2021).

Hydropsini Dowling, 1975 (Clade A; Fig. 2)
Type-genus: *Hydrops* Wagler, 1830.
Content: (three genera). *Pseudoeryx* Fitzinger, 1826; *Hydrops* Wagler, 1830; *Helicops* Wagler, 1828.

Pseudoeryx (Clade B; Fig. 2 and 3)
Content: (one species). *Pseudoeryx plicatilis* (Linnaeus, 1758).
Comments: We recovered two lineages for *P. plicatilis*, which is currently divided into two subspecies (Fig. 3; Fig. S2). Linnaeus described *Pseudoeryx plicatilis* with the wrong type locality (Ternataeis). Based on a single specimen Cope (1885) described *P. mimeticus* from Marmoré, Bolivia. Ten years later, Bocourt (1895) described the subspecies *Pseudoeryx p. anomalepis* (MNHN-RA-0.6165), based on a single specimen from Colombia, according to the original description. Although on the MNHN website (https://www.mnhn.fr/fr/reptiles) inform that the type material of *P. p. anomalepis* was collected in Napo, Colombia, they still report a second type material (MNHN-RA-1881.519, collection date 1881-12-01). Possibly, M. Wiener collected these two specimens around 1880 and Bocourt used only one to describe *P. p. anomalepis*. The type locality of this subspecies is imprecise. Bocourt informed only that it is from Colombia, while the MNHN website informs Napo, Colombia. Currently, the Napo department is located in Ecuador and there is no province or department in Colombia named Napo. However, there was a possible confusion between Colombia and Gran-Colombia by the author. Gran Colombia was a country established in 1819 with the union of Venezuela, New Granada, Panama and Ecuador, and was dissolved in 1831, and therefore, with this consolidation Napo belonged to that country. Although at the time of collection (~1880) Gran-Colombia had already been dissolved and the countries (Venezuela, Colombia, Ecuador) were arranged as currently known. Roze (1957a) points out that apparently *P. p. anomalepis* would be a valid subspecies, but the genus needed a revision. Roze (1957a) stated that two valid species of *Pseudoeryx* may exist: *P. plicatilis* and *P. mimeticus*, but a detailed taxonomic revision study would be necessary. Mertens (1965) described *P. p. ecuadorensis* based on a single specimen from Ecuador without an exact type locality. The author made a brief taxonomic review of the genus dealing with taxonomic issues involving the other subspecies, besides he synonymized *P. p. anomalepis* with *P. p. plicatilis*. Mertens analyzed five

individuals from Brazil and Paraguay and four specimens from British Guyana from the British Museum via Boulenger 1894: 186 of *P. p. plicatilis*. Mertens, based on these nine specimens of *P. p. plicatilis* and on the plates of Seba (1734: 92, Pl. 57, Fig. 4B) and Jan and Sordelli (1868: 7, Pl. 5 Fig. 2–3; SMF34576, Brazil) considered the male *P. plicatilis* type from ventral (131) and subcaudal (47) scales. Likewise, Mertens separates the three subspecies through chromatic pattern and geographic distribution, *P. p. plicatilis* occurring from northern and eastern South America (from Colombia to northern Argentina). On the other hand, the other subspecies inhabited only areas east of the Andes, *P. p. mimeticus* occurred only in Bolivia and *P. p. ecuadorensis* only in Ecuador. Furthermore, Mertens considered that it would be very unlikely that these three subspecies would occur in sympatry, and that it would be impossible that these subspecies would be connected by intergrades. However, he based his geographic decisions on a small sampling (nine individuals).

With this scenario in place, Mertens (1965) designated that the type locality of *P. p. plicatilis* is Suriname ("Für *Coluber plicatilis* LINNAEUS wird als terra typica Holländisch - Guayana designiert"), although many subsequent works have not observed such information (e.g., Peters & Orejas-Miranda 1970; Dixon & Soini 1986; Cunha & Nascimento 1993; Wallach et al. 2014). However, we emphasize that these geographic and phenotypic limits are not corroborated on the basis of molecular data. The overlap of quantitative and qualitative characters among *Pseudoeryx* subspecies has already been observed in several studies, which makes it difficult to establish geographic and nomenclatural limits (e.g., Hoge & Nina 1960–1962; Dixon & Soini 1986). Because of this, Dixon and Soini (1986) synonymized *P. p. ecuadorensis* with *P. p. mimeticus*. For these reasons, we decided to call *Pseudoeryx plicatilis* lineage 1 (L1) and *Pseudoeryx plicatilis* lineage 2 (L2). *Pseudoeryx* L2 has a distribution near to Suriname, it occurs from French Guiana to Peru, so therefore what Mertens defined does not work (Fig. 3; Fig. S2). Thereby, we will not consider Suriname the type locality of *P. p. plicatilis* and we emphasize that a taxonomic and systematic review is essential, mainly on the type locality, in order to reveal which of the lineages is really the nominal species and which of the subspecies could be elevated to species level. All this confusion of various subspecies was caused by color pattern and low sampling size. The authors who described the subspecies relied only on a single specimen. A large sample size is critical for integrative and systematic taxonomy. Even our study, being the largest to date, using 12 specimens from 10 different locations, was not possible to resolve all these taxonomic issues.

Hydrops (Clade C; Fig. 2 and 3)

Content: (four species). *Hydrops caesurus* Scrocchi, Ferreira, Giraudo, Avila and Motte, 2005; *Hy. martii* (Wagler, 1824); *Hy. relictualis* comb. nov. (Schargel, Rivas-Fuenmayor, Barros, Péfaur and Navarette, 2007); *Hy. triangularis* (Wagler, 1824).

Generic replacement of *Pseudoeryx relictualis* (Clade D; Fig. 2 and 3)

Comments: According to our topology, the species *Pseudoeryx relictualis* is not monophyletic, and given its position, we decided to relocate *Pseudoeryx relictualis* to the genus *Hydrops* (Fig. 3). Therefore, named here *Hy. relictualis* **comb. nov**. In addition, we highlight some morphological characters present in literature that are shared between species of *Hydrops*, and that are not found in *Pseudoeryx*. *Hydrops relictualis* presents the last two maxillary teeth considerably larger and separated from the others by a diastema and nuchal collar (Schargel et al. 2007). Probably the bandless color pattern was decisive for Schargel et al. (2007) discarding the possibility of being a species belonging to the genus *Hydrops*. In their study, Schargel et al. (2007) compare *P. plicatilis* and *Hy. relictualis* using various coloring characters. In addition, the authors point out that the diastema and the nuchal collar are the two main differences between the species, but such characteristics are precisely observed in *Hydrops* (Roze 1957b; Albuquerque 2000; Albuquerque & Lema 2008). The hemipenis of *Hy. relictualis* was not described, since *Pseudoeryx* and *Hydrops* have very distinct hemipenial morphologies, this phenotypic evidence may reinforce the relocation of *Hy. relictualis* within *Hydrops*. It is worth mentioning that, previously, *Hydrops* was already synonymized with *Pseudoeryx* by Bocourt (1895). Even though they are two very different genera, Dunn (1944) described *Hy. lehmanni* based on a single specimen, which Roze (1957a) later identified as *P. plicatilis*.

Hydrops caesurus and *Hydrops martii* (Clade E and F; Fig. 2 and 3)

We recovered *Hy. caesurus* and *Hy. martii* two successive outgroups to *Hy. triangularis*, a monophyletic clade with maximum support in both analyses. This is the first study that includes *Hy. caesurus* in a phylogeny. The species was previously called *Hy. t. bolivianus*, until its formal description as a new species by Scrocchi et al. (2005). *Hydrops caesurus* is more closely related to *Hy. martii* than *Hy. triangularis*. The taxonomic history of *Hydrops* was confusing mainly because the species and subspecies were described from variations in polymorphic characters of a limited number of specimens (Roze 1957b). Subsequent studies have managed to solve in some way the taxonomic enigma of *Hydrops* (Albuquerque 2000; Albuquerque & Lema 2008). We reinforce the results of previous studies on the validity of the species *Hy. martii* and *Hy. triangularis* (Albuquerque 2000; Albuquerque & Lema 2008). Both species have

widely overlapped geographic distributions, and are often found in syntopy (von May et al. 2019). Despite this, our results show that *Hy. martii* and *Hy. triangularis* are valid species, and we highlight that *Hy. triangularis* harbors more than one species under this name.

Hydrops triangularis (Clade G; Fig. 2 and 3)

Comments: We recovered two well-supported lineages for *Hydrops triangularis*. Since the type locality of *Hy. triangularis* is in Lake Tefé, Rio Amazonas, Amazonas, Brazil, it was not possible to designate which lineage it belongs due to the gap in our sampling for the state of Amazonas. *Hydrops triangularis* L1 occurs in the eastern region of the Amazon and *Hy. triangularis* L2 is found in the western region (Fig. 3; Fig. S2). The nearest distance from the type locality within our data (eastern and western population) is 1000 and 580 km, respectively. Since we do not have topotypes of the subspecies named by Roze (1957b), it was unreasonable to indicate which possible taxa can be considered for the unnamed lineage. We have a sample (LSUMZ-H 3105) from Loreto, Peru that is ~125 km southwest of the type locality of *Hy. t. bassleri*, which represents the western clade of *Hy. triangularis* and another sample MZUSP 19497 from UHE Jirau, Rondônia which is ~300 km southwest of the type locality of *Hy. t. bolivianus* representing the eastern clade of *Hy. triangularis*.

Helicops (Clade H; Fig. 2, 4, and 5)

Content: (22 species). *He. acangussu* Moraes-da-Silva, Walterman, Citeli, Sales-Nunes and Curcio, 2022; *He. angulatus* (Linnaeus, 1758); *He. apiaka* Kawashita-Ribeiro, Ávila and Morais, 2013; *He. boitata* Moraes-da-Silva, Amaro, Sales-Nunes, Strüssmann, Teixeira, Andrade, Sudré, Recoder, Rodrigues and Curcio, 2019; *He. carinicaudus* (Wied-Neuwied, 1824); *He. danieli* Amaral, 1938; *He. gomesi* Amaral, 1922; *He. hagmanni* Roux, 1910; *He. hogei* Lancini 1964; *He. infrataeniatus* Jan, 1865; *He. leopardinus* (Schlegel, 1837); *He. modestus* Günther, 1861; *He. nentur* Costa, Santana, Leal, Koroiva and Garcia, 2016; *He. pastazae* Shreve, 1934; *He. petersi* Rossman, 1976; *He. phantasma* Moraes-da-Silva, Amaro, Sales-Nunes, Rodrigues and Curcio, 2021; *He. polylepis* Günther, 1861; *He. scalaris* Jan, 1865; *He. tapajonicus* Frota, 2005; *He. trivittatus* (Gray, 1849); *He. yacu* Rossman and Dixon, 1975.

Comments: Given the great diversity of species within this genus, several clades strongly supported, and aiming to indicate a better phylogenetically supported taxonomic organization, we allocated the species into four groups and two complexes as follows: *Helicops leopardinus* group, *Helicops carinicaudus* group, *Helicops hagmanni* group, *Helicops angulatus* group.

Furthermore, *Helicops trivittatus*, *Helicops danieli* and *Helicops polylepis* were not assigned to any species group.

The *Helicops leopardinus* group (Clade I; Fig. 2 and 4A)

Content: (five species). *He. infrataeniatus* Jan, 1865; *He. leopardinus* (Schlegel, 1837); *He. modestus* Günther, 1861; *He. phantasma* Moraes-da-Silva, Amaro, Sales-Nunes, Rodrigues and Curcio, 2021 and *He. tapajonicus* Da Frota, 2005.

Comments: Our topologies revealed that *Helicops* with a green back color pattern (smooth or striated) do not form a natural group, which corroborates previous molecular studies (Moraes-da-Silva et al. 2019; 2021). Overall, the color pattern within Hydropsini needs to be carefully analyzed, as it is a polychromatic characteristic. Besides, our results corroborate the findings of Moraes-da-Silva et al. (2021), which recovered *He. phantasma* as sister taxa of the remaining species of the *He. leopardinus* complex.

The *Helicops leopardinus* complex (Clade J; Fig. 2 and 4A)

Our analyzes revealed that the species *He. modestus*, *He. infrataeniatus*, *He. tapajonicus* and *He. leopardinus* formed one clade, and we decided named *He. leopardinus* complex. None of those species was recovered as monophyletic, which could represent a single high polymorphic lineage (i.e., a single species) (Fig. 4A; Fig. S3. However, due to the long now history of the names available for these species, a proper phylogeographic study is paramount to understand the evolutionary history and, consequently, the species taxonomy. To better understand the paraphyly of these species, an integrative taxonomic review must be done to clarify the status of the species within this clade. *Helicops leopardinus* has priority over the other names, however the type locality of *He. leopardinus* is unknown, whereby we decide to not make any taxonomic decision, until further information appears. Previously, it was pointed out that the species *Helicops leopardinus* presented a paraphyletic/polyphyletic group (Moraes-da-Silva 2019; 2021), something we also found in our analysis. In the description of *He. tapajonicus* (Frota 2005), was pointed out that the resembled with *He. leopardinus* in the number of dorsal scales, ventral scales and teeth, but which had smaller lobes on the hemipenis and a very distinct color pattern (no spotted). Still, *He. tapajonicus* is sympatric and syntopic with *He. leopardinus*, occurring in the same microhabitat in the municipalities of Belterra and Santarém, Pará, Brazil. Thus, possibly the different coloration patterns (polychromatism) may be the only characteristic that differentiates these species. Because it has such plasticity, the color pattern has to be used

with caution in this tribe to delimit species. We emphasize the importance of perform phylogeography study of *He. leopardinus* complex to understand their evolutionary history.

Helicops trivittatus (Gray, 1849) (Clade K; Fig. 2 and 4B)

Comments: The phylogenetic relationship of *He. trivittatus* had not been tested before. *Helicops trivittatus* was described by Gray (1849) as *Myron* Gray 1849, a genus of water snake found in Asia in the family Homalopsidae, due to incorrect location information. In fact, several species of this family considerably resemble *Helicops* in external morphology due to evolutionary convergence exerted by the aquatic environment (Rossman 2010).

The *Helicops carinicaudus* group (Clade L; Fig. 2 and 4B)

Content: (three species). *He. boitata* Moraes-da-Silva, Amaro, Sales-Nunes, Strüssmann, Teixeira, Andrade, Sudré, Recoder, Rodrigues and Curcio, 2019; *He. carinicaudus* (Wied-Neuwied, 1824); *He. nentur* Costa, Santana, Leal, Koroiva and Garcia, 2016.

Comments: *Helicops boitata* was recovered as a sister species of *He. carinicaudus* and *He. nentur* with well support, in concordance with previous studies by Moraes-da-Silva et al. (2019, 2021). *Helicops carinicaudus* and *He. nentur* occurs in the south and southeast of Brazil, and their relationship is strong geographically structured (Fig. S4). The other species of the group, *He. boitata*, is endemic to the Pantanal (Fig. S4) with no phylogenetic proximity to the species that inhabit the same region (*He. angulatus*, *He. leopardinus* and *He. polylepis*) (Moraes-da-Silva et al. 2019). We recovered *He. carinicaudus* as paraphyletic (Fig. 4B), the nominal species coincides with *He. carinicaudus* L1, because the type locality is Rio Itapemirim, state of Espírito Santo, Brazil (Fig. S4), while the other two candidate species would not have available names (*He. carinicaudus* L2 and L3). Although *He. carinicaudus* has already been considered a subspecies of *He. infrataeniatus* (Lema 1958), both species are not phylogenetically related.

Helicops danieli Amaral, 1938 (Clade M; Fig. 2 and 4C)

Comments: *Helicops danieli* as sister species of the *He. hagmanni* group, *He. polylepis* and *He. angulatus* group (Fig. 4C). We tested for the first time the phylogenetic relationship of *He. danieli*. We highlight that the Cis-Andean *Helicops* species, *He. scalaris* and *He. danieli*, did not form a clade. Amaral (1937–1938), when he described this species, postulated that *He. danieli* would be phylogenetic close to *He. angulatus* and *He. scalaris*. Medem (1968) and Dugand (1975) mentioned that *He. danieli* might be synonymous with *He. scalaris* because

they consider that both species are morphologically similar. However, no later studies synonymize them (Rossman 2002a), and according to our results *He. danieli* is a valid species.

The *Helicops hagmanni* group (Clade N; Fig. 2 and 4C)

Content: (five species). *He. acangussu* Moraes-da-Silva, Walterman, Citeli, Sales-Nunes and Curcio, 2022; *He. hagmanni* Roux, 1910; *He. pastazae* Shreve, 1934; *He. petersi* Rossman, 1976 and *He. yacu* Rossman and Dixon, 1975.

Comments: *Helicops hagmanni* group was recovered with well support in both analyzes (PP=1.00; BS=100%). *Helicops hagmanni* species is closely related to *He. pastazae* complex (Fig. 4C). *Helicops polylepis* was not recovered in BI as belonging to *He. pastazae* complex (see discussion of *He. polylepis* below). Therefore, due to the contrasting result between both methods (BI and ML; Fig. 2, 4C and 5; Fig. S1), we decided to remove *He. polylepis* from the section defined by Rossman (1976) and include *He. hagmanni*, renaming it *Helicops hagmanni* group. Rossman (1976) when coined the *polylepis* section grouping highlighted that *He. hagmanni* had some characteristics with the species this section, nevertheless the author decides not allocated the *He. hagmanni* with this grouping. In previous studies, *He. hagmanni* has been recovered as a sister species of *He. infrataeniatus*, *He. carinicaudus*, *He. gomesi* and *He. angulatus* (Grazziotin et al. 2012; Pyron et al. 2013; Figueroa et al. 2016). The most current inference using only 11 species of *Helicops* (Moraes-da-Silva et al. 2021) recovered *He. hagmanni* as the sister group of the clade composed by, *He. polylepis He. gomesi*, *He. angulatus*. However, these relationships are an artifact of the lack of sampling of other *Helicops* species, mainly species that also occur outside Brazil. Our results differ substantially from the previous studies, and our inference recovered *He. hagmanni* sister species to *He. pastazae* complex. The specimen of *He. hagmanni* IAVH-CT21340 from Colombia is morphologically similar to *He. hagmanni* (N. Citeli pers. comm.), but we consider it *affinis He. hagmanni*, which separate it from the other individuals of *He. hagmanni* (Fig. S5) because this sample has five of the six genes sequenced, lacking only the Cmos gene, this differentiation is not an artifact of missing data.

Furthermore, an integrative taxonomic review is urgent to assess the status of *He. hagmanni* as, for example, dorsal scales vary between 23–29 in different populations. It was not possible to test the phylogenetic relationship of *He. acangussu* because we did not have access of tissue samples. Morphologically, *He. acangussu* is more similar to *He. hagmanni*, and occurs in sympatry with *He. hagmanni*, *He. leopardinus* and *He. polylepis* and can be confused with these species due of the spotted color pattern (Moraes-da-Silva et al. 2022). Nevertheless, *He.*

acangussu is oviparous, like *He. hagmanni*, while *He. leopardinus* and *He. polylepis* are viviparous. For that reason, we tentatively include *He. acangussu* in *He. hagmanni* group.

The *Helicops pastazae* complex (Clade O; Fig. 2 and 4C)

Content: (three species) *He. pastazae* Shreve, 1934; *He. petersi* Rossman, 1976 and *He. yacu* Rossman and Dixon, 1975.

Comments: This is the first time that the phylogenetic relationship of *He. pastazae* and *He. yacu* are considered. It is noteworthy that Rossman and Abe (1979) observed that *He. yacu* and *He. pastazae* shared several qualitative and quantitative characteristics, and pointed out that more specimens from northeastern Peru would likely reveal that *He. yacu* is a subspecies of *He. pastazae* from the Upper Amazon Basin. Rossman and Dixon (1975) in the description of *He. yacu* points out that the dorsal color pattern of this species resembles in parts *He. hagmanni*. We decided to consider *He. yacu* is a valid species pending an integrative taxonomic review. However, the clade that includes *He. yacu* and *He. pastazae* is monophyletic with maximum support values in both analyses. Therefore, we decided to include *He. yacu* in *He. pastazae* complex (Fig. 4C; Fig. S5). We opt to name *He. pastazae* complex in order to keep their actual taxonomy. However, we highlight the need for a taxonomic revision within this clade, given the molecular and variation found.

It was not possible to include in our analysis *He. pertersi*, an endemic species of the upper watershed of the Napo River in eastern Ecuador (Arteaga 2021). This species is commonly confused with *He. pastazae* due to sympatry and for having several overlapping morphological characteristics (Rossman 1976). The specimens IBSP90604 do not present the morphological diagnosis of *He. petersi* (N. Citeli pers. comm.). We highlight the need for an integrative systematic review and a more comprehensive sampling of *He. hagmanni* group to solve the systematics of this group.

Helicops polylepis Günther, 1861 (Clade P; Fig. 2 and 5).

Comments: *Helicops polylepis* was the sole species with contrasting placement in the two topologies. In BI it is a sister species of *He. angulatus* group with moderate support value (PP=0.90) (Fig. 5), while in ML it was recovered with low support value as sister group of *He. hagmanni* group (BS=35%). Even though the support value is moderate in BI, we decided to abandoned the name *polylepis* section coined by Rossman (1976). The most current inferences from Hydropsini recovered *He. polylepis* as the sister group of *He. gomesi* and *He. angulatus* with high support values (Moraes-da-Silva et al. 2019). However, the lack of a good sampling

probably hampers the solution for the positioning of *He. polylepis*. Rossman (1976) was the only author to propose a phylogenetic hypothesis between *Helicops* species and coined the following groups: *polylepis* section (*He. pastazae*, *He. petersi*, *He. polylepis* and *He. yacu*) and the *He. pastazae* complex (*He. pastazae* and *He. petersi*). The author considered phenotypic characteristics such as scale count and color pattern to propose their relationships. This relationship was not supported by molecular data among *He. polylepis*, *He. pastazae*, *He. petersi* and *He. yacu*, and demonstrates that several qualitative and quantitative characters in Hydropsini are plastic and can cause instability in the systematics of the tribe.

