Doubly hidden biodiversity: cryptic troglobitic species unveiled in the Brazilian semiarid region

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ABSTRACT

Troglobites are exclusively subterranean organisms, often with restricted distribution and high levels of endemism. Paleoclimatic events, such as the glaciations that occurred in the Northern Hemisphere and the simultaneous aridification in tropical regions, as well as oceanic transgressions and regressions in coastal regions, are pointed out as the main responsible for the origin of these species. In the west of the Jandaíra Formation, Northeast Brazil, there are extensive karstic areas, an enormous concentration of caves and underground aquifers in a currently semi-arid region with marked paleoclimatic changes (including oceanic transgressions and regressions). There is also a great variety and concentration of troglobitic species, some with relatively wide distribution in geologically and hydrologically distinct areas. Through phylogenetic and phylogeographic analyses, using sequences of the mitochondrial gene Cytochrome oxidase 1 (cox1), we aimed to evaluate the patterns of genetic variation in aquatic (Isopoda and Amphipoda) and terrestrial (Hemiptera) troglobitic taxa, investigate possible cryptic diversity and delimit Operational Taxonomic Units (OTUs), as well as indicate the factors that possibly influenced their diversification. With the exception of Cirolanidae sp.1 (Isopoda), the other groups presented cryptic lineages. Besides the identification of three OTU's for Cirolanidae sp.2 and five for *Potiberaba* (Amphipoda), *Kinnapotiguara troglobia* (Hemiptera) probably consists of a complex with seven other species, indicating greater structure in terrestrial organisms than in aquatic ones. Furthermore, it was identified that drainages are determinant for the differentiation of lineages in aquatic taxa (Cirolanidae sp.2 and *Potiberaba*) and for *Kinnapotiguara*, and in this case, the *lajedos* determined an even greater structuring. Only small portions of the lineages are found in protected areas, while the majority are in areas exposed to anthropogenic threats. The OTU's delimited here are presented as probable new species, recommending integrative taxonomic studies to formally describe them, thus contributing to their conservation.

Keywords: Arthropoda, Caves, Lineage delimitation, Cox 1, Comparative phylogeography, Troglobites.

INTRODUCTION

Caves have always aroused the curiosity of researchers, mainly due to troglobitic organisms, which are exclusively subterranean (Juan et al., 2010). Such species often exhibit morphological (e.g. anophthalmia or reduction of ocular structures and pigmentation), physiological, and behavioral specializations resulting from isolation and evolution in the subterranean environment, or as a function of the absence of pressures typical of surface environments (Culver and Pipan, 2009). However, such specializations can also lead to a loss in dispersive ability, favoring lineage isolation and speciation. Thus, troglobites often exhibit restricted distribution and high levels of endemism, regardless of the scale of assessment (Gilbert and Deharveng, 2002; Mammola et al., 2015).

Paleoclimatic events have been pointed out as primarily responsible for the isolation of ancestral lineages in the subterranean environment and subsequent origin of troglobitic species. Events such as the glaciations that occurred in the Northern Hemisphere in the Pliocene and Pleistocene (White and Pipan, 2019), and the simultaneous establishment of arid climates in tropical regions, such as Australia (Humpreys, 2019). In coastal regions, oceanic transgressions and regressions that occurred at different periods have also been noted as responsible for the origin of diverse lineages of stygobites (aquatic troglobites) many of which are considered oceanic relicts (Fisĕr et al., 2013; Alvarez et al., 2015; Souza et al., 2018; Humphreys, 2019; Delic et al., 2020).

The Caatinga, the only exclusively Brazilian biome, is the largest seasonally dry forest in South America (Silva et al., 2017). This biome is the result of several paleoclimatic changes that have occurred since the Miocene. Changes that are responsible for the diversification and adaptation of several current fauna and flora groups to the increasingly arid and markedly seasonal climate (Werneck, 2011; Dupont et al., 2013; Costa et al., 2018; Silva and Souza, 2018). Additionally, the region north of the Brazilian semiarid has a history of oceanic transgressions and regressions that have been occurring since the upper Cretaceous, with major events in the Miocene (Rosseti et al., 2013; Bagni et al., 2020).