Helicops hogei Lancini, 1964

Lancini (1964) described *He. hogei* based on a single specimen (MCNC 462, female) from Rio Autana, Amazonas, Venezuela, and later Pérez-Bravo (1976–1977) reported a second specimen (MBUCV 8849, female) from Rio Cuybini, Cerro La Paloma, Sierra Imataca, Delta Amacuro, Venezuela. Rossman (2002b) contested the taxonomic status of *Helicops hogei*, which he compared *He. scalaris* with a photo of the back of *He. hogei* (provided by Vanda Ferreira and Rubens Yuki) as well as descriptions of the literature (Lancini 1964 and Pérez Bravos 1976–1977). Apparently, the two females of *He. hogei* are morphologically indistinguishable from females of *He. scalaris*. Rossman (2002b) declared to be convinced that *He. hogei* represents a junior synonym of *Helicops scalaris* ("To my mind, the evidence seems convincing that *Helicops hogei* Lancini is, in fact, a junior synonym of *He. scalaris* Jan"), however, he did not fulfill the taxonomic needs to perform the synonym. The International Code of Zoological Nomenclature (ICZN) demand that the taxonomist be clearer, stating that the researcher is synonymizing the species. Moreover, Rossman reports his concern about the gap in the geographic distribution of *He. scalaris*, in case that *He. hogei* was a synonym of *He. scalaris*, which is only found in the western Andes, in the Lake Maracaibo region. He considered that there may have been a possible mislabeling of the specimen collection initially known as *He. hogei*. Probably because of this ambiguous quote by Rossman (2002b) some studies consider *He. hogei* synonymous of *He. scalaris* (Frota 2005; Kawashita-Ribeiro et al. 2013; Costa et al. 2016; Moraes-da-Silva 2019; 2021; 2022), while other studies disregarded this information, but point out that the species needs a reassessment (Molina et al. 2004 "2003"; Rivas et al. 2012; Natera-Mumaw et al. 2015). This taxonomic issue will not be easily resolved, *He. hogei* also needs to be compared to *He. polylepis* and *He. hagmanni*, as these two species occur on the eastern side of the Andes and the number of specimens collected increased substantially. Although, Natera-Mumaw et al. (2015) stated that the two specimens identified as *He. hogei*

(MCNC 462 and MBUCV 8849) are missing, and they found two more unidentified specimens from Puerto Ayacucho, Queseras, Amazonas collected on September 21, 1967 (USNM 21786) and Puerto Ayacucho, Caño Pavones, Amazonas collected on September 2 February 1981 (MBUCV 7039), which are also lost. The authors suggest that all specimens (four) were borrowed and, so far, have not returned (approximately 22 years).

The *Helicops angulatus* group (Clade Q; Fig. 2 and 5)

Content: (four species). *He. angulatus* (Linnaeus, 1758); *He. apiaka* Kawashita-Ribeiro, Ávila and Morais, 2013; *He. gomesi* Amaral, 1922, and *He. scalaris* Jan, 1865.

Comments: *Helicops angulatus* group was recovered with well support in both analyzes (PP=1.00; BS=100%) (Fig. 5). *He. scalaris* has been suggested as a subspecies of *He. polylepis* (Amaral 1935). Nonetheless, our results show that *He. scalaris* is embedded within the *He. angulatus* group, as sister species of one *He. angulatus* lineages (L1).

Helicops angulatus was recovered as paraphyletic and divided into four sub-clades with *He. gomesi* nested in them (Fig. 5; Fig. S6). Given the wide distribution of *He. angulatus*, besides to its bimodal reproduction, which is allopatric, some authors suspected that this species would be a complex of cryptic species (e.g., Kawashita-Ribeiro et al. 2013). Our results revealed that the *He. angulatus* L1 has populations occurring in Trinidad, Venezuela, Colombia, Guyana and Roraima (Brazil). This finding corroborated Murphy et al. (2020), which first revealed the paraphyly of *He. angulatus*. However, to keep the monophyly, the authors named the populations of Venezuela and Trinidad as *He. angulatus* and revalidated *Helicops cyclops* Cope, 1868, whose type locality is Bahia, Brazil to encompass the other populations. Although *He. angulatus* (*sensu lato*) is a species with continental distribution, and the authors did not analyze a significant sampling along its distribution. Their findings and taxonomic decisions relied on small samples from Peru (2), Brazil (4) and Guyana (2). In addition, the distribution limits of the two species and how to distinguish them morphologically were not well established due to overlapping characters.

Furthermore, *Natrix aspera* (Wagler, 1824) has a nominal preference over *He. cyclops*. According to the original description and Vanzolini (1981), *N. aspera* (= *Helicops aspera*) was collected in the expedition of Spix and Martius to Brazil, between 1817–1820 in the province of Bahia (Wagler 1824: 38 said: "*Habitat numerosus in provinciae Bahiae adjacentibus*"). The specimen (ZSM 1528/0 female) was designated as the lectotype of *N. aspera* by Hoogmoed and Gruber (1983) (see Franzen & Glaw 2007 for more information). Murphy et al. (2020) do not mention anything in the study on the type locality of *N. aspera*. Additionally, Murphy et al.

(2020) report that the *He. cyclops* holotype is ANSP 5131, but according to Malnate (1971), who made available the catalog with all primary types from the Academy of Natural Sciences, Philadelphia (ANSP), indicates that this holotype is ANSP 5183 from Bahia, Brazil.

The type locality of *He. angulatus* is unknown (Asian, in error), but apparently there is a high possibility that Linnaeus received specimens from Suriname (Murphy et al. 2020: 145). According to our topology, the population of Suriname (*He. angulatus* L2) was not recovered along with the populations of Trinidad (L1) (Fig. 5; Fig. S6). Therefore, contrasting what Murphy et al. (2020) considered, *He. angulatus* L1 is not the possible nominal clade for *He. angulatus*. Moreover, in our topology we have a sample from Bahia that belongs to *He. angulatus* L4. Therefore, this lineage would possibly be from *Natrix/Helicops aspera*. There are apparently two possible type localities available to harbor the *He. angulatus* (*sensu lato*) lineages, Suriname and Bahia, Brazil (Wagler 1824; Hoogmoed & Gruber 1983; Franzen & Glaw 2007; Murphy et al. 2020). So, this led us to two lineages with no names available. From this point of view, we did not make any taxonomic decisions and chose to keep *He. cyclops* in the synonymy of *He. angulatus*.

It was not possible to assess the phylogenetic relationship of *He. apiaka* because we did not have access to tissue samples of this species. Morphologically this species resembles *He. angulatus* (hemipenis, color pattern and overlapping dorsal, ventral, subcaudal scales) (Kawashita-Ribeiro et al. 2013), yet, both species occur in sympatry from Paranaíta, Mato Grosso, Brazil. Since the greater geographic coverage of *He. angulatus* (*sensu lato*) is present in *He. angulatus* L4, which possibly *He. apiaka* may be closely related. We highlight the need for an integrative taxonomic review of *He. angulatus*, *He. gomesi* and *He. apiaka* in the entire distribution of these species to evaluate each lineage that was revealed here, and also test whether each population (viviparous or oviparous) can actually represent distinct lineages.

2.5. Conclusions

Our results corroborate with previous inference and reveal new relationships and indicate that these snakes need fine scale investigation with respect to diversification. We tested for the first time the phylogenetic relations of several species of Hydropsini, which help to elucidate the taxonomic status and relationships within tribe, and make our work the most complete study of phylogenetic affinities of the tribe. Our phylogenetic tree does not reflect some current taxonomic arrangements for Hydropsini. We highlight the problematic taxonomy of the tribe, and do not make changes due to the lack of taxonomic revision and population

analysis (i.e., phylogeography) of all species of the tribe, except *Hy. relictualis* **comb. nov.** which we rearrange it to *Hydrops*, maintain the monophyly of the genus. Besides, we highlight the possibility of new species or synonymizations, which would reflect in the current knowledge of the systematic of Hydropsini. Therefore, to establish a congruent classification based on monophyletic clades we recommend that all these species have integrative taxonomic revisions to elucidate the cryptic diversity, especially within *P. plicatilis*, *Hy. triangularis*, *He. hagmanni*, *He. pastazae* and *He. angulatus* group. On the other hand, such integrative taxonomic revisions could also result in synonymizations as in the *He. leopardinus* group. Since several species of the tribe do not have a defined type locality (e.g., *P. plicatilis*, *He. angulatus*, *He. leopardinus*), or because we do not have topotype samples for some specimens, it was difficult to indicate possible solutions. One way to solve this problem would be to use ancient DNA approaches on deposited type specimens and try to identify which lineages revealed here they belong, thus facilitating taxonomic decision making. Our study has a robust sample covering all of South America, but in order to better understand the systematics of *P. plicatilis* and *Hy. triangularis*, some geographic gaps need to be sampled (i.e., topotypes of some of the subspecies, and more sampling from Bolivia and Peru). Here, we consider the concept of species according to de Queiroz (2007), therefore, species are lineages with distinct evolutionary history, and they do not necessarily need to have morphological differences. Thus, *Helicops angulatus* represents a species complex that needs taxonomic revision. Although our results provide resolution for several clades, others remain problematic.

Our results shed light on several evolutionary questions, understanding evolutionary relationships in Hydropsini can generate insights that will facilitate the understanding of evolution of adaptations and evolutionary processes. Future analyzes should pay special attention to the following issues: to test hypotheses about the evolutionary pressures that favor the origin of viviparity in the tribe and reproductive bimodality in *He. angulatus*, center of origin and dispersion of the tribe, morphological polymorphisms and polychromatisms. Furthermore, it would be interesting to do a species delimitation, DNA barcode study in Hydropsini to molecularly verify the threshold of genetic differentiation between the species of this tribe, since there is so much overlap and polymorphism in some morphological characters. Possibly, each clade will have a different barcode gap.

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Supplementary information

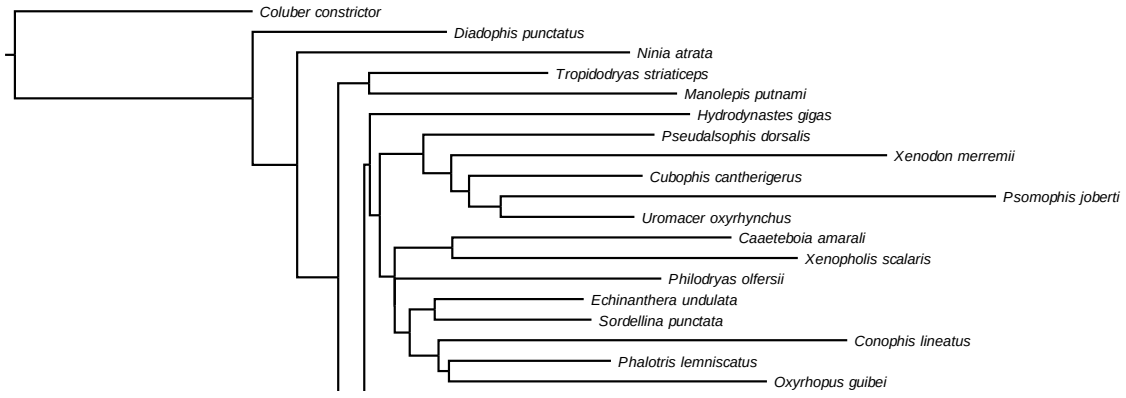


Figure S1. Maximum likelihood (ML) phylogenetic inference of Hydropsini tribe based on concatenated mtDNA (16S and Cytb) and nuDNA (Cmos, NT3, BDNF, and R35). Numbers below nodes are bootstrap values (BS) ≥ 70% (we present only well-supported node values).

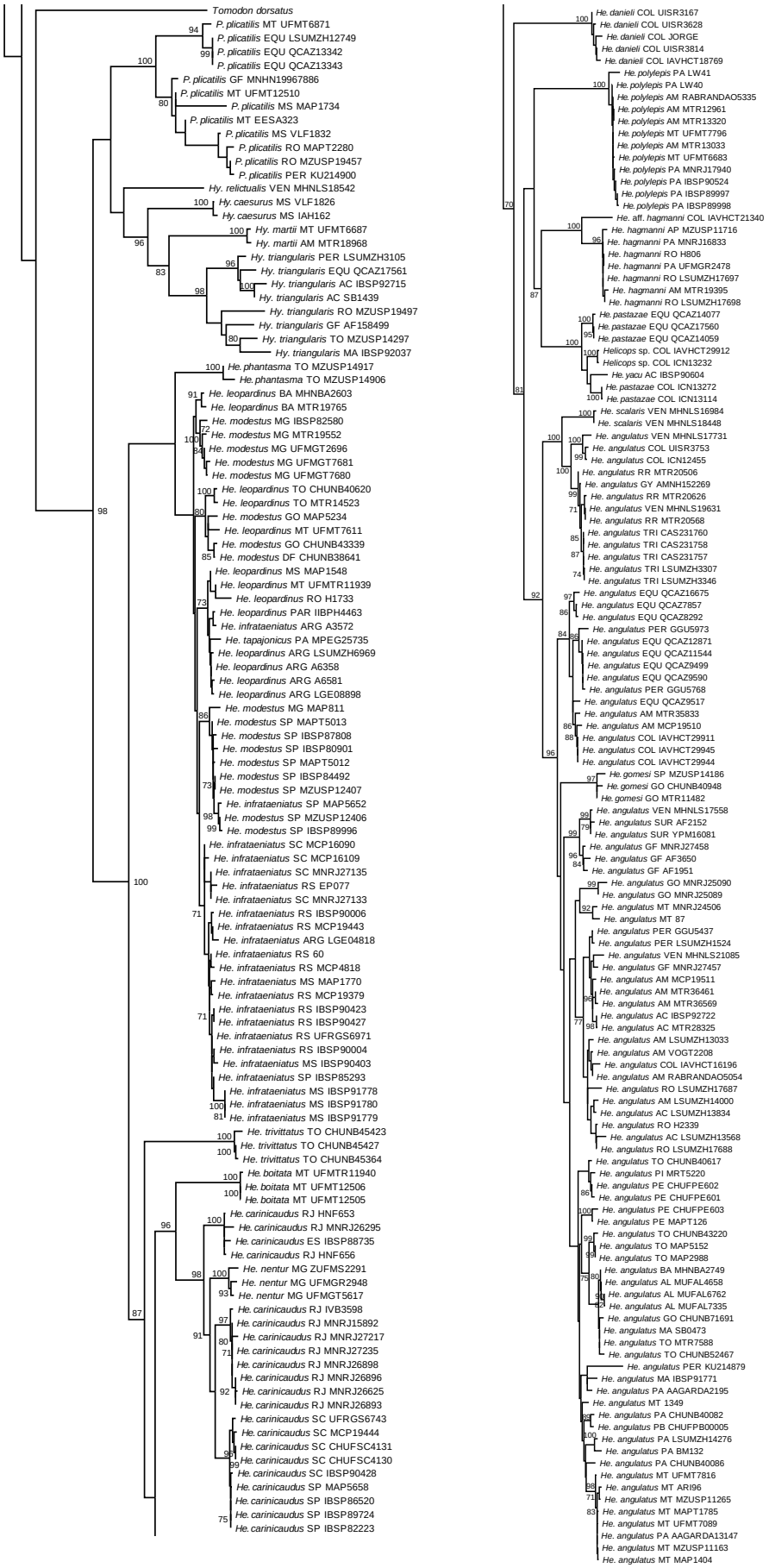


Figure S1. (to be continued)

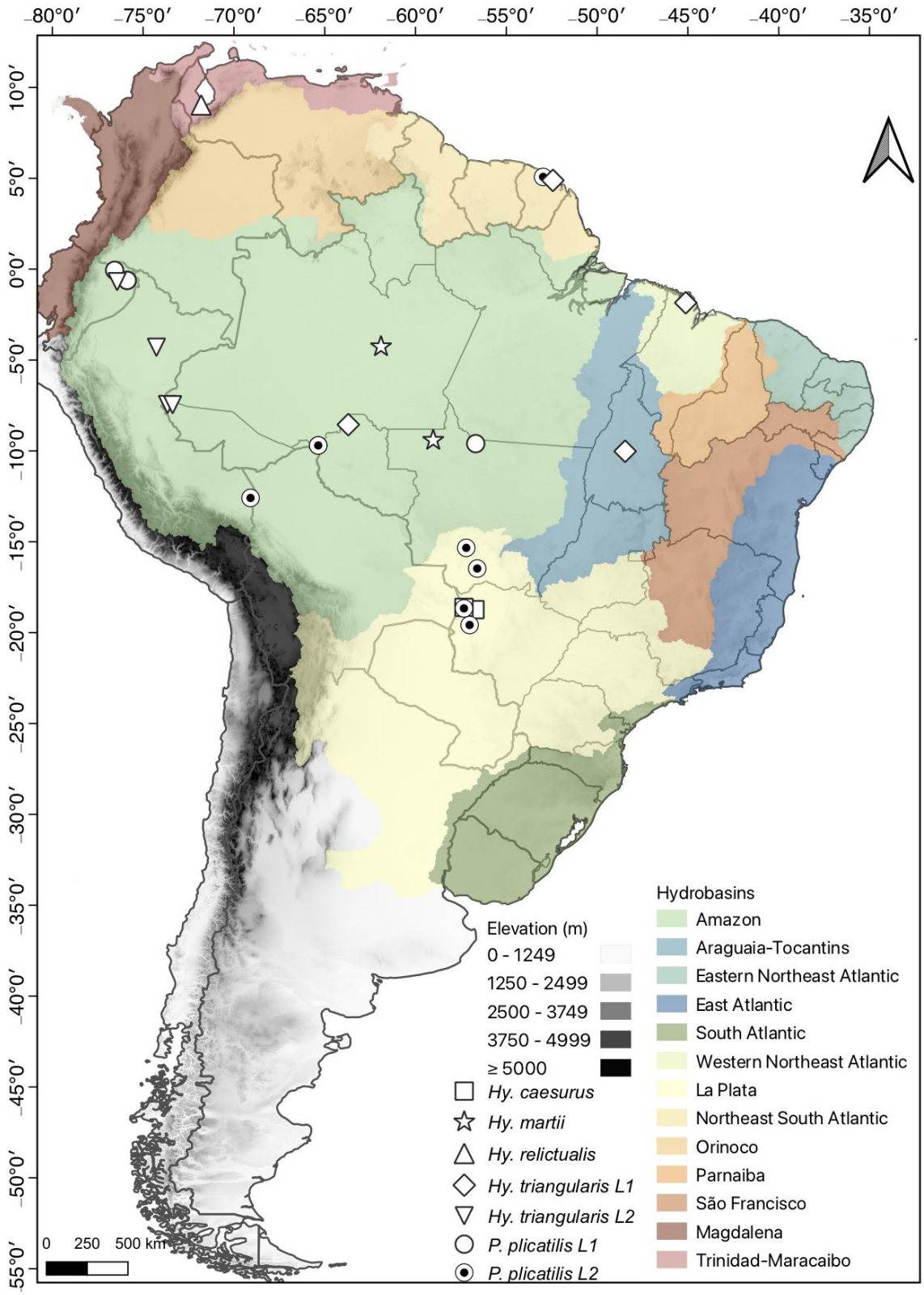


Figure S2. Geographic distribution of *Pseudoeryx* and *Hydrops* samples from this study along six watersheds across South America.

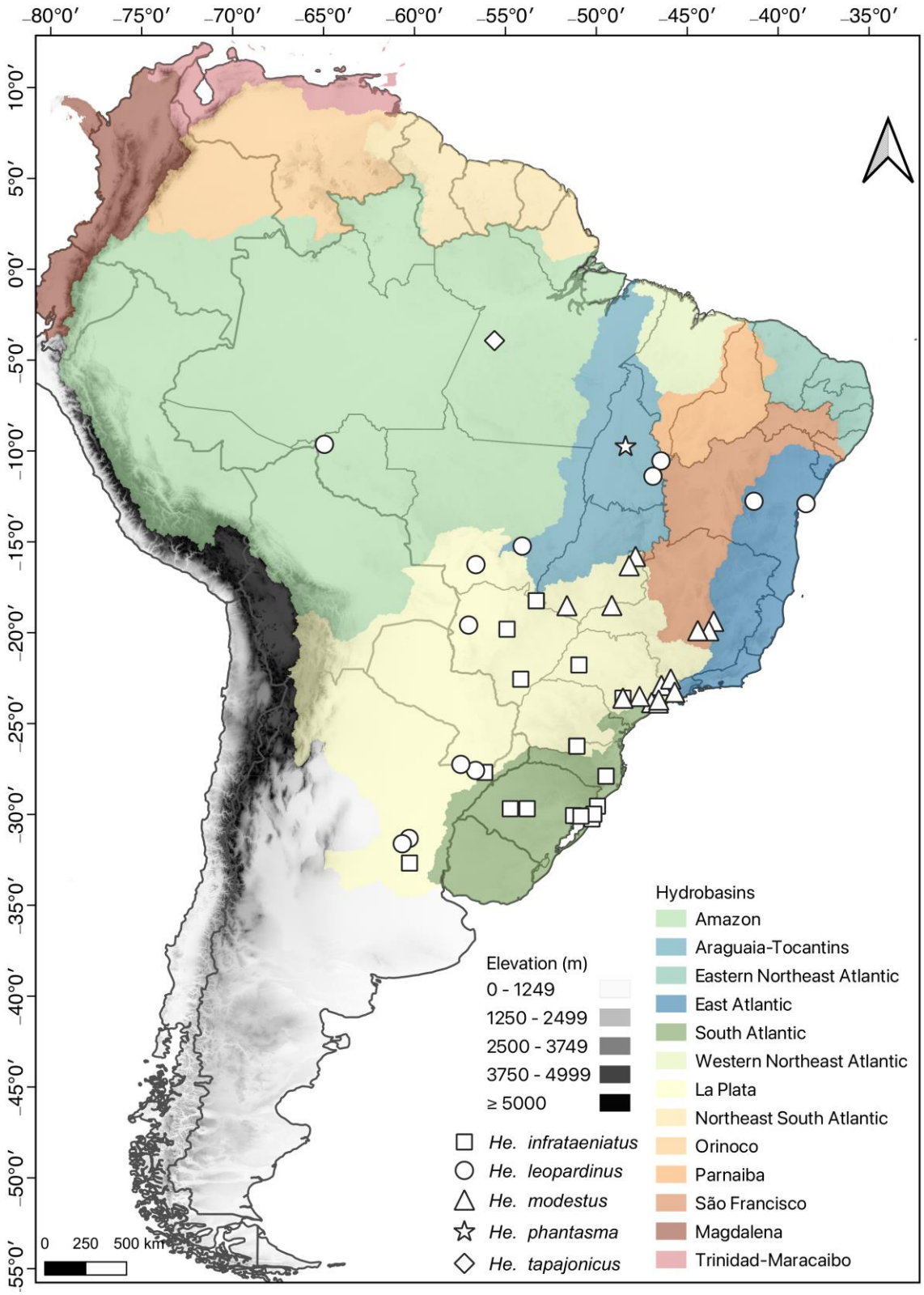


Figure S3. Geographic distribution of *Helicops leopardinus* group and *He. leopardinus* complex samples from this study along six watersheds across South America.

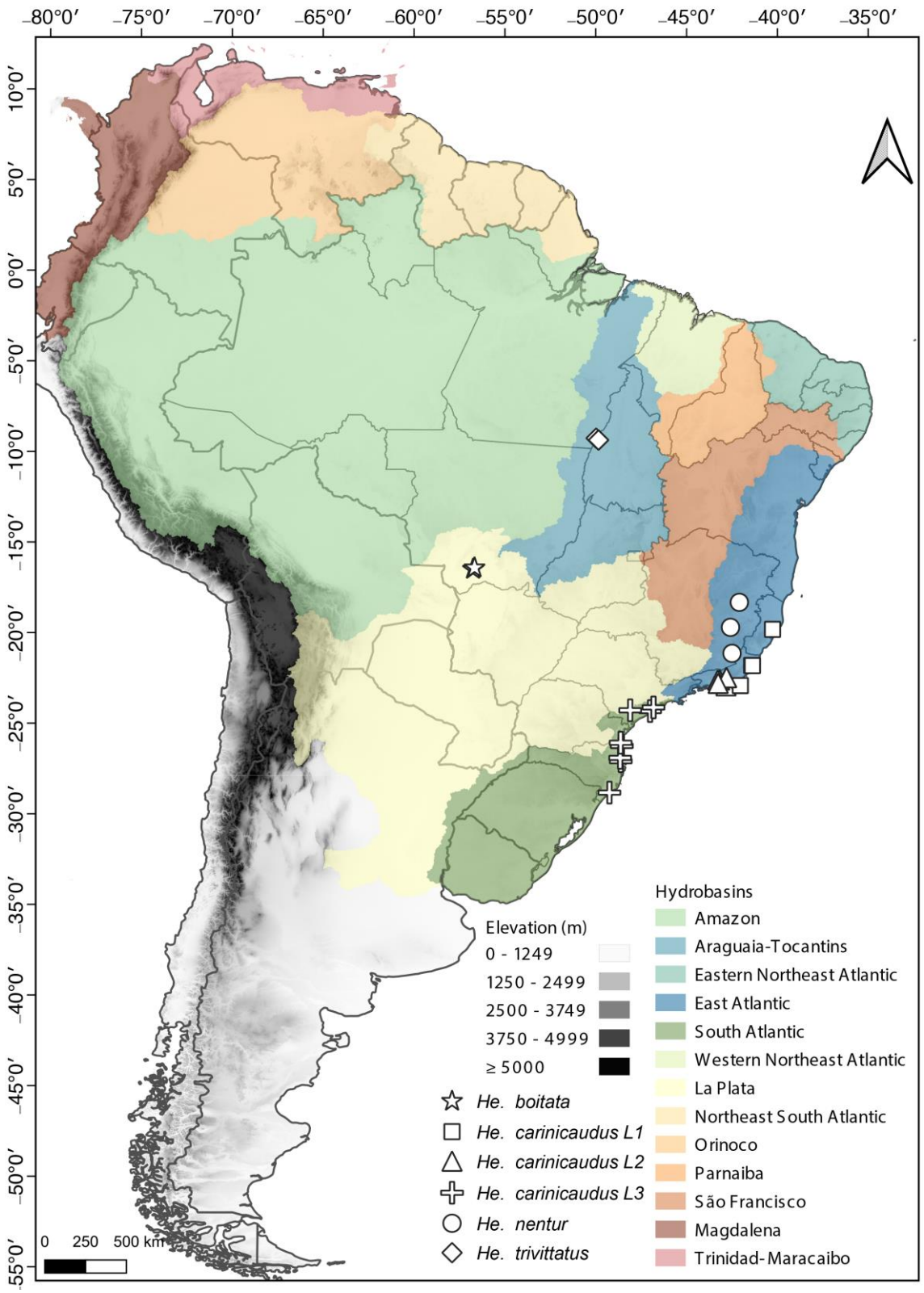


Figure S4. Geographic distribution of *Helicops trivittatus* and *He. carinicaudus* group samples from this study along of four watersheds across South America.

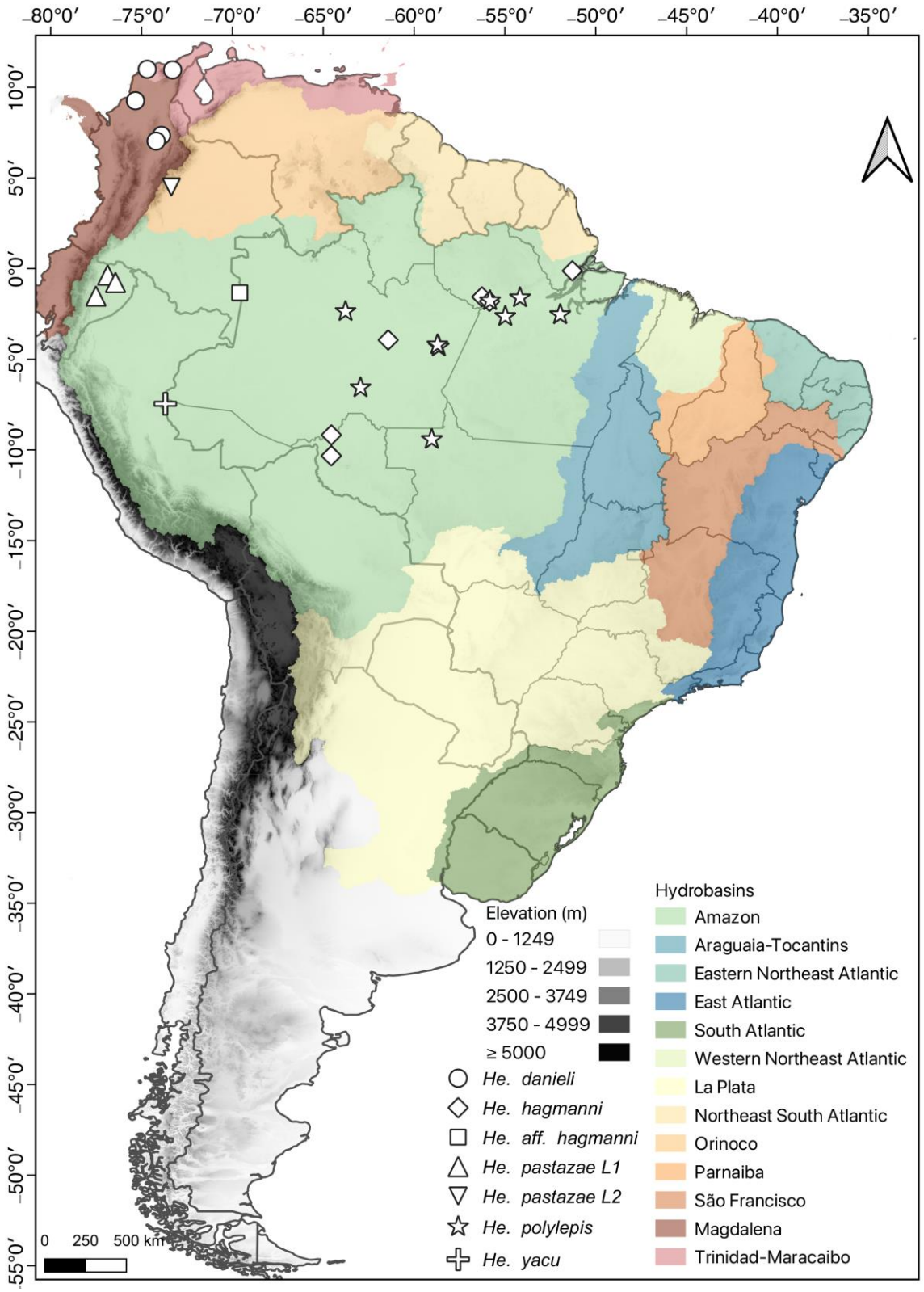


Figure S5. Geographic distribution of *Helicops danieli*, *He. hagmanni* group, *Helicops pastazae* complex and *He. polylepis* samples from this study along five watersheds across South America.

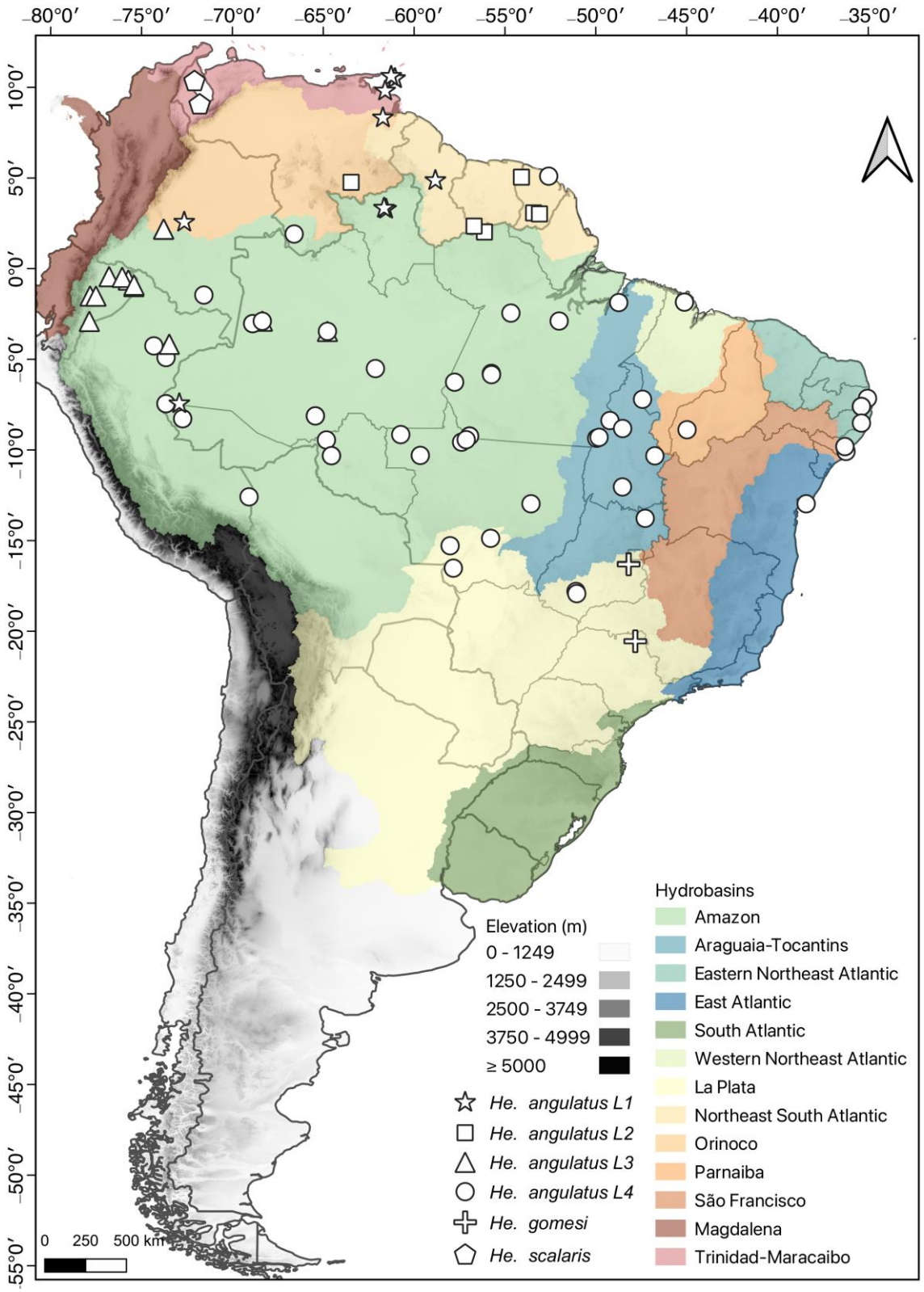


Figure S6. Geographic distribution of *Helicops angulatus* group samples from this study along 10 watersheds across South America.

Table S1. Taxa, vouchers, locality data, genetic markers and GenBank accession numbers used in this study. Absent acronyms in Sabaj (2022): MTR or MRT: researcher field number Miguel Trefaut U. Rodrigues (University of São Paulo, USP); A: researcher field number Vanessa Arzamendia (Faculty of Humanities and Sciences, National University del Litoral); AAGARDA: researcher field number Adrian Antonio Garda; AF: researcher field number Antoine Fouquet; GGU: researcher field number Giussepe Gagliardi Urrutia (Universidad Nacional de la Amazonía Peruana, Facultad de Ciencias Biológicas); LW: field number of the Federal University of Western Pará; MAP and MAP-T: field and tissue number of the Mapinguari laboratory at the Federal University of Mato Grosso do Sul; MHNBA: Bahia Natural History Museum voucher; RABRANDÃO: researcher field number Reuber Brandão (University of Brasilia) SB: project field number scales of biodiversity; VLF: researcher field number Vanda Lucia Ferreira (Federal University of Mato Grosso do Sul. Other museum acronyms follow Sabaj (2022). L: lineages.

This table is presented separately (in another document).

Table S2. List of outgroup taxa and GenBank accession numbers of specimens used in this study.

Terminal	16S	Cytb	Cmos	NT3	BDNF	R35
Cubophis cantherigerus	AF158475[1]	AF544669[2]	AF544694[2]	FJ434100[3]	FJ433999[3]	EF144082[4]
Pseudalsophis dorsalis	JQ598892[5]	JQ598946[5]	JQ598994[5]	-	-	-
Caaeteboia amarali	GQ457747[6]	JQ598921[5]	GQ457867[6]	-	-	-
Conophis lineatus	JQ598865[5]	JQ598924[5]	-	-	JQ599016[5]	-
Echinanthera undulata	-	JQ598929[5]	JQ598978[5]	-	JQ599022[5]	-
Phalotris lemniscatus	GQ457756[6]	JQ598941[5]	GQ457877[6]	-	JQ599039[5]	-
Hydrodynastes gigas	GQ457743[6]	-	GQ457863[6]	-	JQ599031[5]	-
Philodryas olfersii	AF158484[1]	JQ598945[5]	JQ598993[5]	-	JQ599041[1]	-
Oxyrhopus guibei	JQ627295[*]	JQ598938[5]	JQ598989[5]	-	JQ599038[5]	-
Psomophis joberti	GQ457768[6]	KX694887[7]	GQ457889[6]	KX695043[7]	KX694755[7]	-
Tomodon dorsatus	GQ457777[6]	KX694882[7]	GQ457897[6]	KX695055[7]	KX694768[7]	-
Tropidodryas striaticeps	GQ457778[6]	-	-	-	JQ599060[5]	-
Xenodon merremii	JQ598911[5]	JQ598963[5]	-	-	JQ599062[5]	
Manolepis putnami	JQ598881[5]	JQ598936[5]	JQ598988[5]	-	JQ599035[5]	-
Sordellina punctata	JQ598903[5]	JQ598956[5]	JQ599000[5]	-	JQ599052[5]	-
Xenopholis scalaris	-	GQ895897[8]	GQ895837[8]	-	-	-
Uromacer oxyrhynchus	FJ416712[9]	FJ416716[9]	-	-	-	-
Coluber constrictor	-	MF402779[10]	MF402186[10]	MF402515[10]	-	MF402633[10]
Diadophis punctatus	-	EU193763[11]	AF471122[12]	EU390917[13]	EU402637[13]	JN703085[14]
Ninia atrata	KX694675[7]	KX694854[7]	-	KX695034[7]	KX694742[7]	-

[1] sequences from Vidal, N., Kindl, S. G., Wong, A., & Hedges, S. B. (2000). Phylogenetic relationships of xenodontine snakes inferred from 12S and 16S ribosomal RNA sequences. Molecular Phylogenetics and Evolution, 14(3), 389-402.

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Table S3. PartitionFinder 2 model of nucleotide substitution. Best-fitting partitioning scheme model of nucleotide substitution for 16S, Cytb, Cmos, NT3, BDNF and R35 genes.

Partitioning scheme	Model
16S	GTR+I+G
CYTB1	GTR+G
CYTB2	HKY+I+G
CYTB3	HKY+I+G
CMOS1, CMOS3, NT33, R353, R351, CMOS2	HKY+I+G
NT31	K80+G
BDNF3, NT32, R352	K80+I+G
BDNF2, BDNF1	JC+I

Supplementary Material 1. Laboratory protocols and primers used in this study.

PCR protocols

We used two different protocols for PCR techniques in an attempt to maximize available samples.

1) Polymerase Chain Reaction (PCR) amplification of gene fragments was performed in a final volume of 25 µl reactions using 5X PCR Buffer (–Mg), dNTP at 0.2 mM, each primer at 0.2 mM, MgCl2 at25 mM, 0.1 U/µl Promega® Taq DNA Polymerase, and 2 µl of template DNA.

2) Protocol consisted of Master Mix PCR Ampliqon Taq, each primer at 10 mM and 2 µl of template DNA, in a total reaction volume of 23 µl.

The PCR cycling program was as follows to first protocol to mtDNA gene: 94°C for 2 min, followed by 34 cycles of 45 s denaturation at 94° C, 45 s annealing at X° C, and 1 min extension at 72° C, concluding with a 7 min extension at 72° C. To nuDNA gene: 94°C for 2 min, followed by 34 cycles of 30 s denaturation at 94° C, 1 min annealing at X° C, and 1 min extension at 72° C, concluding with a 7 min extension at 72° C. PCR products were analyzed on 2% agarose gels and were purified by ammonium acetate/ethanol.

Gene	Name	Primer	Annealing temperature	Reference
16S	16SF	5'- CGC CTG TTT ATC AAA AAC AT -3'	48° to 51°	Palumbi et al. 2002
	16SBR	5'- CTC CGG TTT GAA CTC AGA TCA -3'		
CYTB	Cytb_F	5'- TCA AAY ATC TCA ACC TGA TGA AAY TTY GG -3'	58°	Pook et al. 2000
	Cytb_R	5'- GGC AAA TAG GAA GTA TCA YTC TGG YTT -3'		
CYTB	L14910_cytbF	5' -GAC CTG TGA TMT GAA AAC CAY CGT TGT- 3'	50°	Burbrink et al. 2000
	H16064_cytbR	5' -CTT TGG TTT ACA AGA ACA ATG CTT TA- 3'		
C-MOS	S77_c-mos	5'-CATGGACTGGGATCAGTTATG-3'	54°	Lawson & Slowinski 2005
	S78_c-mos	5'-CCTTGGGTGTGATTTTCTCACCT-3'		
NT3	NT3_F3	5'- ATA TTT CTG GCT TTT CTC TGT GGC -3'	54°	Noonan & Chippindale 2006
	NT3_R4	5'- GCG TTT CAT AAA AAT ATT GTT TGA CCG G -3'		
BDNF	BDNF_F	5'- GAC CAT CCT TTT CCT KAC TAT GGT TAT TTC ATA CTT -3'	50°	Noonan & Chippindale 2006
	BDNF_R	5'- CTA TCT TCC CCT TTT AAT GGT CAG TGT ACA AAC -3'		
R35	R35_L75F	5'- TCTAAGTGTGGATGATYTGAT - 3'	50°	Fry et al. 2006
	R35_H792R	5'- CATCATTGGRAGCCAAAGAA - 3'		

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3 CHAPTER 2:

Spatial-temporal evolution of water snakes in South America driven by Pebas System during the Early Miocene

Abstract

The South American hydrographic and landscape evolution has a complex history in the last 23 Mya due to the Andean uplift, which provided new aquatic habitats, involving incursions and regressions in marine and continental lakes, and established the current drainages of the Amazon, Maracaibo, Magdalena, and Orinoco rivers. Using a dated phylogeny for 22 of the 26 valid species and the 'BioGeoBEARS' dispersal models, we estimated ancestral areas of the Hydropsini in South America to (1) correlate the diversification of *Pseudoeryx*, *Hydrops* and *Helicops* with hydrological and geological events and (2) provide insights into the biogeography of this tribe. Hydropsini arose around 21 Mya in the Amazonian ancestral scenario in the early Miocene. The Northwest Amazonia is the biogeographic unit with the highest species richness of the Hydropsini and the majority of the speciation events that lead to the current diversity within of tribe occurred ca six million years ago. We propose that the origin and the subsequent diversification of *Pseudoeryx*, *Hydrops*, and *Helicops* is consistent with major hydrological and geological events that occurred during the Amazon Miocene such as Pebas System, which acted as a cradle and disperser for diversification of the lineages. Furthermore, after colonization of adjacent basins, the mountain ranges in Colombia and Venezuela acted as a vicariant barrier, separating the species *Hy. relictualis*, *He. scalaris*, and *He. danieli* from her Andean cis sister taxa. The easternmost colonization of the continent was also brought about by mid-Miocene dispersal, when the current drainages were already established. Still, most of the more recent diversifications took place during the Plio-Pleistocene. Therefore, our results reinforce that the dynamism of the Miocene landscape in the Amazon had a great impact on the aquatic fauna, given the ecological association of Hydropsini with watercourses.

Keywords: Andean uplift, Historical biogeography, Hydrographic basin, Marine introgression, Xenodontinae.

3.1 Introduction

Evolution is the cornerstone of biology (Dobzhansky 1973), phylogenies are indispensable to all comparative evolutionary studies, and a large-scale sampling of taxa is paramount for such studies (e.g., Bergmann & Morinaga 2019; Furness & Capellini 2019). Together with species distribution, phylogenies' help to elucidate central biogeographic and evolutionary questions (Hortal et al. 2015). Nevertheless, there are many knowledge gaps that hamper robust evolutionary studies, such as Linnean, Darwinian and Wallacean Shortfall (Hortal et al. 2015), which correspond to the lack of basic information, concerning taxonomy, evolutionary relationships and geographic distribution, making it difficult to understand evolutionary patterns and processes.

The Neotropical region presents an exceptional climate and habitat diversity, and the organisms' evolutionary trajectories are influenced by historical and environmental factors (Rull 2008; 2011; Hoorn et al. 2010a; b; Rull & Carnaval 2020). In that regard, biogeographic and evolutionary patterns of organisms are deeply linked to geomorphological processes, such as the mountain ranges uplift (Antonelli et al. 2009), watershed formations (Lundberg et al. 1998; Hoorn et al. 2010a; Hoorn et al. 2022), habitats fragmented through Pleistocene climatic changes (Haffer 1969), and the emergence of the diagonal of open vegetations (Werneck 2011; Azevedo et al. 2020). In order to understand the long and complex formation history of South American landscape and the river systems, is fundamental consider the Andes uplifting through time (Lundberg et al. 1998). Remarkably during the Neogene, the Andean uplift due to tectonic movement, provided new aquatic habitats, recurrent marine incursions and regressions, and changes in river courses, which also promoted biotic diversification (Hoorn et al. 2010a, b). Thus, it is difficult to disassociate the evolution of the landscape with the evolution of biota, since these processes are interconnected. Therefore, this geological dynamism is related to the origins of current diversity (e.g., Antonelli et al. 2018). Species associated to aquatic environments are affected at some level by the hydrological and ecological processes connected with the basins where they live, which influence a broad biogeographic pattern (e.g., Farias et al. 2019; Piatti et al. 2019; Coelho et al. 2022).

One of the main events in South America that shaped its hydrography and landscape occurred during the Early and Middle Miocene. There about 23 to 10 Mya, the formation of the Pebas system in the western Amazon, an epicontinental system covering more than one million km^2, configured a vast landscape with several lakes and wetlands with fluvial and marine influences (Hoorn et al. 2010a, b; Hoorn et al. 2022). This system affected the biogeographic patterns of aquatic and non-aquatic organisms that occurred in that region. Studies have proposed the biogeographic hypothesis that the Pebas System was a barrier or a cradle for organisms during the Miocene (e.g.,

Wesselingh & Salo, 2006; Salas-Gismondi et al. 2015). Recently, Hoorn et al. (2022) proposed that the Pebas System was a semi-permeable large-scale barrier, and species dispersal was selectively favored or inhibited in similar proportions. However, the cradle, barrier, permeable hypotheses are not mutually exclusionary and may be relevant for different taxa, where one or more than one of these hypotheses may represent remnants of diversification patterns that persist to this day.

Still in the Miocene, the final uplift of the Brazilian Central Plateau (Del'Arco & Bezerra 1989) was another important factor that shaped the current diversity in South America. This event promoted the compartmentalization of the landscape and was also decisive in the diversification of several taxa (e.g., Antonelli et al. 2018; Oliveira et al. 2018; Rull & Carnaval 2020; Damasceno et al. 2021; Fontenelle et al. 2021). In addition, it also delimits ecoregions that correspond to the distribution of distinct climatic areas in South America. Therefore, the geomorphological history of South America, such as the uplift of the Andes, the formation of the Pebas system, the establishment of hydrographic basins, marine introgressions and climatic oscillations during the Miocene-Pliocene-Pleistocene were fundamental to determine the current distribution of the biota (e.g., Carnaval et al. 2014; Werneck et al. 2015; Pirani et al. 2019). Among the different organisms that are influenced by these events, snakes are known to inhabit different types of habitats, and such distribution can be determined by climate (Dal Vechio et al. 2018), geomorphological barriers (Hamdan et al. 2019), or even by aquatic environments (Carvalho et al. *in press*). Consequently, snakes are an excellent model for investigating evolutionary and ecological hypotheses, as they have adapted to a wide variety of environments, including terrestrial, fossorial, arboreal, marine, and freshwater habitats (Vitt and Cadwell 2013).