In this region there are extensive karstic areas due to the occurrence of limestones of the Jandaíra Formation, which were deposited under a marine transgressive regime in the upper Cretaceous (Bezerra et al., 2007). The Jandaíra

Formation corresponds to a carbonate ramp that outcrops in almost the entire emersed portion of the Potiguar Basin, occupying an area of more than 13,000 km^2 in northern Rio Grande do Norte and northeastern Ceará (Bezerra et al., 2007). There are also several intensely karstified limestone outcrops (locally known as *lajedos*) that concentrate the great majority of the more than 1,000 known caves in the region (Cruz et al., 2010; CANIE/CECAV, 2020; Rabelo et al., 2020).

The western region of the Jandaíra Formation has great biospeleological relevance due to the richness and concentration of invertebrate troglobites species, including the only oceanic relict stygobites currently recorded in Brazil (crustaceans and planaria) (Fisĕr et al., 2013; Souza et al., 2018). As observed for other regions of the world (Holsinger, 2000), most of the more than 70 currently known troglobitic species for the area have restricted distributions, including several extreme endemics. However, four taxa have a relatively wide occurrence (Ferreira et al., 2010; Hoch and Ferreira, 2013; Fisĕr et al., 2013; Bento et al., 2016; Bento et al., 2021). Two species of cirolanid isopods, not yet described, with occurrence in several caves over an area of more than 1,400 km^2 (Ferreira et al, 2010; Bento et al., 2016), including four municipalities in the states of Rio Grande do Norte and Ceará. The amphipod genus *Potiberaba* Fišer, Zagmajster & Ferreira, 2013 (Amphipoda: Mesogammaridae), with occurrence in several caves in the same region as the cirolanids (Ferreira et al., 2010; Bento et al., 2016). The only described species of this genus, *Potiberaba porakuara* Fišer, Zagmajster & Ferreira, 2013, occurrs in a cave in the extreme south of the distribution (Três Lagos cave, in Felipe Guerra/RN) (Fišer et al, 2013). In addition, the troglobitic leafhopper *Kinnapotiguara troglobia* Hoch & Ferreira, 2013 (Hemiptera: Kinnaridae), which occurs in several caves in an area of about 50 km^2 in the municipalities of Felipe Guerra/RN and Governador Dix-Sept Rosado/RN (Hoch and Ferreira, 2013), area that is crossed by the Apodi-Mossoró River the main one in the region.

Cirolanid and amphipod crustaceans are considered oceanic relicts (Ferreira et al., 2010; Fišer et al., 2013), and occur in geologically and hydrologically distinct areas (Cruz et al., 2010; Bento et al., 2021). Because of the possibility of connection via underground aquifers, stygobites generally exhibit greater dispersal capacity and wide distribution compared to terrestrial troglobites (Culver et al., 2009). Therefore, the gene structure of aquatic taxa is expected to correspond to the different basins and underground aquifers found in the region, with structured populations or distinct species depending on the time of isolation.

In the case of *K. troglobia*, small variations in male genital morphology were identified among individuals from different caves that may be evidence of speciation (Hoch and Ferreira, 2013). The Apodi-Mossoró River may represent a biogeographic barrier to be tested (Hoch and Ferreira, 2013), as populations have been found in caves on both banks of this river. Similarly, different tributaries of this drainage may also play a role as a barrier to dispersal.

There is possibly connectivity in the terrestrial environment between caves in the region, which would be maintained by a system of micro- and mesocavities that would enable gene flow between populations (Hoch and Ferreira, 2013). Nevertheless, this is expected to occur only on a local scale, as observed in other regions of the world (Mammola et al., 2016; Pipan and Culver, 2017). In this context, it is possible that *lajedos* function as caves clusters connected by micro- and mesocavities, allowing restricted flow of individuals of troglobitic species between nearby caves of the same *lajedo* (Mammola et al., 2020).

The diversity of these arthropods troglobites and their relatively wide distribution, in a currently semi-arid region marked by paleoclimatic changes, make them excellent models for testing evolutionary and diversification patterns in subterranean, aquatic and terrestrial environments in the Caatinga. Therefore, we aimed to evaluate the patterns of genetic variation of the different taxa throughout their known distribution, investigate potential cryptic diversity and delimit operational taxonomic units (OTUs), as well as indicate the environmental factors that possibly influenced their diversification.

The current distribution of target groups' lineages should mainly reflect allopatric events, but also the level of connectivity of the subsurface environment and the dispersal potential of each taxon. It is likely that the deepest divergences correspond to late Miocene events: the last major oceanic regression (Rosseti et al., 2013) for aquatic groups (oceanic relicts), and the emergence of the Apodi-Mossoró river valley (Bagni et al., 2020) for *K. troglobia*.