South America presents the second highest occurrence of freshwater water snakes, surpassed only by Asia (Pauwels et al. 2008; Murphy 2012). This is a pattern that follows biogeographic regions with a rich hydrological web of both fresh and brackish water (Murphy 2012). Snakes have independently invaded the aquatic milieu many times, since several families have representatives that habit this environment (Acrochordidae, Boidae, Colubridae, Dipsadidae, Elapidae, Homalopsidae, Natricidae and Viperidae) (Pauwels et al. 2008; Murphy 2012). Approximately half of the diversity of South American water snakes belongs to the Hydropsini, a tribe composed of *Pseudoeryx* (two lineages), *Hydrops* (five lineages), and *Helicops* (22 lineages). Given their aquatic habit, this group presents morphological variations, such as dorsally oriented eyes and nostrils, and a fish-based diet (Roze 1957a; b).

Hydropsini harbors both widespread and geographically restricted taxa, providing a powerful system for identifying processisthat influence species distribution and evolution. Even though they are associated with the aquatic environment, these snakes are distributed in contrasting

ecoregions (Scartozzoni et al. 2010; Nogueira et al. 2019; May et al. 2019; Moraes-da-Silva et al. 2019; 2021; 2022; Acosta-Ortiz et al. 2020; Arteaga et al. 2021a; b; Citeli et al. 2022). In addition, little has been discussed about how and which historical processes drove their diversification and distribution. On the other hand, testing hypotheses about biogeography that deal with the complex Neotropical geological history depends on incorporating a well-resolved phylogeny, which currently does not exist for hydropsids. The Amazon region harbors the greatest diversity of Hydropsini, in which the three genera occur in this region, and 18 out of 26 species (69%). Given the high diversity of Hydropsini semi-aquatic snakes in Amazonia, an origin in the region would be supported, and rivers would facilitate their dispersion colonization for adjacent areas. Moreover, studies have supported the hypothesis that normally the most diverse region for some taxa is where their diversification began (Wiens et al. 2011; Moraes et al. 2022). Accordingly, in this study, our goal is to estimate the hydropsids biogeographic evolution, and to correlate the evolution of this tribe with the geomorphologic dynamics of the continent.

3.2 Material and Methods

Sampling and biogeographic units

To perform the biogeographic analyses described below, we used the molecular dataset provided by Carvalho (2022) to generate a dated molecular phylogeny for Hydropsini. In this study they generated a phylogeny with two mitochondrial (16S and Cytb) and four nuclear genes (Cmos, NT3, NDNF, and R35) concatenated for Hydropsini, and revealed an unobserved/described diversity and taxonomic inflation. The dataset contains 1080 sequences for 241 specimens of Hydropsini throughout South America (see Table S1 for the list of samples and localities).

We defined six biogeographic units by combining Neotropical ecoregions *sensu* Dinerstein et al. (2017), with the basin hydrographic Lehner and Grill (2013) and the distribution pattern of Hydropsini species. We considered the historical continuity of the land masses from the Late Cretaceous to the Holocene, adding to factors that may act as dispersal barriers for snakes to define the biogeographic units. The geographic distributions of each species were obtained from Scartozzoni et al. (2010); Kawashita-Ribeiro et al. (2013); Costa et al. (2016); Nogueira et al. (2019); May et al. (2019); Moraes-da-Silva et al. (2019; 2021; 2022); Acosta-Ortiz et al. (2020); Arteaga et al. (2021a; b); Citeli et al. (2022).

Species tree and divergence times

We used PartitionFinder 2 to identify partitioning schemes and the most appropriate nucleotide substitution models (Lanfear et al. 2017). According to our concatenated alignment, we found eight partitions evaluated by Bayesian Inference Criterion (BIC). We used startBEAST v.2.6.3 (Bouckaert et al. 2019) to estimate the species tree and lineage divergence times. Analyses were time-calibrated using a mean rate of evolution for the Cytb (1.3% rate/site/million years) calculated by Daza et al. (2009). We assumed a strict clock model and a birth-death model of diversification as a tree prior. We ran the MCMC chain for 500,000,000 iterations, sampling every 50,000 iterations, and discarded the initial 10% iterations as burn-in. We checked for stationarity by visually inspecting trace plots and ensuring that all ESS values were above 200 in Tracer v1.7.1 (Rambaut et al. 2018).

Biogeographical Analysis

The geographic distribution and dispersal models over time for Hydropsini tribe in South America was estimated using BioGeoBEARS package (Matzke 2013; 2014) in the R v4.1.2 (R Core Team 2022). Hydropsini distribution has been subdivided into six geographical areas representing biogeographic units named as follow: A) Transandean, B) Northwest Amazonia, C) Southeast Amazonia, D) Northeast Diagonal, E) Southwest Diagonal, and, F) Atlantic Forest.

The BioGeoBEARS package implements Maximum Likelihood (ML) methods that replicate the main premises of the three methods most commonly used in historical biogeography, namely, DEC (Dispersion-Extinction Cladogenesis; Ree & Smith 2008), DIVA (Analysis of Dispersion-Vicariance; Ronquist 1997) and BayArea (Bayesian Inference from Historical Biogeography for Discrete Areas; Landis et al. 2013). Collectively, these models allow for a wide range of processes, including speciation within the area, vicariance, range expansion (dispersion to a new area), and range contraction (extinction in an area). Additionally, we tested the same three models plus a founder effect parameter on each model named "J": DEC+J, DIVALIKE +J, and BAYAREALIKE+J (Matzke 2014). From the model with the lowest Akaike information criterion (AIC), we estimate the probabilities of the ancestral area along the phylogeny (Burnham & Anderson 2003).

In general, the three "+ *j*" models significantly improve the fit of the model to the corresponding model without the inclusion of "+ *j*". We acknowledge the critique of DEC/DEC + *j* statistical comparisons put forward by Ree and Sanmartín (2018), but we decided to maintain this parameter based on the replies done by Matzke (2022).

3.3 Results

We recovered that the most recent common ancestor (MRCA) of Hydropsini was estimated around 22.93 Mya (HPD 95%: 19.64–26.52 Mya), between the end of the Oligocene and the beginning of the Miocene. The majority of the speciation events that lead to the current diversity within Hydropsini occurred between 21 and 1.5 million years ago, with most diversifications occurring along the Late Miocene to Pleistocene (see Fig. 1; Fig. S1; Table 1). The clade containing *Pseudoeryx* and *Hydrops* diverged in the early Miocene (~21.56 Mya; 95% HDP: 16.74–24.42 Mya) (Table 1). The *P. plicatilis* lineages diversified around 3.09 Mya (95% HDP: 0.24–7.15 Mya). The MRCA of *Hydrops* and origin of *He. relictualis* was estimated at around 20.53 Mya, in the early Miocene (95% HDP: 14.9–23.63 Mya). Besides, the MRCA of *Helicops* was estimated at around 18.99 Mya (95% HDP: 16.32–21.63). Within *Helicops*, the clade formed by *He. trivittatus* and *He. carinicaudus* group had started around 15.37 Mya (95% HDP: 10.44–19.32), and specifically the *He. carinicaudus* group started its diversification around 11.76 Mya (95% HDP: 6.54–15.61 Mya), which both diversifications occur in the middle Miocene. The other *Helicops* clade had its MRCA established around 17.54 Mya, in the early Miocene, and from there, the *He. leopardinus* group diverged (~8.51 Mya; 95% HDP: 5.62–11.55 Mya).

The MRCA of the clade containing *He. danieli*, *He. hagmanni* group, *He. polylepis* and *He. angulatus* group arose at about 16.23 Mya (95% HDP: 13.01–18.53 Mya). The *He. hagmanni* group diversification around (~12.23 Mya; 95% HDP: 8.81–14.56 Mya) and *Helicops pastazae* complex begin its diversification around 3.10 Mya (95% HDP: 2.03–4.19 Mya). While the MRCA of *He. polylepis* and *He. angulatus* group split about 11.89 Mya; 95% HDP: 9.02–14.36 Mya. The MRCA of *He. angulatus* group was estimated in the late Miocene (10.15 Mya; 95% HDP: 8.15–12.01 Mya). Within this group, the diversification of *He. scalaris* and *He angulatus* L1 were estimated at around 5.37 Mya (95% HDP: 3.29–7.2 Mya) in the early Pliocene. Almost simultaneously, the other clade within *He. angulatus* composed of *He. gomesi* and three lineages of *He. angulatus* started their diversification around 6.12 Mya (95% HDP: 4.76–7.51 Mya) in the late Pliocene. Table 1 summarizes the divergence times, geological periods and possible putative events.

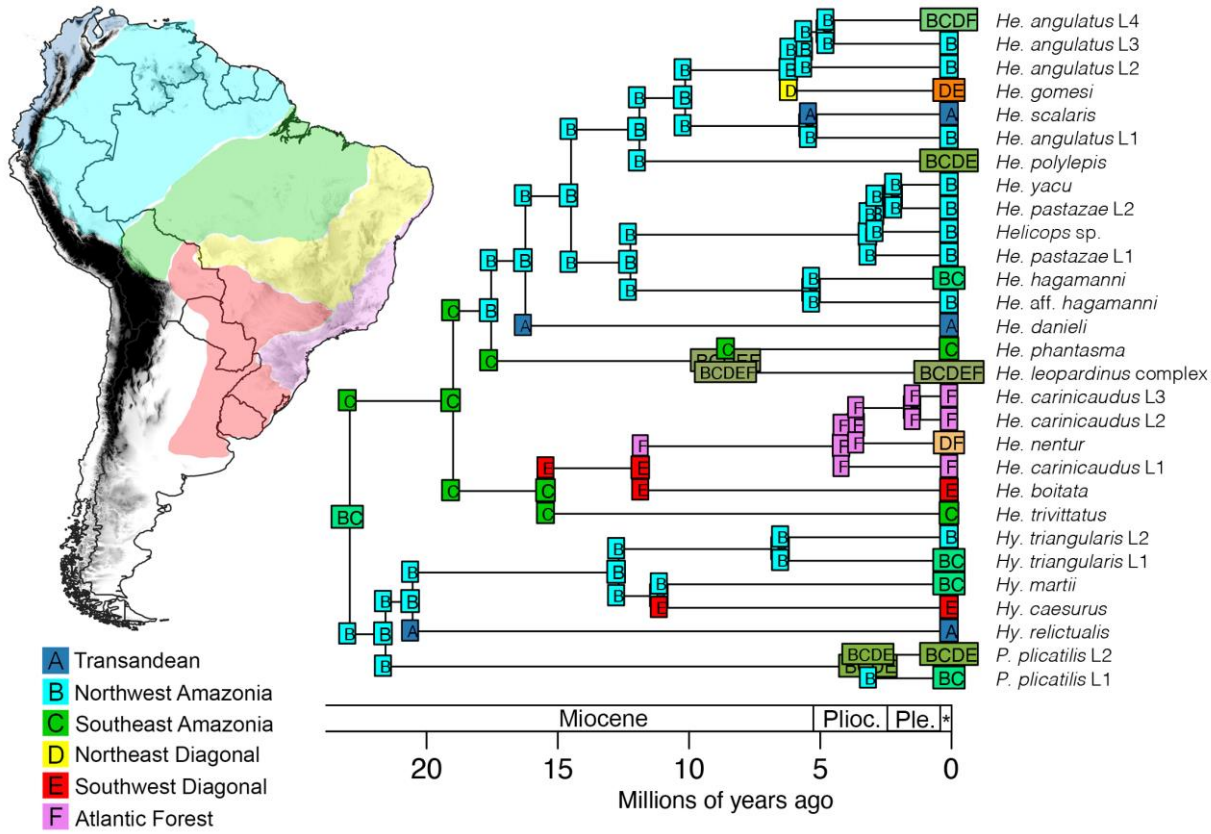


Figure 1. Ancestral geographic scenario of the Hydropsini tribe reconstructed under DEC+j model by BioGeoBEARS. The biogeographic areas defined based on the combination of Neotropical ecoregions, with the basin hydrographic, and distribution pattern of Hydropsini species. Single capital letters and colors indicate different biogeographic units used in this study. Mixed letters and colors represent combinations of such units.

Table 1. Summary of the temporal and spatial main events that influenced the dispersal/vicariance for the Most Recent Common Ancestor (MRCA) of Hydropsini lineages. (A) Transandean; (B) Northwest Amazonia; (C) Southeast Amazonia; (D) Northeast Diagonal; (E) Southwest Diagonal and (F) Atlantic Forest. We follow the stratigraphy International Chronostratigraphic Chart (ICS).

MRCA	ANCESTRAL AREA	DIVERGENCE TIMES MEAN (95% HDP)	GEOLOGICAL PERIODS	PUTATIVE EVENTS
Hydropsini	B and C	22.93 Mya (19.64–26.52 Mya)	Early Miocene and late Oligocene	Pebas system
Pseudoeryx / *Hydrops*	B	21.56 Mya (16.74–24.42 Mya)	Early Miocene	Pebas system
Hydrops	B	20.53 Mya (14.9–23.63 Mya)	Early Miocene	Pebas system
Hy. relictualis	A	20.53 Mya (14.9–23.63 Mya)	Early Miocene	Pebas system; final uplift of Cordillera de Mérida and Sierra de Perijá
Hy. caesurus	E	11.05 Mya (4.18–14.05 Mya)	Early Pliocene and middle Miocene	Connection between the Paleo-Amazonia-Orinoco and La Plata basins; Petaca/Yecua Formation

MRCA	ANCESTRAL AREA	DIVERGENCE TIMES MEAN (95% HDP)	GEOLOGICAL PERIODS	PUTATIVE EVENTS
Hy. martii	B	11.05 Mya (4.18–14.05 Mya)	Early Pliocene and middle Miocene	Pebas system
Hy. triangularis	B	6.43 Mya (2.39–10.37 Mya)	Late Pliocene and Late Miocene	Acre system (current establishment of the Amazonian drainages)
Pseudoeryx	B, C, D, and E	3.09 Mya (0.24–7.15 Mya)	Late Pleistocene and Late Miocene	Acre system (current establishment of the Amazonian drainages); Petaca/Yecua Formation
Helicops	C	18.99 Mya (16.32–21.63 Mya)	Early Miocene	Pebas system
He. trivittatus	C	15.37 Mya (10.44–19.32 Mya)	Late Miocene and early Miocene	Pebas system
He. carinicaudus group	E	11.76 Mya (6.54–15.61 Mya)	Late Miocene and middle Miocene	Connection between the Paleo-Amazonia-Orinoco and La Plata basins; Petaca/Yecua Formation
He. boitata	E	11.76 Mya (6.54–15.61 Mya)	Late Miocene and middle Miocene	Connection between the Paleo-Amazonia-Orinoco and La Plata basins; Petaca/Yecua Formation
He. carinicaudus lineages / *He. nentur*	F	4.11 Mya (2.43–5.97 Mya)	Pliocene	Final uplift of the Brazilian Central Plateau; Pleistocene Climatic Event; Marine introgression
He. nentur	F	3.55 Mya (1.67–4.75 Mya)	Early Pleistocene and early Pliocene	Final uplift of the Brazilian Central Plateau; Pleistocene Climatic Events; Marine introgression
He. carinicaudus L2 and L3	F	1.41 Mya (0.24–2.63 Mya)	Late Pleistocene and early Pleistocene	Pleistocene Climatic Events; Marine introgression
He. leopardinus group	B, C, D, E, and F	8.51 Mya (5.62–11.55 Mya)	Late Miocene	?
He. phantasma	C	8.51 Mya (5.62–11.55 Mya)	Late Miocene	?
He. danieli	A	16.23 Mya (13.01–18.53 Mya)	Middle Miocene and early Miocene	Pebas system; final uplift Eastern Cordillera
He. hagmanni group	B	12.23 Mya (8.81–14.56 Mya)	Late Miocene and middle Miocene	Pebas system
He. hagmanni	B	5.25 Mya (3.33–7.22 Mya)	Late Pliocene and late Miocene	Acre system (current establishment of the Amazonian drainages)
He. pastazae complex	B	3.10 Mya (2.03–4.19 Mya)	Early Pleistocene and early Pliocene	Acre system (current establishment of the Amazonian drainages)

MRCA	ANCESTRAL AREA	DIVERGENCE TIMES MEAN (95% HDP)	GEOLOGICAL PERIODS	PUTATIVE EVENTS
He. pastazae L2 / *He. yacu*	B	2.13 Mya (0.21–3.06 Mya)	Pleistocene	Acre system (current establishment of the Amazonian drainages); Climate oscillation
He. polylepis	B	11.89 Mya (9.02–14.36 Mya)	Late Miocene and middle Miocene	Pebas system
He. angulatus group	B	10.15 Mya (8.15–12.01 Mya)	Late Miocene and middle Miocene	Pebas system; Acre system (current establishment of the Amazonian drainages); Marine introgression
He. scalaris	A	5.37 Mya (3.29–7.2 Mya)	Late Pliocene and late Miocene	Final uplift of Cordillera de Mérida and Sierra de Perijá
He. angulatus L1	B	5.37 Mya (3.29–7.2 Mya)	Late Pliocene and late Miocene	Acre system (current establishment of the Amazonian drainages); Marine introgression
He. gomesi	D	6.12 Mya (4.76–7.51 Mya)	Early Pliocene and late Miocene	Connection between the Paleo-Amazonia-Orinoco and La Plata basins
He. angulatus L2/L3/L4	B, C, D, and F	5.56 Mya (4.2–6.55 Mya)	Early Pliocene and late Miocene	?

Overall, Hydropsini is widely distributed throughout South America across the biogeographic units adopted here. Three lineages are Trans Andean restricted (unit A), 17 occur in the Northwest Amazonia (unit B; highest richness), 10 in the Southeast Amazonia (unit C), six in the Northeast Diagonal (unit D), six in the Southwest Diagonal (unit E), and six in the Atlantic Forest (unit F) (Fig. 1). Within *Pseudoeryx*, there is a widely distributed taxon (*P. plicatilis* L2) and a taxon restricted to the Amazon unit (*P. plicatilis* L1). Likewise, within *Hydrops* we observe *Hy. martii* and *Hy. triangularis* L1 occurs widely in the two Amazon units, while *Hy. relictualis* and *Hy caesurus* are restricted to the Trans Andean and Southwest Diagonal units, respectively. Furthermore, within *Helicops* only *He. polylepis*, *He. leopardinus* complex and *He. angulatus* L4 are widely distributed, while most species only occur in one biogeographic unit and three occur in two units (Fig. 1).

Among the six tested models based on the six units, DEC+J was the best fit model for our data (AIC = 169.26; Table 2). Our results indicate that the most likely area distribution of the MRCA of Hydropsini was at the Amazon region, between the Northwest Amazonia and the Southeast Amazonia units (B and C), around 22.93 Mya, in the early Miocene (Fig. 1). According to the DEC+J model, both main clades started their diversification in Amazon units (B or C), although still in the recent history of the tribe diversification (early Miocene), *Hy. relictualis* dispersed to the Trans Andean (unit A) and remained restricted to the Lake Maracaibo area, Venezuela. At

approximately 16.23 Mya the MRCA of *He. danieli* also dispersed from Northwest Amazonia to Trans Andean (unit A), and until the present time, its populations are restricted to the west side of the Andes. Within *Helicops* there was a new dispersion of the common ancestor from the Southeast Amazonia (unit C) to the Southwest Diagonal (unit E) in the Middle Miocene, around 15.37 Mya. The common ancestor of the *He. carinicaudus* group has its origin in Southwest Diagonal (unit E) and spread most of its lineages to the Atlantic Forest (unit F). However, *He. boitata* remains restricted to Southwest Diagonal (unit E), and *He. nentur* more recently scattering to Northeast Diagonal (unit D). Likewise, in the later Miocene, there was another scattering event within *Hydrops* from Northwest Amazonia (unit B) to Southwest Diagonal (unit E). Thenceforth, *Hy. caesurus* lasting restricted to Southwest Diagonal (unit E). Several species originated and remained in the same biogeographic unit, such as *He. carinicaudus* (all lineages), *He. danieli*, *He. pastazae* complex, *He. scalaris* and *He. angulatus* (L1, L2, L3).

Table 2. Comparison of the BioGeoBEARS model for Hydropsini within six areas based on the log-likelihood (LnL) and the Akaike information criterion (AIC); N, parameters number; d, dispersion rate; e, extinction rate; J, relative probability of speciation between founding events. The best model is shown in bold.

Model	**LnL**	**N**	***d***	***e***	***J***	**AIC**
DEC	-84.77	2	0.02	0.02	0.00	173.54
DEC+J	**-81.63**	**3**	**0.01**	**0.00**	**0.05**	**169.26**
DIVALIKE	-83.84	2	0.02	0.00	0.00	171.68
DIVALIKE+J	-82.80	3	0.01	0.00	0.03	171.59
BAYAREALIKE	-87.81	2	0.01	0.07	0.00	179.62
BAYAREALIKE+J	-87.81	3	0.01	0.07	0.00	181.62

3.4 Discussion

We revealed that the most likely ancestral scenario for Hydropsini diversification occurred in the Amazon region, around 21 Mya in the early Miocene. There were at least three dispersal events for the Magdalena and Maracaibo regions throughout the Miocene. Cordilleras in Colombia and Venezuela may act as a vicariant event, which separated the species *Hydrops relictualis*, *Helicops scalaris* and *He. danieli* from her Andean cis sister taxa. The easternmost colonization of the continent was also caused by distinct dispersal events within the *He. angulatus* group, *He. leopardinus* group and *He. carinicaudus* group. The majority of the speciation events that lead to the current diversity within Hydropsini occurred ca six million years ago.

Our ancestral area estimate suggests that the MRCA of *Pseudoeryx*, *Hydrops* and *Helicops* originated in Amazonia (Northwest Amazon and Southeast Amazon units) during early Miocene

between ~21 e 18 Mya, coincides with the initial period of the Pebas system, generated and sustained by the Andean uplift (Hoorn et al. 2010a; b; Hoorn et al. 2022) (Table 1). The paleoenvironment of western Amazonia in the Miocene was characterized by a large wetland known as the Pebas System, which was composed of a vast and well-irrigated floodplain, with freshwater rivers, lakes, swamps and even marine-influenced water bodies, located among Brazil, Bolivia, Colombia, Ecuador and Peru (Hoorn et al. 2010a; 2010b; 2022). The Pebas system was formed by the paleo Amazon-Orinoco River (east-west drainage), that was sporadically invaded by the Caribbean Sea from the north and had a contribution of sediments from the Andes and emptied into the Caribbean Sea (Hoorn 1993; Hoorn et al. 1995). Several studies have reported the irradiation influenced by this paleoenvironment in various extinct (e.g., plants, invertebrates, reptiles, fish, mammals) and extant (e.g., amphibians, stingrays) organisms (e.g., Behling et al. 2010; Waddell et al. 2018; Fontenelle et al. 2021; Suarez et al. 2021; Stutz et al. 2022). The Pebas system also harbored a huge diversity of reptiles, including water snake species as recorded by fossils of *Eunectes* (Anacondas) (Hsiou & Albono 2009). Such diversity could have represented one of the highest during the Miocene (Riff et al. 2010; Salas-Gismondi et al. 2015). However, the period of extinction of most taxa corroborates the shift from the Pebas System to the Acre System at the end of the Miocene (Hoorn et al. 2010a; b; Hoorn 2022).

The separation of the trans Adean lineages from the cis Andeans of *Helicops* and *Hydrops* occurred due to processes of dispersal followed by vicariance. The paleo western Amazon-Orinoco, Maracaibo and Magdalena were connected until the middle of the Miocene (~8-10 Mya: Hoorn et al. 2010a; b; Hoorn et al. 2022), which possibly led to the dispersion of the ancestors of *Hy. relictualis*, *He. danieli* and *He. scalaris* to the northmost area of the continent. The final uplift of the Colombian Eastern Cordillera and the Serra de Perijá and the Venezuelan Cordillera de Mérida close the Caribbean passage of the paleo Amazonas-Orinoco Oeste (Hoorn 1993; Hoorn et al. 1995; Lundberg et al. 1998) (Table 1). These uplift processes isolated the *He. danieli* ancestors in the Magdalena basin from the *Hy. relictualis* and *He. scalaris* lineages of the Lake Maracaibo basin and further separated them from the other cis Andean lineages. In addition, these geological events established the current Magdalena, Maracaibo, Amazonas and Orinoco drainages (Hoorn 1993; Hoorn et al. 1995). This pattern of vicariance due to the uplift of the Andes that subdivided closely related ancestral clades is also recovered, for example, in fish, caimans, porpoises (Lundberg et al. 1998; Albert et al. 2018).

The Pebas System was paramount for biota diversification, and is considered a cradle for several groups (e.g., Salas-Gismondi et al. 2015; Fontenelle et al. 2021). On the other hand, it also limited the distribution of several clades, for example, non-aquatic mammals and plants

(Wesselingh & Salo, 2006; Antonelli et al. 2009; Antoine et al. 2017). However, the Pebas System may have been a permeable biogeographic system (Hoorn et al. 2022). Given the characteristics, such as the heterogeneity of the landscape and the cyclical periods of inundation during a long period of time, dispersion for some taxa would be possible (i.e., permeability), while at the same time serving as a barrier to dispersion for others. The permeability and cradle hypothesis corroborate our prediction that river corridors favor the dispersion and speciation of aquatic snakes in South America, including Hydropsini. Taxa are able to adapt to the variability of the cyclic landscape (Hoorn et al. 2022), possibly this is the biogeographic pattern of Hydropsini, given that they are not strictly aquatic species and can move and forage out of water. Biogeographic patterns in organisms restricted to the aquatic environment are often related to drainage and capture of waterheads and in part to climate and vegetation types, exhibiting a hierarchy of filters (Tonn 1990). But for aquatic snakes, these mechanisms/factors are weakly involved. They are organisms that can forage outside the environment.

There were two independent dispersal events between 15 and 11 Mya from Northwest and Southwest Amazonia to the Southwest Diagonal (E unit) of *Hydrops* and *Helicops* ancestors, respectively. A connection between the Paleo-Amazonia-Orinoco basin and La Plata during the Miocene has been supported by geological and fossil evidence (Lundberg et al. 1998; Hulka et al. 2006; Albert et al. 2018). The watershed of the Pebas system extended south to the Madre de Dios-Beni basins in the middle Miocene (~15 Mya). Additionally, during the Middle-Late Miocene (14–7 Mya) in the Petaca/Yecua Formation, Bolivia, there was a marshy and lacustrine environment (Hulka et al. 2006). Probably this connection in the central region of the continent made possible the dispersion of these ancestors to the Southwest Diagonal (E unit) (Table 1). *Hydrops caesurus* and *He. boitata* have diversified in the La Plata basin (Southwest Diagonal unit), and are closely related to the Pantanal floodplain, a wetland that experiences extensive and prolonged seasonal flooding. Although this environment has evolved recently (Pleistocene) compared to ancestral dispersal (~15 and 11 Mya), so far, these two species are restricted to this watershed (Assine 2004).

Still during the late Miocene, the final establishment of the Central Brazilian Plateau occurred, which secondarily functioned to promote the diversification of lineages in the Brazilian shield. This event is also known to have influenced other vertebrate groups (e.g., amphibians, lizards Maciel et al. 2010; Prado et al. 2012; Werneck et al. 2015; Oliveira et al. 2018). In addition, the São Francisco basin was influenced by the uplift of mountains in the central region of Brazil and successive headwater capture events were responsible for the current course of this basin (Rosa et al. 2003). It is possible that these events, even secondarily to the water snakes of the Hydropsini

tribe, have promoted the diversification and dispersion of the lineages for *He. angulatus* and *He. carinicaudus* groups.

Once both Amazon units (B and C) are regions of Hydropsini origin, it is expected that they harbor the biggest species diversity. In fact, 18 of the 26 species of Hydropsini occur in Amazonia (Table 1). Most current diversifications within Hydropsini were around 6 Mya (Late Miocene): *He. angulatus* group, *He. pastazae* group, and the *Pseudoeryx* and *Hydrops* lineages. Possibly, the two phases of the western Amazon (Pebas and Acre System) promoted the diversification of this tribe (e.g., Fontenelle et al. 2021), which drainages had different courses, caused extensive landscape change throughout the Miocene. According to Hoorn et al. (2010a), the change from a lacustrine environment (Pebas System) to a fluvial environment (Acre System), led to a particularly rapid diversification in the Western Amazon, as the diversity of this area far exceeds the diversity in the cratonic areas. Moreover, adjacent units have fewer species, with a maximum of six species. The observed differences in diversity levels between basins are probably not related to evolutionary time. Since the Northwest Amazonia unit has lineages as young as the other units. Nevertheless, the speciation of Hydropsini lineages certainly were due to more than one single event, and to proper understand the diversification of each lineage on a finer scale, phylogeographic analyzes at population scale are necessary.

3.5 Conclusions

This is the first biogeographic hypothesis for a large group of South American aquatic snakes for which the effects of geological events at continental-scale can be evaluated. We propose that the extensive area and connectivity of the Pebas System made it possible for Hydropsini to disperse to adjacent basins, driven by adequate conditions of long duration (time) in a large region (area). Herein, we provide phylogenetic evidence of the influence of time, biogeography and ecology on the diversification and distribution patterns of the tribe that holds nearly half of the diversity of aquatic snakes in South America. Our results show evidence that the dispersals in Hydropsini were unidirectional, with no lineage returning the Amazonian units. Much of the diversification of the Hydropsini (at least 18 lineages) took place in the dynamically changing rivers and watersheds of South America in the Amazon region, mainly the Pebas System, during the middle Miocene to Pleistocene.

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Supplementary information

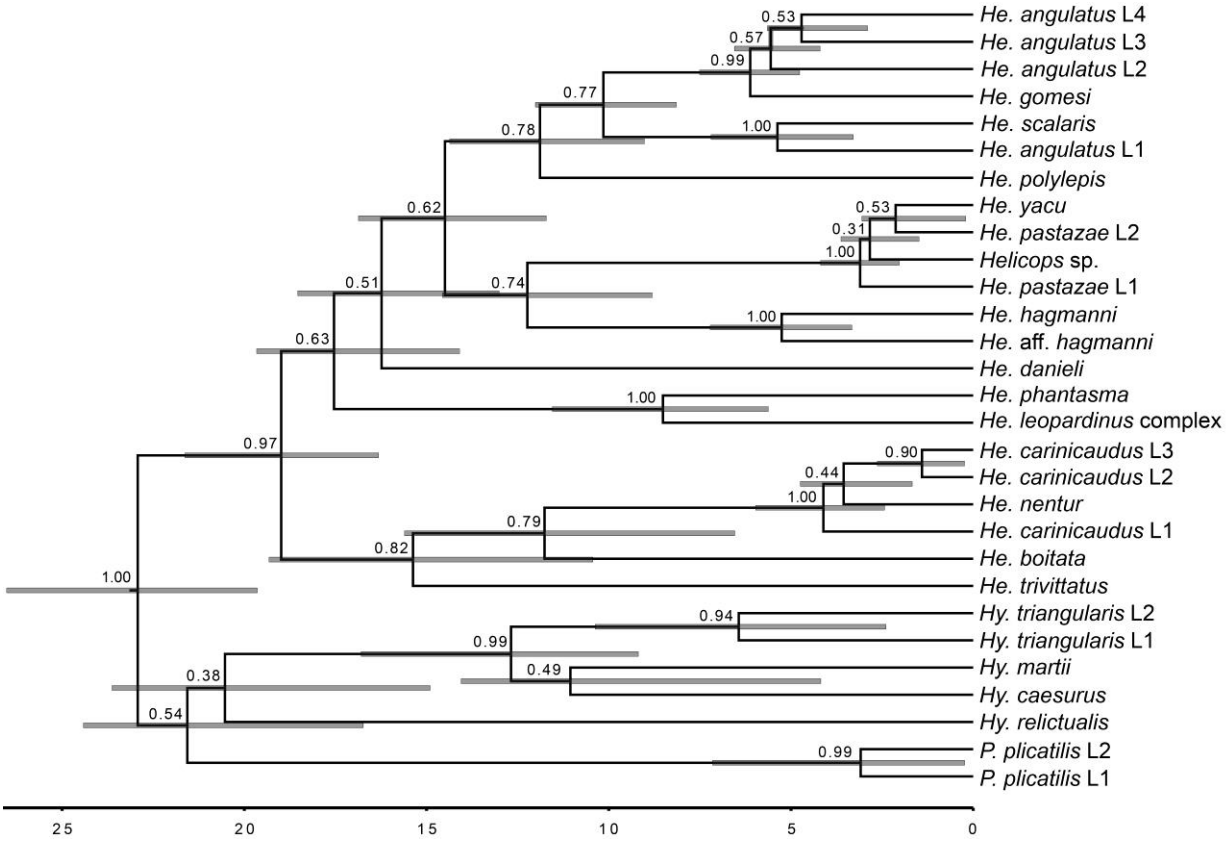


Figure S1. Dated Species Tree (*BEAST) of the Hydropsini based on mtDNA (16S and Cytb) and nuDNA (Cmos, NT3, BDNF and R35). Values above the nodes indicate posterior probabilities. The bars represent the 95% HDP.

Table S1. Taxa, vouchers, locality data, genetic markers and GenBank accession numbers used in Carvalho 2022 (PSC 2022). L: lineages. Absent acronyms in Sabaj (2022): MTR or MRT: researcher field number Miguel Trefaut U. Rodrigues (University of São Paulo, USP); A: researcher field number Vanessa Arzamendia (Faculty of Humanities and Sciences, National University del Litoral); AAGARDA: researcher field number Adrian Antonio Garda; AF: researcher field number Antoine Fouquet; GGU: researcher field number Giussepe Gagliardi Urrutia (Universidad Nacional de la Amazonía Peruana, Facultad de Ciencias Biológicas); LW: field number of the Federal University of Western Pará; MAP and MAP-T: field and tissue number of the Mapinguari laboratory at the Federal University of Mato Grosso do Sul; MHNBA: Bahia Natural History Museum voucher; RABRANDÃO: researcher field number Reuber Brandão (University of Brasilia) SB: project field number scales of biodiversity; VLF: researcher field number Vanda Lucia Ferreira (Federal University of Mato Grosso do Sul.

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
87	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
1349	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
AAGARDA13147	PSC 2022	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Pará
AAGARDA2195	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pará
BM132	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pará
CHUFPB00005	-	PSC 2022	PSC 2022	-	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Paraíba
CHUFPE601	-	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pernambuco
CHUFPE602	-	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pernambuco
CHUFPE603	-	PSC 2022	PSC 2022	-	-	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pernambuco
CHUNB40082	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pará
CHUNB40086	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pará
CHUNB40617	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Tocantins
CHUNB43220	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Tocantins
CHUNB52467	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Tocantins
CHUNB71691	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Goiás
H2339	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Rondônia
IBSP91771	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Maranhão
IBSP92722	PSC 2022	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Acre

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
LSUMZH13033	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Amazonas
LSUMZH13568	PSC 2022	-	PSC 2022	-	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Acre
LSUMZH13834	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Acre
LSUMZH14000	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Amazonas
LSUMZH14276	PSC 2022	PSC 2022	PSC 2022	-	-	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pará
LSUMZH17687	PSC 2022	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Rondônia
LSUMZH17688	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Rondônia
MAP1404	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
MAP2988	PSC 2022	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Tocantins
MAP5152	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Tocantins
MAP-T126	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Pernambuco
MAP-T1785	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
MCP19510	-	PSC 2022	-	-	PSC 2022	-	L3	*Helicops*	*angulatus*	Brazil	Amazonas
MCP19511	-	PSC 2022	-	-	PSC 2022	-	L4	*Helicops*	*angulatus*	Brazil	Amazonas
MHNBA2749	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Bahia
MNRJ24506	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
MNRJ25089	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Goiás
MNRJ25090	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Goiás
MRT5220	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Piauí
MTR20506	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Brazil	Roraima
MTR20568	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Brazil	Roraima
MTR20626	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Brazil	Roraima
MTR28325	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Acre
MTR35833	-	PSC 2022	PSC 2022	-	PSC 2022	-	L3	*Helicops*	*angulatus*	Brazil	Amazonas

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
MTR36461	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Amazonas
MTR36569	-	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Amazonas
MTR7588	MN038116	-	MN032472	MN032485	MN032459	-	L4	*Helicops*	*angulatus*	Brazil	Tocantins
MUFAL4658	-	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Alagoas
MUFAL6762	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Alagoas
MUFAL7335	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Alagoas
MZUSP11163	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
MZUSP11265	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
RABRAD5054	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Amazonas
UFMT7089	PSC 2022	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
UFMT7816	PSC 2022	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
VOGT2208	-	PSC 2022	PSC 2022	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Amazonas
ARI96	PSC 2022	PSC 2022	-	-	-	-	L4	*Helicops*	*angulatus*	Brazil	Mato Grosso
SB0473	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	L4	*Helicops*	*angulatus*	Brazil	Maranhão
IAVH-CT16196	-	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Colombia	Leticia
IAVH-CT29911	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L3	*Helicops*	*angulatus*	Colombia	Meta
IAVH-CT29944	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	L3	*Helicops*	*angulatus*	Colombia	Meta
IAVH-CT29945	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L3	*Helicops*	*angulatus*	Colombia	Meta
ICN12455	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Colombia	Guaviare
UIS-R3753	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Colombia	Casanare
QCAZ11544	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Orellana
QCAZ12871	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Orellana
QCAZ16675	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Morona Santiago
QCAZ7857	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Pastaza

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
QCAZ8292	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Pastaza
QCAZ9499	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Orellana
QCAZ9517	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Sucumbios
QCAZ9590	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L3	*Helicops*	*angulatus*	Ecuador	Orellana
AF1951	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*angulatus*	French Guiana	
AF3650	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*angulatus*	French Guiana	
MNRJ27457	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	L4	*Helicops*	*angulatus*	French Guiana	Kourou
MNRJ27458	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*angulatus*	French Guiana	Saint-Laurent-du-Maroni
AMNH152269	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Guyana	
GGU5437	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Peru	Loreto
GGU5768	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L3	*Helicops*	*angulatus*	Peru	Loreto
GGU5973	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	L3	*Helicops*	*angulatus*	Peru	Loreto
KU214879	GQ457738	-	GQ457857	-	-	-	L4	*Helicops*	*angulatus*	Peru	Madre de Dios
LSUMZH1524	-	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	L4	*Helicops*	*angulatus*	Peru	Loreto
AF2152	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*angulatus*	Suriname	Sipaliwini
YPM16081	PSC 2022	-	-	-	-	-	L2	*Helicops*	*angulatus*	Suriname	Sipaliwini
CAS231757	MT951599	MT951608	MT951605	-	-	-	L1	*Helicops*	*angulatus*	Trinidad	Sangre Grande
CAS231758	MT951597	MT951607	MT951603	-	-	-	L1	*Helicops*	*angulatus*	Trinidad	Sangre Grande
CAS231760	MT951600	MT951606	MT951604	-	-	-	L1	*Helicops*	*angulatus*	Trinidad	Sangre Grande
LSUMZH3307	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Trinidad	Tunapuna-Piarco
LSUMZH3346	PSC 2022	PSC 2022	AF471160	PSC 2022	PSC 2022	-	L1	*Helicops*	*angulatus*	Trinidad	Tunapuna-Piarco
MHNLS17558	PSC 2022	-	-	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*angulatus*	Venezuela	Bolívar
MHNLS17731	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*angulatus*	Venezuela	Delta Amacuro
MHNLS19631	PSC 2022	-	-	PSC 2022	PSC 2022	-	L1	*Helicops*	*angulatus*	Venezuela	Delta Amacuro

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
MHNLS21085	PSC 2022	-	PSC 2022	PSC 2022	-	PSC 2022	L4	*Helicops*	*angulatus*	Venezuela	Amazonas
UFMT12505	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*boitata*	Brazil	Mato Grosso
UFMT12506	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*boitata*	Brazil	Mato Grosso
UFMT11940	MN038124	-	MN032471	MN032484	MN032458	-	-	*Helicops*	*boitata*	Brazil	Mato Grosso
IAH162	-	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	-	*Hydrops*	*caesurus*	Brazil	Mato Grosso do Sul
VLF1826	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Hydrops*	*caesurus*	Brazil	Mato Grosso do Sul
CHUFSC4130	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	L3	*Helicops*	*carinicaudus*	Brazil	Santa Catarina
CHUFSC4131	PSC 2022	PSC 2022	-	-	-	-	L3	*Helicops*	*carinicaudus*	Brazil	Santa Catarina
HNF653	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	L1	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
HNF656	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
IBSP82223	PSC 2022	PSC 2022	-	-	-	-	L3	*Helicops*	*carinicaudus*	Brazil	São Paulo
IBSP86520	PSC 2022	PSC 2022	-	-	-	-	L3	*Helicops*	*carinicaudus*	Brazil	São Paulo
IBSP88735	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*carinicaudus*	Brazil	Espiríto Santo
IBSP89724	PSC 2022	PSC 2022	-	-	-	-	L3	*Helicops*	*carinicaudus*	Brazil	Santa Catarina
IBSP90428	PSC 2022	PSC 2022	-	-	PSC 2022	PSC 2022	L3	*Helicops*	*carinicaudus*	Brazil	Santa Catarina
IVB3598	PSC 2022	PSC 2022	-	-	-	-	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
MAP5658	PSC 2022	PSC 2022	-	-	-	-	L3	*Helicops*	*carinicaudus*	Brazil	São Paulo
MCP19444	-	PSC 2022	PSC 2022	-	PSC 2022	-	L3	*Helicops*	*carinicaudus*	Brazil	Santa Catarina
MNRJ15892	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
MNRJ26295	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
MNRJ26625	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
MNRJ26893	PSC 2022	PSC 2022	-	-	-	-	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
MNRJ26896	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
MNRJ26898	PSC 2022	PSC 2022	-	-	-	-	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
MNRJ27217	PSC 2022	PSC 2022	-	-	-	-	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
MNRJ27235	-	PSC 2022	-	-	-	-	L2	*Helicops*	*carinicaudus*	Brazil	Rio de Janeiro
UFRGS6743	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	L3	*Helicops*	*carinicaudus*	Brazil	Santa Catarina
IAVH-CT18769	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*danieli*	Colombia	Cesar
JORGE	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*danieli*	Colombia	Sucre
UIS-R3167	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*danieli*	Colombia	Santander
UIS-R3628	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*danieli*	Colombia	Santander
UIS-R3814	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*danieli*	Colombia	Cesar
CHUNB40948	-	PSC 2022	PSC 2022	-	PSC 2022	-	-	*Helicops*	*gomesi*	Brazil	Goiás
MTR11482	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*gomesi*	Brazil	Goiás
MZUSP14186	GQ457739	-	GQ457858	-	-	-	-	*Helicops*	*gomesi*	Brazil	São Paulo
H806	PSC 2022	-	PSC 2022	-	PSC 2022	-	-	*Helicops*	*hagmanni*	Brazil	Rondônia
LSUMZH17697	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	-	*Helicops*	*hagmanni*	Brazil	Rondônia
LSUMZH17698	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	*Helicops*	*hagmanni*	Brazil	Rondônia
MNRJ16833	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*hagmanni*	Brazil	Pará
MTR19395	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	-	*Helicops*	*hagmanni*	Brazil	Amazonas
MZUSP11716	MW219602	PSC 2022	JQ598985	MW218477	MW218475	PSC 2022	-	*Helicops*	*hagmanni*	Brazil	Amapá
UFMG-R2478	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*hagmanni*	Brazil	Pará
IAVH-CT21340	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	affHag	*Helicops*	*aff. hagmanni*	Colombia	Amazonas
A3572	PSC 2022	-	PSC 2022	-	-	PSC 2022	-	*Helicops*	*infrataeniatus*	Argentina	Victoria
LGE04818	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	*Helicops*	*infrataeniatus*	Argentina	Corrientes
60	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
EP077	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
IBSP85293	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	São Paulo
IBSP90004	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
IBSP90006	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
IBSP90403	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Mato Grosso do Sul
IBSP90423	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
IBSP90427	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
IBSP91778	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Mato Grosso do Sul
IBSP91779	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Mato Grosso do Sul
IBSP91780	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Mato Grosso do Sul
MAP1770	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Mato Grosso do Sul
MAP5652	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	São Paulo
MCP16090	PSC 2022	-	PSC 2022	-	PSC 2022	-	-	*Helicops*	*infrataeniatus*	Brazil	Santa Catarina
MCP16109	PSC 2022	-	PSC 2022	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	Santa Catarina
MCP19379	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	-	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
MCP19443	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
MCP4818	GQ457740	PSC 2022	GQ457859	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
MNRJ27133	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*infrataeniatus*	Brazil	Santa Catarina
MNRJ27135	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	Santa Catarina
UFRGS6971	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*infrataeniatus*	Brazil	Rio Grande do Sul
A6358	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*leopardinus*	Argentina	Garay
A6581	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*leopardinus*	Argentina	La Capital
LGE08898	PSC 2022	PSC 2022	-	PSC 2022	-	PSC 2022	-	*Helicops*	*leopardinus*	Argentina	Corrientes
LSUMZH6969	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*leopardinus*	Argentina	
CHUNB40620	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	*Helicops*	*leopardinus*	Brazil	Tocantins
H1733	MN038120	-	MN032464	MN032477	MN032451	-	-	*Helicops*	*leopardinus*	Brazil	Rondônia
MAP1548	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*leopardinus*	Brazil	Mato Grosso do Sul
MHNBA2603	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*leopardinus*	Brazil	Bahia
MTR14523	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*leopardinus*	Brazil	Tocantins
MTR19765	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*leopardinus*	Brazil	Bahia
UFMT7611	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*leopardinus*	Brazil	Mato Grosso
UFMT-R11939	MN038121	-	MN032465	MN032479	MN032452	-	-	*Helicops*	*leopardinus*	Brazil	Mato Grosso
IIBP-H4463	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*leopardinus*	Paraguay	Ñeembucú
MTR18968	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Hydrops*	*martii*	Brazil	Amazonas
UFMT6687	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Hydrops*	*martii*	Brazil	Mato Grosso
CHUNB38641	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	Distrito Federal

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
CHUNB43339	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	Goiás
IBSP80901	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*modestus*	Brazil	São Paulo
IBSP82580	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	Minas Gerais
IBSP84492	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	São Paulo
IBSP87808	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*modestus*	Brazil	São Paulo
IBSP89996	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*modestus*	Brazil	São Paulo
MAP5234	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	Goiás
MAP811	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	Minas Gerais
MAP-T5012	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*modestus*	Brazil	São Paulo
MAP-T5013	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*modestus*	Brazil	São Paulo
MTR19552	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*modestus*	Brazil	Minas Gerais
MZUSP12406	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	São Paulo
MZUSP12407	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*modestus*	Brazil	São Paulo
UFMG-T2696	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*modestus*	Brazil	Minas Gerais
UFMG-T7680	PSC 2022	PSC 2022	-	-	-	-	-	*Helicops*	*modestus*	Brazil	Minas Gerais
UFMG-T7681	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*modestus*	Brazil	Minas Gerais
UFMG-R2948	PSC 2022	PSC 2022	-	-	PSC 2022	-	-	*Helicops*	*nentur*	Brazil	Minas Gerais
UFMG-T5617	KT453992	PSC 2022	KT453991	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*nentur*	Brazil	Minas Gerais
ZUFMS-REP2291	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*nentur*	Brazil	Minas Gerais
ICN13114	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*pastazae*	Colombia	Cundinamarca
ICN13272	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Helicops*	*pastazae*	Colombia	Cundinamarca
QCAZ14059	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	-	L1	*Helicops*	*pastazae*	Ecuador	Orellana
QCAZ14077	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L1	*Helicops*	*pastazae*	Ecuador	Pastaza
QCAZ17560	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L1	*Helicops*	*pastazae*	Ecuador	Orellana

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
MZUSP14906	MW219601	PSC 2022	MW218473	MW218476	MW218474	PSC 2022	-	*Helicops*	*phantasma*	Brazil	Tocantins
MZUSP14917	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*phantasma*	Brazil	Tocantins
EESA323	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Pseudoeryx*	*plicatilis*	Brazil	Mato Grosso
MAP1734	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Pseudoeryx*	*plicatilis*	Brazil	Mato Grosso do sul
MAPT2280	PSC 2022	PSC 2022	PSC 2022	-	-	PSC 2022	L2	*Pseudoeryx*	*plicatilis*	Brazil	Rondônia
MZUSP19457	MN038115	-	MN032460	MN032473	MN032447	-	L2	*Pseudoeryx*	*plicatilis*	Brazil	Rondônia
UFMT12510	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L2	*Pseudoeryx*	*plicatilis*	Brazil	Mato Grosso
UFMT6871	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L1	*Pseudoeryx*	*plicatilis*	Brazil	Mato Grosso
VLF1832	-	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L2	*Pseudoeryx*	*plicatilis*	Brazil	Mato Grosso do sul
LSUMZH12749	PSC 2022	PSC 2022	-	-	-	PSC 2022	L1	*Pseudoeryx*	*plicatilis*	Ecuador	Sucumbios
QCAZ13342	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L1	*Pseudoeryx*	*plicatilis*	Ecuador	Orellana
QCAZ13343	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	-	L1	*Pseudoeryx*	*plicatilis*	Ecuador	Orellana
MNHN1996.7886	AF158500	-	-	-	-	-	L2	*Pseudoeryx*	*plicatilis*	French Guiana	
KU214900	GQ457765	GQ895885	GQ457886	-	-	-	L2	*Pseudoeryx*	*plicatilis*	Peru	Madre de Dios
IBSP89997	-	PSC 2022	PSC 2022	PSC 2022	-	-	-	*Helicops*	*polylepis*	Brazil	Pará
IBSP89998	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Pará
IBSP90524	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Pará
LW40	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Pará
LW41	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Pará
MNRJ17940	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Pará
MTR12961	MN038118	PSC 2022	MN032467	MN032481	MN032456	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Amazonas
MTR13033	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*polylepis*	Brazil	Amazonas
MTR13320	MN038119	-	MN032466	MN032482	MN032457	-	-	*Helicops*	*polylepis*	Brazil	Amazonas

Voucher	16S	Cytb	Cmos	NT3	BDNF	R35	L	Genus	Speies	Country	Federative Unit/Department
RBRAND5335	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Amazonas
UFMT6683	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*polylepis*	Brazil	Mato Grosso
UFMT7796	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*polylepis*	Brazil	Mato Grosso
MHNLS18542	-	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	-	*Hydrops*	*relictualis*	Venezuela	Zulia
MHNLS16984	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*scalaris*	Venezuela	Zulia
MHNLS18448	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	-	*Helicops*	*scalaris*	Venezuela	Zulia
IAVH-CT29912	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L3	*Helicops*	*sp.*	Colombia	Meta
ICN13232	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L3	*Helicops*	*sp.*	Colombia	Caquetá
MPEG25735	PSC 2022	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*tapajonicus*	Brazil	Pará
IBSP92037	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	L1	*Hydrops*	*triangularis*	Brazil	Maranhão
IBSP92715	-	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	L2	*Hydrops*	*triangularis*	Brazil	Acre
MZUSP14297	GQ457744	-	GQ457864	-	JQ599032	-	L1	*Hydrops*	*triangularis*	Brazil	Tocantins
MZUSP19497	MN038114	PSC 2022	MN032461	MN032474	MN032448	PSC 2022	L1	*Hydrops*	*triangularis*	Brazil	Rondônia
SB1439	-	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	L2	*Hydrops*	*triangularis*	Brazil	Acre
QCAZ17561	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	L2	*Hydrops*	*triangularis*	Ecuador	Orellana
Genbank	AF158499	-	-	-	-	-	L1	*Hydrops*	*triangularis*	French Guiana	
LSUMZH3105	PSC 2022	AF471039	AF471158	-	PSC 2022	PSC 2022	L2	*Hydrops*	*triangularis*	Peru	Loreto
CHUNB45364	-	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*trivittatus*	Brazil	Tocantins
CHUNB45423	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	PSC 2022	-	*Helicops*	*trivittatus*	Brazil	Tocantins
CHUNB45427	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	PSC 2022	-	*Helicops*	*trivittatus*	Brazil	Tocantins
IBSP90604	-	PSC 2022	PSC 2022	PSC 2022	-	PSC 2022	-	*Helicops*	*yacu*	Brazil	Acre

4 CHAPTER 3:

Phylogeography of *Helicops leopardinus* complex (Serpentes: Dipsadidae: Xenodontinae)

Abstract

The *Helicops leopardinus* complex is composed by four species of aquatic snakes (*H. leopardinus*, *H. infrataeniatus*, *H. modestus*, and *H. tapajonicus*) widespread throughout the South America, diagnosed by two chromatic patterns with no pholidose differentiation. In this paper, we characterized the genetic diversity and structure in the *H. leopardinus* complex and investigated which factors influenced the genetic differentiation across their distribution. Our analyses are based on the largest dataset ever assembled for these species of a wide geographic coverage (<3,000 km), using two mitochondrial and four nuclear genes. We presented the first phylogenetic relationship to *H. leopardinus* complex and we found that the four species are paraphyletic. The *Helicops leopardinus* group (including *H. phantasma*) originated at the Plio-Pleistocene (ca. 2.6 Mya), while the diversification within the complex occurred around 1.2 Mya. We found five genetically and spatially structured populational clusters, except Cluster 4, which is widespread. Moreover, we found that gene flow is unevenly shared among clusters. The genetic differentiation of the *H. leopardinus* complex is mainly explained by the interaction of geographic distance (IBD), climatic variation (IBE), and hydrographic basins. We provided new insights about diversification patterns and processes for a species complex of a broadly distributed group of watersnakes along South America. Once the dorsal chromatic patterns were not recovered as groups/clusters, this trait need to be better investigated for species diagnostic proposes.

Keywords: Hydropsini, Isolation by distance, Isolation by environment, Phylogeography, Polychromatism, Polymorphism, Squamata, South America, Taxonomy, Watersnake, Watersheds.

4.1 Introduction

Phenotypic diversity does not necessarily reflect genetic diversity (Mângia et al. 2020; Teixeira et al. 2022) and may also lead to bias in taxonomic and biogeographic decisions (Carvalho et al. 2020; Prates et al. 2020; Novaes-e-Fagundes et al. 2022). Spatial patterns of genetic diversity are more related to being shaped by abiotic and biotic characteristics (Dal Vechio et al. 2018; Oliveira et al. 2018a; Vasconcellos et al. 2019; Damasceno et al. 2021). For example, abiotic factors can permit or restrict gene flow and create opportunities for regional adaptations (Grazziotin et al. 2006; Shepard & Burbrink 2009; Prates et al. 2018). Historical and contemporary factors (ecological and/or geoclimatic) can also exert a force on the generation of genetic diversity, which may or may not cause speciation (Oliveira et al. 2018b; Camurugi et al. 2022), independently of polymorphic patterns (Leaché & Fujita 2010; Oliveira et al. 2015; 2018b). Knowledge of how genetic diversity is structured in space, as well as the phylogenetic relationships between taxa and populations on temporal scales, contribute to our understanding of organism's evolutionary history (Avise 2000; Rull & Carnaval 2020).

More recently, studies have incorporated climate and landscape data to clarify patterns of spatial genetic structure, although this influence is also mediated by population processes and biotic factors (e.g., Oliveira et al. 2018a; Pirani et al. 2019; Vasconcellos et al. 2019). Since environmental and landscape features may influence connectivity or vicariance among populations, and consequently promote genetic divergence, Isolation by Distance (IBD) is one of the patterns and is based on the premise that genetic differentiation increases with geographical distance (Wright 1943). However, populations may adapt due to environmental variation between regions, which can also provide genetic divergence, independent of geographic distance (Wang & Bradburd 2014; Camurugi et al. 2021). In this case, the pattern is known as Isolation by Environment (IBE). Rivers are important evolutionary agents, influencing the processes of diversification of lineages (e.g., Pirani et al. 2019; Coelho et al. 2022). Recently, several studies have shown that the hypothesis of rivers as barriers is not so dualistic and vicariant, and a certain permeability and dynamics depending on several intrinsic characteristics of each species and that it does not always generate allopatric speciation. (e.g., Pirani et al. 2019).

Systematic approaches that use genetic data help to overcome morphological issues, and are particularly useful for investigating the diversity of those organisms for which morphological identification is challenging (McVay et al. 2015; Bernstein et al. 2021; Damasceno et al. 2021). Within mud snakes (Homalopsidae), an aquatic snake group from Southeast Asia and the Indo-Australian Archipelago, taxonomic inflation was observed based on molecular data in species

usually diagnosed by external morphology, in taxa with allopatric (e.g., *Brachyorrhos raffrayi* and *B. albus*; *Cerberus dunsoni*, *C. microlepis,* and *C. schneiderii*) and sympatric distribution (e.g., *Homalopsis mereljcoxi* and *H. semizonata*) (Bernstein et al. 2021). According to the authors, this taxonomic inflation is probably due to morphological polymorphism and overlap in some characters such as ventral and subcaudal scales and prefrontal head plates. In the Neotropics, a similar scenario is observed in another genus of water snakes of the tribe Hydropsini.

Helicops represent a genus formed by 22 described species distributed from Trinidad to Uruguay throughout South America (Guedes et al. 2017; Nogueira et al. 2019). The *Helicops leopardinus* complex is composed by four species (*H. infrataeniatus*, *H. leopardinus*, *H. modestus,* and *H. tapajonicus*) with a wide distribution across the hydrographic basins of South America, that may represent a unique polychromatic lineage based on recent phylogenetic analysis (Moraes-da-Silva et al. 2019; 2021; Carvalho 2022). Among these four phylogenetically close species there are two dorsal chromatic patterns, spotted and green, the latter being either striated or smooth, which are used to delimit and diagnose them, although there is overlap among species (Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021). Accordingly, overlaps in certain morphological traits in *Helicops* (*sensu lato*) can make it difficult to delimit species based on external phenotypes, which can inflate the taxonomy of the Hydropsini tribe (e.g., Rossman 1976; Rossman & Abe 1979; Kawashita-Ribeiro et al. 2013).

Helicops leopardinus presents wide distribution in South America, occurring from Suriname to Uruguay (Guedes et al. 2017; Nogueira et al. 2019), and has a spotted dorsal pattern. This species is closely related to *H. tapajonicus*, *H. modestus* and *H. infrataeniatus*, the latter two occur mainly in the basins of the east coast of Brazil and Uruguay, South Atlantic Sul, and La Plata, but can also be found in other basins to the east of the continent and present a smooth/striated dorsal pattern (Guedes et al. 2017; Nogueira et al. 2019). On the other hand, *H. tapajonicus* is known for a few localities in the Amazon basin, with a smooth dorsal without stripes or spotted patterns (Frota 2005; Guedes et al. 2017; Nogueira et al. 2019). Within *H. leopardinus* complex there is overlap in morphological characters such as dorsal, supra and infralabial, ventral scales, and the number of maxillary teeth (Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021). Moreover, *H. infrataeniatus* and *H. leopardinus* have moderate keels on dorsal scales, while *H. modestus* and *H. tapajonicus* are weakly keeled (it is worth mentioning that this characteristic is subjective), and all are oviparous (the reproductive mode of *H. tapajonicus* is unknown) (Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021).

Numerous phylogeographic studies in South American snakes have focused on terrestrial taxa, mainly in the Viperidae family (e.g., Grazziotin et al. 2006; Dal Vechio et al. 2018; Hamdan

et al. 2020; Dal Vechio et al. 2020), and much less emphasis has been given to semi-aquatic and aquatic snakes. The distribution, biology, ecology, evolutionary history, demography, and population structure of water snakes remain largely understudied. However, some molecular studies have revealed some phylogenetic relationships and taxonomic issues (inflation and/or underestimation) in Hydropsini (Moraes-da-Silva et al. 2019; 2021; Carvalho 2022). Nevertheless, few studies have evaluated the evolutionary and biogeographic history of aquatic snake species, especially widely distributed species, such as the *H. leopardinus* complex (Braz et al. 2016; 2018; Carvalho et al. *in press*). Considering that these species are found in different watersheds, ecoregions, and contrasting landscapes, such factors supposedly could affect their dispersal capacities differently.

Our aim in this study is to evaluate which processes were important for the evolutionary history of these taxa, which we aim to (a) infer the phylogenetic relationships among *H. leopardinus*, *H. infrataeniatus*, *H. modestus*, and *H. tapajonicus*; (b) verify how their genetic diversity is geographically partitioned; and (c) test which abiotic factors (e.g., geographic, environment, and hydrologic) have driven their current genetic structure. Afterwards, we tested whether the pattern found can be explained by environmental, geographic and/or watershed drivers. We applied phylogenetic analyses (Bayesian inference and Maximum Likelihood) and population structure methods (BAPS) and lineages delimintation (GYMC and bPTP analyses) to determine patterns of genetic structure within this species complex.

4.3 Material and Methods

Sampling and molecular data

Different scientific collections provided tissue samples of 26 individuals of *Helicops leopardinus*, 30 of *H. modestus*, 37 of *H. infrataeniatus*, and one of *H. tapajonicus* from 88 localities in South America (Fig. 1; Table S1). We also obtained sequences of the *H. leopardinus* complex downloaded from GenBank (Table S1). We used the phenol-chloroform extraction protocol to extract the DNA from muscle, liver, or scale (Sambrook et al. 1989). We amplified the partial sequences of the two mitochondrial genes (mtDNA; 16S ribosomal (16S) and Cytochrome b (Cytb)) and four nuclear genes (nuDNA; Oocyte maturation factor Mos (Cmos), Neurotrophin-3 (NT3), Brain-Derived Neurotrophic Factor Precursor (BDNF), and RNA fingerprint protein 35 (R35)) using the standard Polymerase Chain Reaction (PCR) technique (for primers and PCR protocols see Supplementary Material 1). We aligned each locus using the Muscle algorithm (Edgar 2004) in Geneious v9.1.8 (Geneious 2022; https://www.geneious.com). We used the species *Helicops*

phantasma to root the tree. We used the PHASE algorithm (Stephens et al. 2001) to recover phase information for each nuDNA gene implemented in DNAsp v5 (Librado & Rozas, 2009). Only samples with the probability of pairs of alleles in heterozygosity higher than 80% were considered in the subsequent analyses.

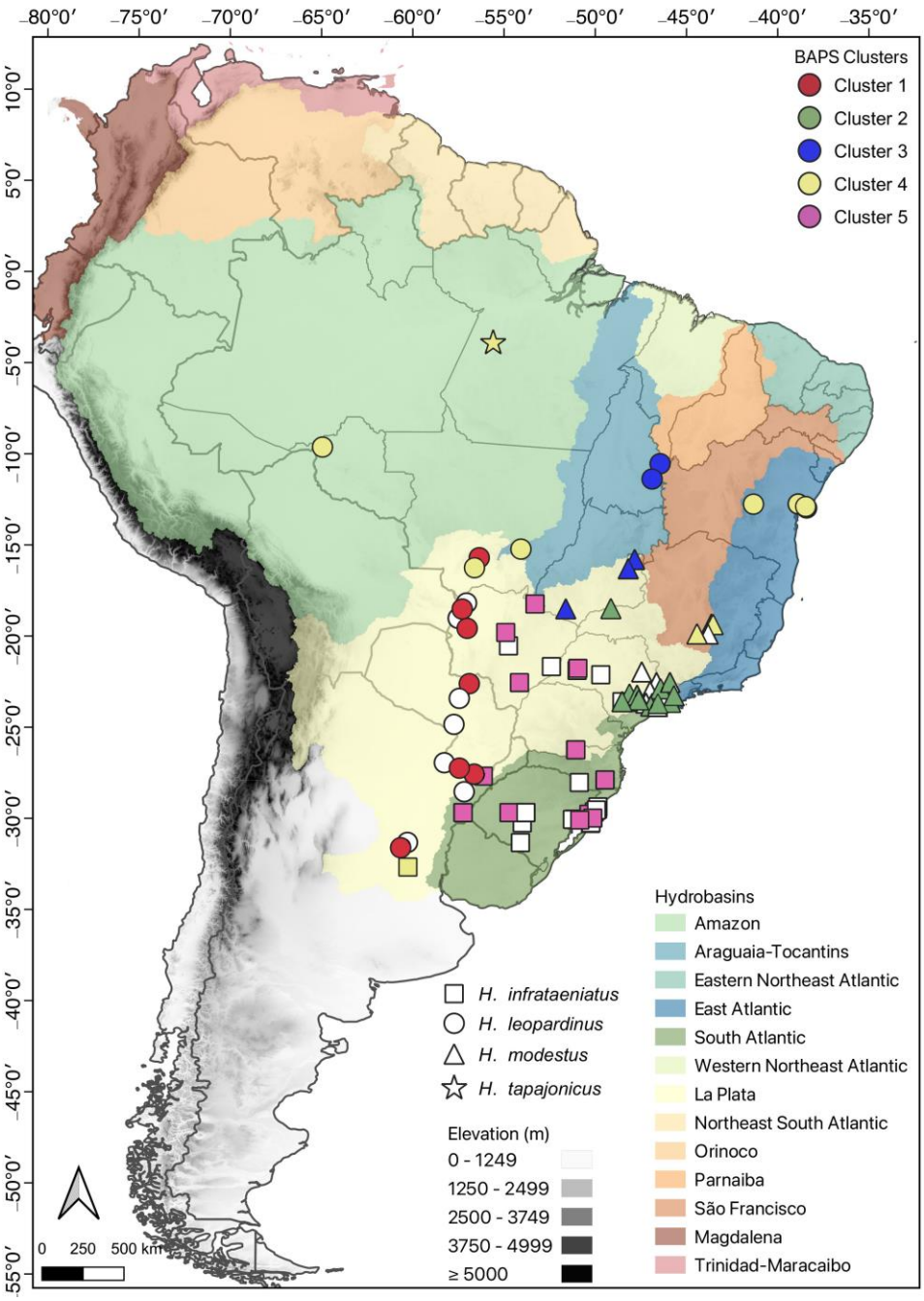


Figure 1. Geographic distribution of *Helicops leopardinus* complex samples (*H. leopardinus*, *H. infrataeniatus*, *H. modestus*, and *H. tapajonicus*) along the major hydrobasins across South America. White symbols indicate samples not used in the BAPS analysis.

Phylogenetic inference and divergence times

We performed a Bayesian inference (BI) analysis with the concatenated alignment of mtDNA loci (16S and Cytb) using BEAST v2.6.6 (Bouckaert et al. 2019). We used PartitionFinder 2 to identify partitioning schemes and the most appropriate nucleotide substitution models evaluated by BIC (Lanfear et al. 2017). According to the most appropriate substitution model for each partition (Table S2), we ran 100,000,000 generations sampled every 10,000 generations using the Yule process tree prior, a strict clock model, and the rate of 1.3% substitutions per million years. This mutation rate was estimated for mitochondrial genes (including Cytb) in Dipsadidae snakes by Daza et al. (2009). We checked visually the effective sample sizes (ESS values > 200) using Tracer v1.7 (Rambaut et al. 2018). We discarded 10% of generations as burn-in. We also implemented a Maximum Likelihood (ML) tree inferred in RAxML (Stamatakis 2014) via raxmlGUI 2.0 (Edler et al. 2021) using concatenated mtDNA and nuDNA loci (all genes). We used PartitionFinder 2 to identify partitioning schemes and the most appropriate nucleotide substitution models evaluated by BIC (Lanfear et al. 2017; see Table S2). For ML analysis, we used partitions restricted to GTR-based models, GTRGAMMA with rapid bootstrap analysis, and 1,000 bootstrap iterations.

Population assignment and genetic diversity

We inferred the spatial genetic structure in *Helicops leopardinus* complex using the Bayesian Analysis of Population Structure (BAPS) implemented in the BAPS v6.0 (Corander et al. 2008). We used individuals that had at least one mtDNA and two nuDNA genes (phased data), which totaled in 47 individuals (Table S3). To detect genetic clusters, we used the option 'spatial clustering of individuals' with a population mixture model, k ranging from 1 to 10, number of iterations of 100, 200 reference individuals from each cluster, and 20 iterations per individual. We used the resulting file of the admixture analysis with the function 'plot gene flow' in BAPS, and set the threshold for the significance of *P* values of the admixture estimates to 0.05. We generated a network of clusters, where gene flows are shown by weighted arrows, so that the weights relating to the amounts of ancestry in the source cluster are assigned to the target cluster (Tang et al. 2009).

We also used two tree-based species delimitation analyses to assess lineage boundaries within the *Helicops leopardinus* complex (single locus delimitation), Bayesian implementation of the Poisson Tree Proces (bPTP) and Generalized Mixed Yule Coalescent (GMYC). We ran a bPTP (Zhang et al. 2013) using the ML tree. This analysis was performed on PTP webserver (http://species.h-its.org/ptp/), with 500,000 MCMC generations, thinning set at 100, and burn-in at 10%. We ran a GMYC (Pons et al. 2006; Fujisawa & Barraclough 2013) using concatenated

mitochondrial BI tree and the package ‘splits’ (Ezard et al. 2017) in the R v 4.1.2 platform (R Core Team 2022).

We calculated different genetic statistics for each cluster recovered in BAPS and for each locus using DNASP (Librado & Rozas, 2009): number of polymorphic sites (S), haplotype number (h), haplotype diversity (Hd), and nucleotide diversity (π). We also estimated tests of Fu's Fs statistics and Tajima's D (using 10,000 coalescent simulations) to test the equilibrium of the population and the p-value for each locus. Next, to check haplotype sharing among clusters generated by BAPS, we built a haplotype network using the median-joining method with PopArt v1.7 (Leigh & Bryant 2015).

Testing genetic differentiation

We tested three plausible hypotheses (explanatory variables) that may explain genetic differentiation patterns of the *Helicops leopardinus* complex: geographic distance, climate, and hydrographic basin. Isolation by distance (IBD) pattern is expected whether the spatial distances affect the levels of gene flow across natural populations, assuming the premise that genetic differentiation increases with geographical distance. To test whether landscape environmental gradient (climate) affected the genetic differentiation of *H. leopardinus* complex, we assessed whether isolation by environment (IBE) is the main predictor of genetic variation in the species, given that different climates can lead to greater genetic differentiation due to local adaptation. To test IBE, we extracted 19 climate variables for each collecting locality based on the 2.5 arc-min resolution layers from the WorldClim bioclimatic dataset (Fick & Hijmans 2017) and removed those highly correlated (Pearson correlation > 0.7), resulting in 10 climatic variables: Mean Diurnal Range (Bio2), Isothermality (Bio3), Temperature Annual Range (Bio7), Mean Temperature of Wettest Quarter (Bio8), Mean Temperature of Driest Quarter (Bio9), Mean Temperature of Warmest Quarter (Bio10), Annual Precipitation (Bio12), Precipitation of Wettest Month (Bio13), Precipitation of Warmest Quarter (Bio18), and Precipitation of Coldest Quarter (Bio19). To test the role of the hydrographic basins, we associated each locality with its hydrographic basin, which resulted in six categories: Amazon, Araguaia-Tocantins, East Atlantic, La Plata, São Francisco, and South Atlantic. To determine if the basins were responsible for promoting interindividual genetic differentiation, is expected to have greater connectivity and gene flow within basins, and consequently greater genetic differentiation between basins.

To test whether IBD, IBE, and hydrographic basins explain part of the genetic differentiation in *Helicops leopardinus* complex, we conducted redundancy analysis (RDA) in the R environment v4.1.2 (R Development Core Team 2022) using the packages ‘raster’,‘rworldmap’, ‘rgdal’, ‘ape’,

and ‘vegan’ (South 2011; Paradis et al. 2019; Hijmans 2020; Oksanen et al. 2020; Bivand et al. 2021). We ran RDA following the approach outlined in Myers et al. (2017). We used only the mtDNA16S gene for this analysis due to missing data for Cytb and nuDNA. We generated the matrix of interindividual genetic distances as response variables using a normalized genetic distance matrix in MEGA v.11 (Tamura et al. 2021), which was then subjected to principal coordinate analysis (PCoA). We conducted a total of seven RDA analyses where the genetic differentiation (as measured by the PCoA matrix of genetic distances) were tested using climate, hydrographic basins, and geographic distance as explanatory variables, and all combinations of these variables including a full model with all three variables. RDA returns an r2 and adj.R2 values and significance were assessed using an ANOVA.

4.3 Results

Phylogenetic inference and divergence times

The final concatenated alignment for the mtDNA 16S and Cytb comprised 1165 bp, while the alignment concatenated for the mtDNA and nuDNA comprised 2754 bp. Both BI (concatenated mtDNA) and ML (concatenated mtDNA and nuDNA) phylogenetic trees generated congruent topologies (Fig. 2; Fig. S1). *Helicops leopardinus* complex is monophyletic and well supported (PP=1.00). However, none of the four currently recognized species are well supported and instead all are paraphyletic. The divergence time between *Helicops leopardinus* complex and *H. phantasma* was about 2.6 Mya (95% HPD: 1.93–3.25), during the Plio-Pleistocene. The diversification within the *H. leopardinus* complex started around 1.3 Mya (95% HPD: 0.96–1.56) and all main clades diversified before 0.8 Mya, suggesting a rapid diversification of these aquatic snakes.

Population assignment and genetic diversity

We found five genetically and spatially structured clusters (except Cluster 4 which occurs spread throughout sampling) based on BAPS results from mtDNA and nuDNA data (Fig. 1; Fig. 3A; Table S3). We observed genetic admixture in five samples (10.64% of sampling) with unequal proportions of admixture (Fig. 3B). Cluster 2, Cluster 3, and Cluster 4 presented one sample with admixture, while Cluster 5 presented two samples. Only Cluster 1 had no admixture individuals (Fig. 3B). The network of clusters indicating the gene flow showed uneven genetic sharing among clusters (Fig. 4). Cluster 1 contributed genetically to all other four clusters (mainly with Cluster 2 and 3), but is not genetically influenced by any of them. Cluster 3 had the higher level of input genetic material transfer. Cluster 2 provided genetic materials to Clusters 4 and 5, while Cluster 4

contributed genetically to Clusters 2 and 5. Despite the genetic clustering found with BAPS, both bPTP and GMYC delimitation analyses supported the existence of only one species in *Helicops leopardinus* complex (Fig. 2).

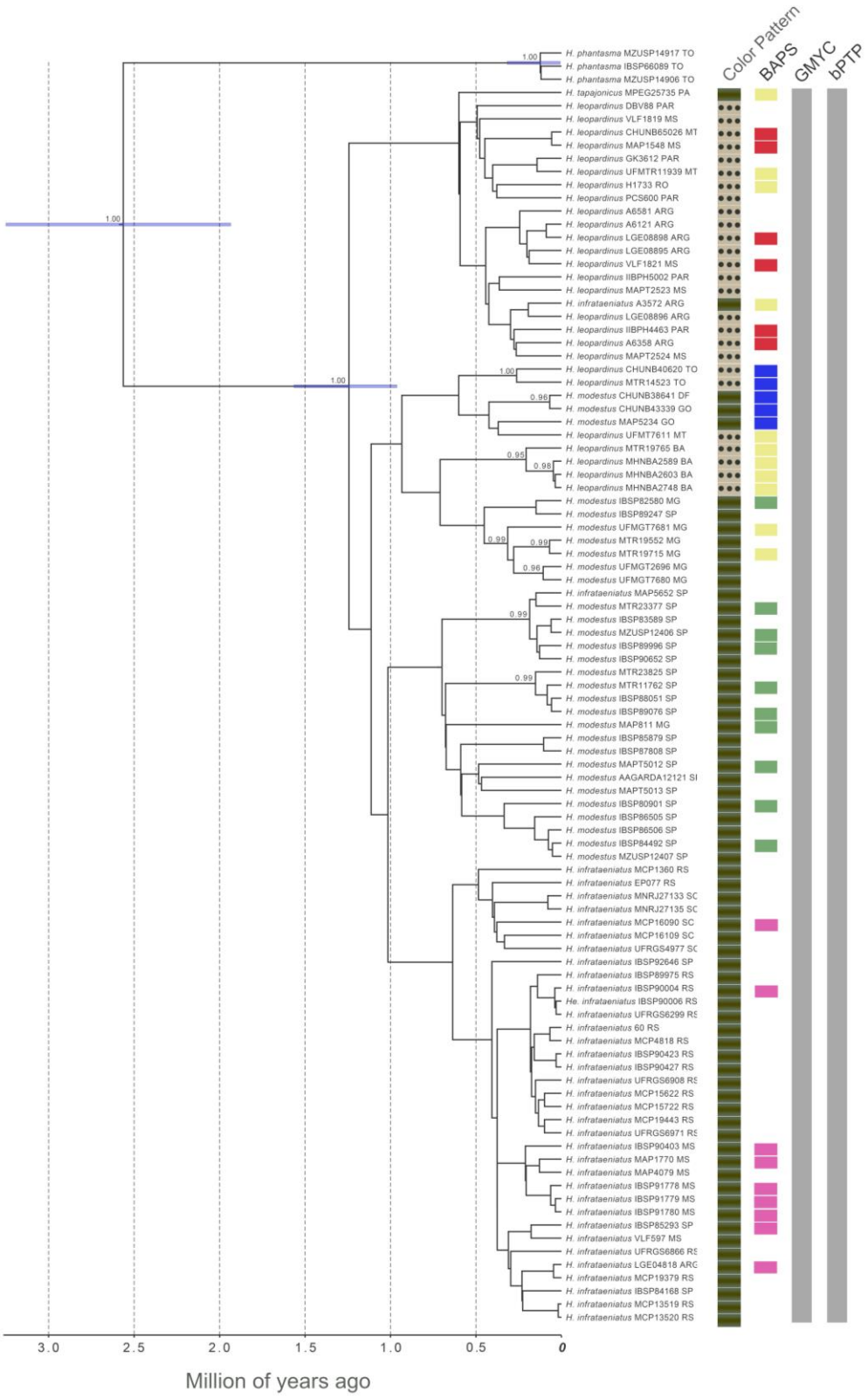


Figure 2. Phylogenetic relationships of *Helicops leopardinus* complex and divergence times by Bayesian Inference in BEAST based on mtDNA 16S and Cytb concatenated genes. Numbers

below nodes are posterior probability values; nodes without values indicate PP < 0.95. Bars represent the 95% Highest Posterior Density (HPD) interval for divergence dates. Taxon name, voucher number, and locality are indicated for each terminal.

A summary of all population genetic statistics for *Helicops leopardinus* complex is presented in Table S4. Haplotypic diversity was higher in mtDNA 16S and Cytb, and nuDNA NT3 and R35 when we considered all samples, and continued high when analyzed considering BAPS clusters (Table S4). Both nuDNA Cmos and BDNF revealed low haplotypic diversity. The haplotype network for mtDNA16S showed that three haplotypes are shared between Clusters 1 and 4, and between Clusters 3 and 4 (Fig. 5). The haplotype network for Cytb find no sharing of haplotypes among clusters, being all exclusive (Fig. 5). Haplotype networks of Cmos and BDNF presented five and four haplotypes, respectively, with the main haplotype being shared with all clusters recovered by BAPS. We observed 29 haplotypes in the NT3 haplotype network (only three are shared) and 18 haplotypes in R35 (only two are shared).

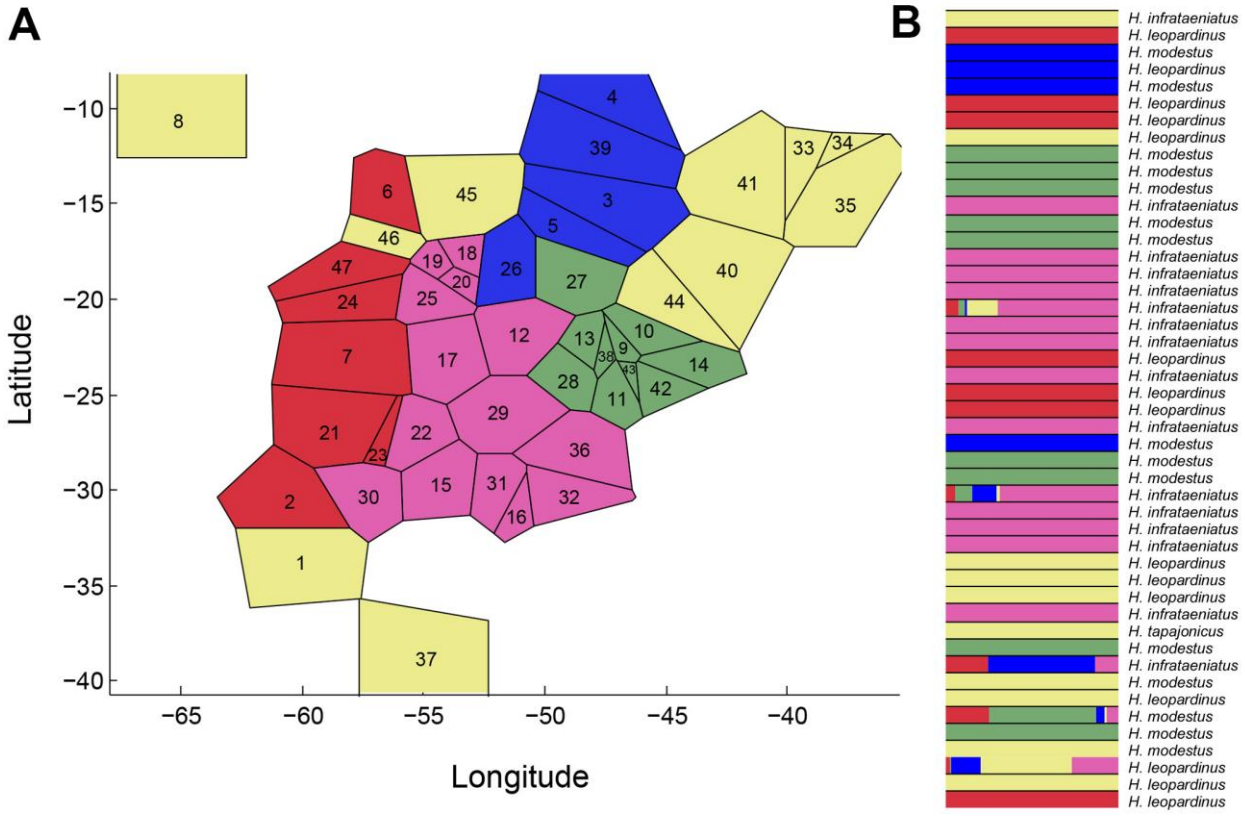


Figure 3. Bayesian Analysis of Population Structure (BAPS) results: A) spatial clustering of individuals. B) Admixture based on mixture clustering. Each color represents one cluster (among the five clusters) generated by the mitochondrial and nuclear gene dataset. Cluster 1: red; Cluster 2: green; Cluster 3: blue; Cluster 4: yellow; Cluster 5: pink.

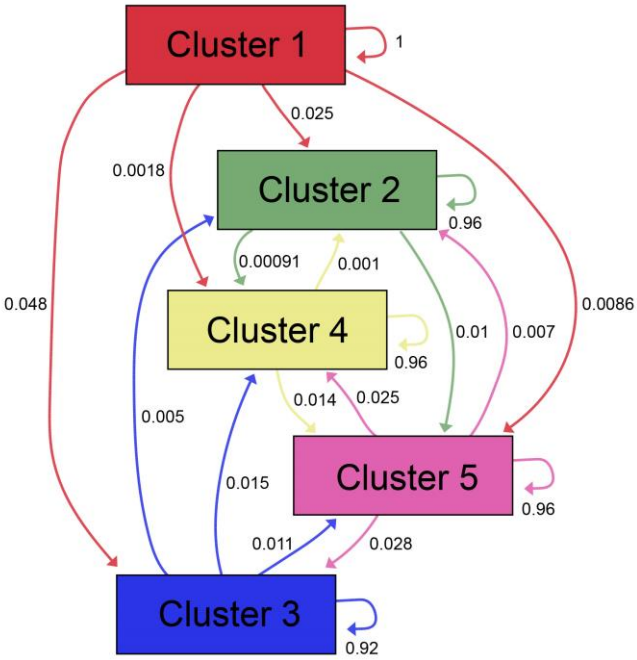


Figure 4. Network of five clusters indicating the gene flow among them by weighted arrows.

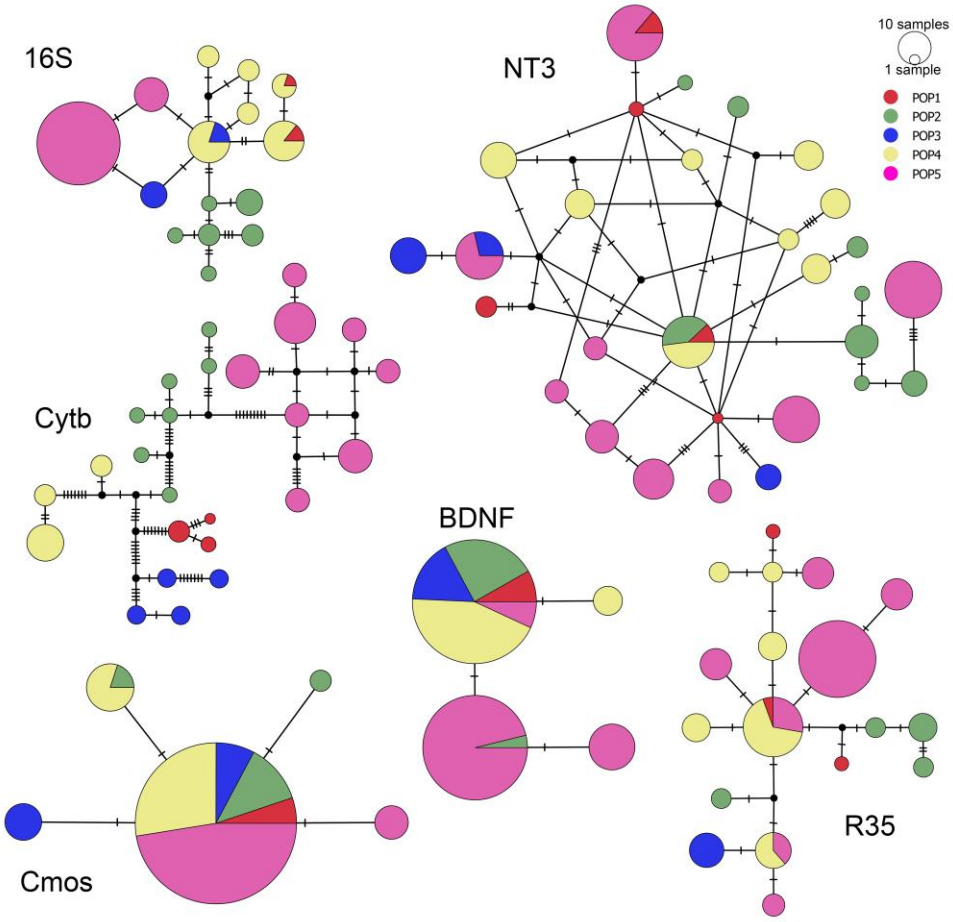


Figure 5. Haplotype networks for each locus within of *Helicops leopardinus* complex. The haplotype colors correspond to the clusters recovered by BAPS, and the size of its area is

proportional to its frequency. Black circles represent unsampled haplotypes and the dashes represent mutational steps.

Genetic differentiation

The RDA analyses demonstrated that genetic differentiation based on mtDNA 16S is significantly correlated to the full model, which includes climatic variation (IBE), geographic distance (IBD), and hydrographic basins (Table 1). The full model explained 62% of genetic differentiation in *Helicops leopardinus* complex (r2 = 0.62, p-value < 0.005). When analyzing each explanatory variable separately and the combinations between them, all models were significant (except for the basins model) (Table 1).

Table 1. Model selection of what variables influence population genetic diversity using redundancy analyses (RDA) for *Helicops leopardinus* complex.

Model	Variance (Adjusted R^2)	Variance (R^2)	*P*-value
	16S	16S	16S
Full	**0.54**	**0.62**	**0.001**
Distance+Environment	0.47	0.53	0.001
Environment+Basins	0.32	0.38	0.001
Environment	0.27	0.31	0.001
Distance	0.11	0.11	0.001
Basins+Distance	0.11	0.11	0.001
Basins	0.00	0.00	0.237

4.4 Discussion

The divergence time between the water snakes of the *H. leopardinus* complex and its sister taxa *H. phantasma* was about 2.6 Mya, while within the *H. leopardinus* complex started ~1.3 Mya ago. Both phylogenetic inferences are congruent and recover that all nominal species are non-monophyletic. Besides, bPTP and GMYC analyses supported the existence of a single lineage in *H. leopardinus* complex. We found five genetically and spatially structured population clusters by BAPS, except Cluster 4, which is widespread. Moreover, we found that the gene flow is unevenly shared among clusters. We observed that in this species complex the genetic differentiation is best explained by the interaction of geographic distance (IBD), climatic variation (IBE), and hydrographic basin.

Phylogenetic inference, divergence times, and genetic structure

Both BI and ML trees recovered the four species of *Helicops leopardinus* complex as paraphyletic (Fig. 2; Fig. S1). The lack of support in all clades makes the relationships among and within species unresolved, and reveals that they can be even more intricate. Many molecular phylogeny studies in watersnakes around the globe have recovered clades and species as non-monophyletic, which were previously identified based solely on morphology. Such contrasting findings between molecules and morphology were noted at species and genus levels (e.g., McVay et al. 2015; Bernstein et al. 2021). Some examples of non-monophyletism in North America include the genus *Regina* (e.g., Price 1983; Rossman 1985; Alfaro & Arnold 2001; McVay et al. 2015). In Asia, within Homalopsidae, Bernstein et al. (2021) revealed 13 non-monophyletic species among five genera. Carvalho et al. (2020) demonstrated that *Hydrodynastes melanogigas* in South America was only a melanic population, and therefore, a junior synonymy of *H. gigas*. In our case, *Helicops* species present overlap and discrepancies in morphological characteristics, both in qualitative and quantitative data, which in many cases make it difficult to delimit and identify species.

The period of diversification of *Helicops leopardinus* complex (1.3 Mya; 95% HPD: 0.96–1.56 in the Pleistocene) took place when the main geomorphological structures in South America were already established as we know them today (mountain ranges and basin drainages). Therefore, there were probably other factors that promoted this diversification. During the Pleistocene, one of the most remarkable events and that also determined the diversification of other organisms in South America were the severe climatic oscillations (Vuilleumier 1971; Carnaval et al. 2014). As climate (see discussion below) was one of the important factors for the genetic differentiation of this species complex, possibly climate may also have been important for diversification.

The species delimitation methods have recovered only one species (single lineage) for the whole complex. However, the population assignment (BAPS) found five clusters with four of them spatially structured, and one widespread (Cluster 4). Besides, we found gene flow among all clusters, which occurs asymmetrically (Fig. 3A), and demonstrates that the evolutionary pattern depends on both abiotic (landscape, climate) and biotic. Gene flow was previously found in other watersnakes (e.g., Brandley et al. 2010; McCartney-Melstad et al. 2012; Asztalos et al. 2021). For example, asymmetric gene flow was revealed between populations of *Eunectes notaeus* (Anaconda) in Argentina, suggesting that this could be a frequent pattern, especially in aquatic species (McCartney-Melstad et al. 2012). Possibly the distance, climate, and hydrographic basins influence these dynamics of gene flow among clusters.

Effect of IBD, IBE, and hydrographic basin on genetic differentiation

Helicops leopardinus complex has a wide geographic distribution, occurring in different ecoregions and watersheds. We found that genetic differentiation in the *H. leopardinus* complex is driven by climate (10 climatic variables; IBE), geographic distance (IBD), and hydrographic basins altogether. Such interaction among predicting variables coincides with the spatial heterogeneity in South America, which can generate different climates across the continent, and may promote genetic differentiation due to local adaptation (Savolainen et al. 2013; Radersma et al. 2020). Phenotypic and genetic differentiation across landscapes (habitats), or contrasting environmental gradients may also be indicative of local adaptation (Conover et al. 2009), which may eventually lead to diversification (Sobel et al. 2010; Nosil 2012). We highlight that the genetic differentiation in the *Helicops leopardinus* complex does not reflect the structure of the nominal species separation. Therefore, widely distributed species can be affected by climates and geographic distances along their distribution limits (Mott 2010), which can act individually as main drivers, or act synergistically. Several studies with other species have shown different populations adapted to local abiotic or biotic conditions (Dal Vechio et al. 2018; Camurugi et al. 2021). In this sense, changes in phenotype induced by environmental gradients (new environment) are, in principle, plastic responses. Whether they will be selected by adaptive evolution in the future is debated (Radersma et al. 2020).

As we are dealing with semiaquatic species, we considered that hydrographic basins could be important factors in the phylogeographic structure of the *Helicops leopardinus* complex, since, in general, hydrographic basins delimit the different lineages found in them. However, it only had influence in synergy with other abiotic factors (i.e., climate and geographic distance). On the other hand, solely, the hydrographic basins had no influence on the phylogeography of this species complex. The phylogeographic patterns in aquatic snakes varies between regions, geographic distributions, and species, even in sister lineages (e.g., Guiher & Burbrink 2008; Brandley et al. 2010; Lukoschek et al. 2011). In other aquatic snakes in North and South America, Europe, and Asia, rivers may or may not act on genetic structure. Species from North and South America exhibited contrasting phylogeographic patterns. The Mississippi River (USA) affected the structure of *Nerodia rhombifer*, while it was not a barrier to *Agkistrodon piscivorus* and *N. erythrogaster* (Guiher & Burbrink 2008; Brandley et al. 2010). In South America, populations of *Eunectes notaeus* (Anaconda) from Argentina showed genetic structure associated with water courses (McCartney-Melstad et al. 2012), while for both species of *Hydrodynastes*, widely distributed throughout the continent, such association was not observed (Carvalho et al. 2020; Carvalho et al. *in press*). Although in Asia, *Enhydris subtaeniata* (mud snake) is structured by watersheds (Lukoschek et al. 2011), *Natrix natrix* (grass snake) in Central Europe did not show evidence of genetic structure in

a habitat highly fragmented by agriculture (Meister et al. 2010). All these examples suggest the potential of different effects of water barriers on the genetic structure of aquatic organisms. The same river/basin may differently be involved in generating and maintaining species diversity. Therefore, it is difficult to generalize the phylogeographic patterns of species with an semiaquatic habit, especially if we consider organisms from South America, a rich region regarding hydrographic networks.

Furthermore, phenotypic traits may influence patterns of species distribution and genetic diversity, like aspects of life history, behavior, and diet, and may be as important as abiotic factors (e.g., Voris et al. 2012; Papadopoulou & Knowles 2016; Zamudio et al. 2016). For example, in *Enhydris subtaeniata*, a Mud Snake from Indochina, data on the size and reproductive traits reflected its genetic differences (Voris et al. 2012). Methods and tools that incorporate these traits may help to elucidate the evolutionary processes involved in the phylogeographic pattern with better accuracy and less generalization (Papadopoulou & Knowles 2016; Zamudio et al. 2016).

Our findings support a dispersion scenario for these watersnakes. The detected genetic structure was observed spatially and geographically, except for the Cluster 4, once all clusters was not congruent with ecoregion boundaries, basins, or other biogeographic barriers. In summary, we were not able to find physical barriers to contain the dispersion and gene flow of this species complex.

Although seems contradictory the high genetic variability found in the *Helicops leopardinus* complex, and the whole sampling was recovered as a single lineage, previous studies show that this may reflect long-distance dispersal (LDD) (Berthouly-Salazar et al. 2013). As previous found in a neotropical lizard from Caatinga (Werneck et al. 2015), the LDD may promote the genetic differentiation that leads to adaptative evolution in new environments (Kremer et al. 2012). Besides, as previous noted, the LDD would overcome the sequential founder effect and mitigate the reduction of genetic diversity associated with short dispersion (Berthouly-Salazar et al. 2013). Therefore, the scenario we have found for these water snakes probably contributed to maintain such high genetic diversity that reflect on the genetic differentiation.

Taxonomic implications

Considering the pattern presented of non-monophyly, genetically and spatially organized clusters but, high diversity, and asymmetric gene flow in *H. leopardinus* complex, we cannot rule out the possibility that they are four distinct species (*H. leopardinus, H. modestus, H. infrataeniatus* and *H. tapajonicus*). We cannot exclude a possible scenario of incomplete lineage sorting (that retention of ancestral polymorphism), which makes it difficult to assess the relationships among the

species. However, incomplete lineage sorting and secondary gene flow produce very similar patterns, making the investigation of speciation history difficult (Mallet et al. 2016). Furthermore, the introgression process may also have created this phylogenetic incongruity. Ruane et al. (2014) showed that mtDNA introgression occurs among distinct species of *Lampropeltis* in the southwestern US. Possibly, *H. leopardinus* complex may represent a single polychromatic species widely distributed throughout South America. Nevertheless, it may be a combination of these mechanisms that are involved in this pattern, but the evolutionary history of this species complex needs to be evaluated in the future using phylogenomic coalescent methods.

The taxonomy of the species belonging to the *H. leopardinus* complex has never been evaluated and, traditionally, they are identified based on geographic patterns and dorsal coloration (Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021). However, there is no external morphological character that diagnoses these species, all qualitative and quantitative characters overlap (Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021). Once that even in the known literature for *Helicops* there is no strong evidence that supports the diagnosis of these four species, our findings corroborate that the lack of diagnose evidence, reveals a population-level genetic structure and not four distinct species. Non-monophyletic species with recent diversification, sympatry and polymorphism (with phenotypic intergrades) suggest the occurrence of local adaptation in *Helicops leopardinus* complex.

Despite the lack of evidence, it is well known that the spotted pattern is used to identify *Helicops leopardinus* in this species complex. However, in the lower La Plata basin, spotted and green patterns can generate an intermediate dorsal pattern (intergrade) composed of these forms (Fig. 2). We sampled this dorsal pattern in our analysis, a sample from Victoria, Entre Rios province, Argentina (A3572). This pattern was historically identified as *H. baliogaster* Cope (1885), currently under the synonym of *H. infrataeniatus*. Due to the geomorphological aspects of the Lower Plata basin, this region where spotted and green patterns co-occur, possibly providing mixed. *Helicops tapajonicus* was compared in its description solely with Amazonian congeners (Frota 2005). However, in its description, is clear that its dorsal coloration pattern and other morphological and hemipenis characteristics are similar to *H. infrataeniatus*, *H. modestus*, and *H. leopardinus* (Costa et al. 2016; Moraes-da-Silva et al. 2019; 2021).

The importance of morphological data for understanding taxonomy, evolution, selection, population dynamics, ecology, and comparative studies is indisputable (e.g., Hillis & Wiens 2000; Wiens 2004; Zamudio et al. 2016). Nevertheless, the description of species based solely on morphology in Hydropsini will likely result in taxonomic problems. Given that, morphological data

can be misleading and do not demonstrate the real diversity and genetic structure of the species, consequently, it may also not reflect the real evolutionary, phylogenetic and biogeographic pattern.

4.5 Conclusions

We have sampled the largest dataset to date for *Helicops* spp. and present the first phylogeographic study of the most diverse tribe of semiaquatic snakes in the Neotropics. Despite the high genetic diversity and relatively recent divergence, the *Helicops leopardinus* complex had a rapid dispersal without physical biogeographical barriers. We observed that in this species complex the genetic differentiation is best explained by the interaction of geographic distance (IBD), climatic variation (IBE), and hydrographic basin. Other likely interpretation for the actual genetic scenario would be a result of the long-distance dispersal. Our results revealed incongruences regarding the current taxonomy for these taxa. Overall, the color pattern within the Hydropsini is a trait that needs to be investigated, as it is a trait that undergoes ecological and evolutionary pressures within aquatic snakes. This spatial chromatically organization is probably a population structuring within the *H. leopardinus* complex, rather than a multispecies structuring, as it shows overlap in dorsal and ventral scale counts and variation in keel pattern. We emphasize the need of a taxonomic revision for these species based on an integrative approach and further geographic, taxonomic, and molecular sampling. Thus, detailed studies in the future focusing on these clusters should try to elucidate the gene flow between them to determine the degree of separation among these clusters. Furthermore, it would be interesting to assess the genetic and phenotypic polymorphism disparity with larger sampling either for localities as well as loci or ideally at the genomic level in conjunction with a detailed morphological examination. Genome-scale sequencing will likely improve our understanding of the broader relationships. Additionally, once watersheds have low explanation on the genetic differentiation, included environment resistance is paramount to fill the landscape influence on the phylogeography of this complex.

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Supplementary information

Table S1. Taxa, vouchers, locality data, genetic markers and GenBank accession numbers used in this study. Absent acronyms in Sabaj (2022): MTR or MRT: researcher field number Miguel Trefaut U. Rodrigues (University of São Paulo, USP); A: researcher field number Vanessa Arzamendia (Faculty of Humanities and Sciences, National University del Litoral); AAGARDA: researcher's field number Adrian Antonio Garda; AF: researcher field number Antoine Fouquet; GGU: researcher field number Giussepe Gagliardi Urrutia (Universidad Nacional de la Amazonía Peruana, Facultad de Ciencias Biológicas); LW: field number of the Federal University of Western Pará; MAP and MAP-T: field and tissue number of the Mapinguari laboratory at the Federal University of Mato Grosso do Sul; MHNBA: Bahia Natural History Museum voucher; RABRANDÃO: researcher field number Reuber Brandão (University of Brasilia) SB: project field number scales of biodiversity; VLF: researcher field number Vanda Lucia Ferreira (Federal University of Mato Grosso do Sul.

This table is presented separately (in another document).

Table S2. PartitionFinder 2 model of nucleotide substitution. Best-fitting partitioning scheme model of nucleotide substitution for 16S, Cytb, Cmos, NT3, BDNF, and R35 genes.

Data set	Partitioning scheme	Model
mitDNA concatenated	16S, CYTB3	GTR+I+G
	CYTB1	HKY
	CYTB2	TRN+G
All genes concatenated	NT32, 16S, CYTB3	GTR+I+G
	R353, R351, R352, CMOS1, CYTB1	HKY+I
	CYTB2	TRN+G
	CMOS3, BDNF1, CMOS2	JC
	BDNF3, NT33, NT31, BDNF2	TRNEF+I

Table S3. Specimens, vouchers, and geographic coordinate of samples used in Bayesian Analysis of Population Structure (BAPS) and which spatial clusters they belong. In bold the specimens that were admixture.

CLUSTER	Taxonomy	Voucher	LONG	LAT	SPATIAL CLUSTERING
1	*H. leopardinus*	A6358	-60.293	-31.321	2
1	*H. leopardinus*	CHUNB65026	-5.635.756	-15.701.839	6
1	*H. leopardinus*	DBV88	-56.901.668	-22.610.612	7
1	*H. leopardinus*	IIBPH4463	-574.479	-272.456	21
1	*H. leopardinus*	LGE08898	-56.637.604	-27.576.399	23
1	*H. leopardinus*	MAP1548	-57.018.704	-19.576.015	24
1	*H. leopardinus*	VLF1821	-57.290.241	-18.518.745	47
2	*H. modestus*	IBSP80901	-46.415.876	-22.914.131	9
2	*H. modestus*	IBSP82580	-45.911.133	-22.564.750	10
2	*H. modestus*	IBSP84492	-46.947.782	-23.849.574	11
2	*H. modestus*	IBSP89076	-48.117.772	-23.168.607	13
2	*H. modestus*	IBSP89996	-45.687.247	-23.257.534	14
2	*H. modestus*	MAP811	-49.140.847	-18.494.203	27
2	*H. modestus*	MAPT5012	-48.528.898	-23.597.412	28
2	*H. modestus*	MTR11762	-47.703.308	-23.242.091	38
2	***H. modestus***	**MTR23377**	-45.866.667	-23.633.333	42
2	*H. modestus*	MZUSP12406	-46.564.848	-23.689.841	43
3	*H. modestus*	CHUNB38641	-47.837.241	-15.798.033	3
3	*H. leopardinus*	CHUNB40620	-46.441.286	-10.542.157	4
3	*H. modestus*	CHUNB43339	-48.187.322	-16.306.977	5
3	*H. modestus*	MAP5234	-516.129	-185.046	26
3	***H. leopardinus***	**MTR14523**	-46.880.111	-11.383.319	39
4	*H. infrataeniatus*	A3572	-602.689	-326.734	1
4	*H. leopardinus*	H1733	-64.950.322	-9.628.578	8
4	*H. leopardinus*	MHNBA2589	-3.887.192	-12.750.745	33
4	*H. leopardinus*	MHNBA2603	-38.461.256	-12.892.578	34
4	*H. leopardinus*	MHNBA2748	-38.411.159	-12.968.826	35
4	*H. tapajonicus*	MPEG25735	-55.594.166	-3.922.388	37
4	*H. modestus*	MTR19715	-43.627.459	-19.340.053	40
4	*H. leopardinus*	MTR19765	-41.323.202	-12.761.809	41
4	*H. modestus*	UFMGT7681	-44.417.455	-19.882.763	44
4	***H. leopardinus***	**UFMT7611**	-541.746	-153.332	45
4	*H. leopardinus*	UFMTR11939	-56.607.544	-16.417.237	46
5	*H. infrataeniatus*	IBSP85293	-50.961.796	-21.763.687	12
5	*H. infrataeniatus*	IBSP90004	-54.684.717	-29.677.590	15
5	*H. infrataeniatus*	IBSP90006	-51.097.008	-30.073.260	16
5	*H. infrataeniatus*	IBSP90403	-54.305.145	-22.479.154	17
5	***H. infrataeniatus***	**IBSP91778**	-53.706.442	-18.316.591	18
5	*H. infrataeniatus*	IBSP91779	-53.706.442	-18.316.591	19
5	*H. infrataeniatus*	IBSP91780	-53.706.442	-18.316.591	20
5	*H. infrataeniatus*	LGE04818	-56.133.332	-27.683.337	22
5	*H. infrataeniatus*	MAP1770	-54.905.619	-19.804.106	25

CLUSTER	Taxonomy	Voucher	LONG	LAT	SPATIAL CLUSTERING
5	***H. infrataeniatus***	**MCP16090**	-51.063.360	-26.243.958	29
5	*H. infrataeniatus*	MCP19379	-57.083.220	-29.744.157	30
5	*H. infrataeniatus*	MCP19443	-51.219.347	-30.021.309	31
5	*H. infrataeniatus*	MCP4818	-50.133.563	-29.982.800	32
5	*H. infrataeniatus*	MNRJ27133	-49.461.667	-27.896.524	36

Table S4. Genetic statistics for each locus for cluster Bayesian Analysis of Population Structure (BAPS) of *Helicops leopardinus* complex. L: length in base pairs (bp); N: sample size; S: number of polymorphic sites; H: number of haplotypes (number of exclusive haplotypes in this lineage); Hd: haplotype diversity; π: nucleotide diversity. neutrality tests Tajima's D and Fu's F. * Phased sequences; ** sampling were not enough to estimate these parameters; *** genetic variation was not enough to estimate these parameters.

Locus	Population	L	N	S	H	H_d	pi π	Tajima's D	P-value	Fu's F	P-value
16S	All	312	40	17	15	0.885	0.01097	-0.08762	0.56030	-0.01436	0.53510
	Cluster 1	312	3	1	2	0.667	0.00214	**	**	0.34967	0.17609
	Cluster 2	311	10	8	6	0.889	0.00857	-0.04424	0.51340	0.23909	0.47520
	Cluster 3	311	3	1	2	0.667	0.00214	**	**	0.35123	0.17083
	Cluster 4	312	10	6	6	0.867	0.00700	-0.05102	0.51700	0.27039	0.48214
	Cluster 5	311	14	1	2	0.264	0.00085	-0.01361	0.74210	0.28551	0.39521
CYTB	All	561	35	62	25	0.975	0.02474	-0.10370	0.57120	-0.15904	0.53480
	Cluster 1	561	7	4	3	0.667	0.00238	-0.04365	0.55330	0.30982	0.47838
	Cluster 2	561	7	20	7	1.000	0.01341	-0.04810	0.50390	0.44978	0.41834
	Cluster 3	561	4	13	4	1.000	0.01307	0.02616	0.65320	0.91362	0.28908
	Cluster 4	561	5	10	3	0.700	0.00784	-0.03861	0.57270	0.52225	0.38616
	Cluster 5	561	12	14	8	0.924	0.00678	-0.05881	0.53450	0.13284	0.50330
Cmos*	All	438	84	4	5	0.301	0.00073	-0.02939	0.66360	-0.03890	0.50467
	Cluster 1	438	12	0	1	0.000	0.00000	***	***	***	***
	Cluster 2	438	18	2	3	0.392	0.00096	-0.02239	0.62730	0.22139	0.48567
	Cluster 3	438	10	1	2	0.533	0.00122	-0.01155	0.58900	0.29798	0.46032
	Cluster 4	438	20	1	2	0.337	0.00077	-0.01577	0.70060	0.20572	0.48304
	Cluster 5	438	24	1	2	0.159	0.00036	0.00581	0.79020	0.21955	0.44571
NT3*	All	431	84	28	29	0.954	0.00957	-0.07900	0.56660	-0.22731	0.55960
	Cluster 1	431	14	6	5	0.824	0.00563	-0.07532	0.54400	0.16597	0.49935
	Cluster 2	431	20	8	8	0.868	0.00498	-0.05136	0.54110	0.07011	0.52696
	Cluster 3	431	8	6	3	0.714	0.00597	-0.04075	0.51420	0.28688	0.46793
	Cluster 4	431	16	10	8	0.917	0.00770	-0.07136	0.53970	0.09903	0.50295
	Cluster 5	431	26	14	9	0.883	0.01209	-0.08765	0.55780	-0.06336	0.53800
BDNF*	All	445	84	3	4	0.490	0.00128	-0.03225	0.58770	-0.04806	0.51857
	Cluster 1	445	12	0	1	0.000	0.0000	***	***	***	***
	Cluster 2	445	20	1	2	0.189	0.00043	0.00023	0.78420	0.24499	0.46773
	Cluster 3	445	8	0	1	0.000	0.0000	***	***	***	***
	Cluster 4	445	18	1	2	0.209	0.01353	0.00047	-0.00773	0.25636	0.47317
	Cluster 5	445	26	2	3	0.394	0.00094	-0.01831	0.63140	0.16689	0.46876
R35*	All	563	56	20	18	0.912	0.00525	-0.08310	0.56410	-0.13004	0.55471
	Cluster 1	563	6	5	3	0.800	0.00474	-0.04016	0.55020	0.34896	0.45541
	Cluster 2	563	10	7	4	0.800	0.00474	-0.05491	0.52350	0.22075	0.48377
	Cluster 3	563	6	2	3	0.600	0.00154	-0.01071	0.63990	0.31750	0.50000
	Cluster 4	563	14	6	6	0.802	0.00291	-0.05719	0.54530	0.16768	0.50087
	Cluster 5	563	20	6	5	0.632	0.00247	-0.04919	0.54710	0.14179	0.51015

Supplementary material 1. Laboratory protocols and primers used in this study.

PCR protocols. We used two different protocols for PCR techniques in an attempt to maximize available samples.

1) Polymerase Chain Reaction (PCR) amplification of gene fragments was performed in a final volume of 25 µl reactions using 5X PCR Buffer (–Mg), dNTP at 0.2 mM, each primer at 0.2 mM, MgCl2 at25 mM, 0.1 U/µl Promega® Taq DNA Polymerase, and 2 µl of template DNA.

2) protocol consisted of Master Mix PCR Ampliqon Taq, each primer at 10 mM and 2 µl of template DNA, in a total reaction volume of 23 µl.

The PCR cycling program was as follows to first protocol to mtDNA gene: 94°C for 2 min, followed by 34 cycles of 45 s denaturation at 94° C, 45 s annealing at X° C, and 1 min extension at 72° C, concluding with a 7 min extension at 72° C. To nuDNA gene: 94°C for 2 min, followed by 34 cycles of 30 s denaturation at 94° C, 1 min annealing at X° C, and 1 min extension at 72° C, concluding with a 7 min extension at 72° C. PCR products were analyzed on 2% agarose gels and were purified by ammonium acetate/ethanol.

Gene	Name	Primer	Annealing temperature	Reference
16S	16SF	5'- CGC CTG TTT ATC AAA AAC AT -3'	48° to 51°	Palumbi et al. 2002
	16SBR	5'- CTC CGG TTT GAA CTC AGA TCA -3'		
CYTB	Cytb_F	5'- TCA AAY ATC TCA ACC TGA TGA AAY TTY GG -3'	58°	Pook et al. 2000
	Cytb_R	5'- GGC AAA TAG GAA GTA TCA YTC TGG YTT -3'		
CYTB	L14910_cytbF	5' -GAC CTG TGA TMT GAA AAC CAY CGT TGT- 3'	50°	Burbrink et al. 2000
	H16064_cytbR	5' -CTT TGG TTT ACA AGA ACA ATG CTT TA- 3'		
C-MOS	S77_c-mos	5'-CATGGACTGGGATCAGTTATG-3'	54°	Lawson & Slowinski 2005
	S78_c-mos	5'-CCTTGGGTGTGATTTTCTCACCT-3'		
NT3	NT3_F3	5'- ATA TTT CTG GCT TTT CTC TGT GGC -3'	54°	Noonan & Chippindale 2006
	NT3_R4	5'- GCG TTT CAT AAA AAT ATT GTT TGA CCG G -3'		
BDNF	BDNF_F	5'- GAC CAT CCT TTT CCT KAC TAT GGT TAT TTC ATA CTT -3'	50°	Noonan & Chippindale 2006
	BDNF_R	5'- CTA TCT TCC CCT TTT AAT GGT CAG TGT ACA AAC -3'		
R35	R35_L75F	5'- TCTAAGTGTGGATGATYTGAT - 3'	50°	Fry et al. 2006
	R35_H792R	5'- CATCATTGGRAGCCAAAGAA - 3'		

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5 Final considerations

The gaps in knowledge about the patterns and processes of South American water snakes are significant due to the lack of scientific attention these taxa receive. We address the systematics and biogeography of Hydropsini (*Pseudoeryx*, *Hydrops* e *Helicops*) based on the largest molecular sampling to date, about 83% of the tribe's diversity (22 of 26 species). The inclusion of more species and specimens resulted in contrasting relationships when compared to previous phylogenetic inferences. We highlight the problematic taxonomy of the tribe and the possibility of new species or synonymizations, which would reflect in the current knowledge of the systematic of Hydropsini. Therefore, to establish a congruent classification based on monophyletic clades we recommend that all these species have integrative taxonomic revisions to elucidate the cryptic diversity. Furthermore, we propose that the landscape dynamism during the Miocene in the Amazon region had a great impact on the origin and diversification of Hydropsini, mainly influenced by the Pebas system, which possible for Hydropsini to disperse to adjacent basins, driven by adequate conditions of long duration (time) in a large region (area). Moreover, we presented the first phylogeographic study of a South American water snake. *Helicops leopardinus* complex may represent a single species although it has two chromatic patterns with no pholidose differentiation. Overall, the color pattern within the Hydropsini is a trait that needs to be investigated, as it is a trait that undergoes ecological and evolutionary pressures within aquatic snakes. We emphasize the need of a taxonomic revision for these species based on an integrative approach and further geographic, taxonomic, and molecular sampling.

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