The expansion of semi-arid conditions (Werneck, 2011; Dupont et al., 2013; Silva et al., 2017; Costa et al., 2018; Silva and Souza, 2018), associated with the intensification of karstification provided by the late exhumation of the limestones of the Jandaíra Formation (Bagni et al., 2020), is expected to have originated several endemic and evolutionarily distinct lineages. Such lineages would be distributed according to the drainage patterns for aquatic taxa, with a wider distribution due to the possibility of dispersion via underground aquifers, if compared to the terrestrial environment. Also according to the limestone outcrops for *K. troglobia*, still having the Apodi-Mossoró River and its tributaries as biogeographic barriers.

This is the first comparative molecular phylogeographic analysis focusing on troglobitic invertebrates from the Caatinga, including phylogenies with divergence time estimates and lineage delimitation methods. The broad sampling, which includes individuals from the entire known geographic distribution of all the target groups, allows the investigation of patterns of diversification and the factors that influenced it. Thus, contributing to the understanding of the evolutionary history not only of the taxa studied, but also of the study area itself, and in the management and conservation of this endemic troglofauna.

MATERIALS AND METHODS

Study Area

The study was carried out in 31 caves and karst springs in the Jandaíra formation, located in the municipalities of Baraúna, Felipe Guerra, Governador Dix-Sept Rosado (GDS Rosado) and Mossoró, Rio Grande do Norte State (RN), and Quixeré, Ceará State, Northeast Brazil (Table 1, Figure 1). Part of the caves are inserted in the Furna Feia National Park (PNFF- *Parque Nacional Furna Feia*), a conservation unit recently created to protect an important concentration of caves between the municipalities of Baraúna and Mossoró (Brasil, 2012).

The region is inserted in the Caatinga and presents a hot and semi-arid climate, predominantly of BSh type, according to the Köppen climate classification (Alvares et al., 2013). The average annual precipitation is around 800mm (however 70% of the precipitation can occur in a single month), which, combined with high solar incidence and low relative humidity leads to high evaporation rates and water deficit throughout the year (Silva et al., 2017).

The area can be subdivided into hydrographically and geologically distinct regions. The caves and springs of Felipe Guerra, GDS Rosado and Mossoró are in the hydrographic basin of the Apodi-Mossoró River and had their genesis conditioned by the base level of this river and its tributaries. Carmo River, in the case of Olho d'Água da Onça; Abreu, Arapuá, Cote and Saco creeks, in the case of the caves on the banks of the Apodi-Mossoró River - Figure 1C (Cruz et al., 2010; Maia et al., 2012). Besides these, the Aposta cave, in Quixeré/CE, and the Olho d'Água do Cedro cave, in

Baraúna/RN, are found in the microbasin of the Bom Sucesso stream, a tributary of the Apodi-Mossoró River. There is also a specificity in relation to the Crotes cave, in Felipe Guerra/RN, which shelters an epikarstic drainage disconnected from the water table (Table 1, Figure 1H).

The caves located in the PNFF are in the Northern Coastal Diffuse Flow Belt (coastal micro basins), an area that corresponds to a tectonic uplift that, acting as a watershed, controls speleogenesis in the region (Cruz et al., 2010; Maia et al., 2012). In this region, there are two micro basins: the Córrego da Mata, where the caves of Lago, Furna Nova and Furna Feia are inserted, and the Córrego do Virgínio, which includes the Pedra Lisa cave (Table 1, Figure 1).

Table 1. Data from the caves and springs with troglobitic species targeted in this study. The first column (ID) indicates the numbers by which the localities are identified in Figure 1. * - Furna Feia National Park; ● - taxon recorded for the first time in this study.

ID	Localidade	Sigla	Município/Estado	Coordenadas (SIRGAS 2000)		Hidrografia		Lajedo	Ocorrência e número de indivíduos sequenciados				Ambiente
				Latitude (S)	Longitude (W)	Bacia	Microbacia		Cirolanidae sp.1	Cirolanidae sp.2	*Potiberaba* sp.	*K. troglobia*	
01	Furna Nova*	FNV	Baraúna/RN	05º 02' 03,21"	37º 34' 16,04"	Microbacias costeiras	Córrego da Mata	Furna Nova	7●				Córrego subterrâneo (freático)
02	Caverna do Lago*	LGO	Baraúna/RN	05º 02' 11,40"	37º 34' 15,24"	Microbacias costeiras	Córrego da Mata	Furna Nova	5				Córrego e lagos subterrâneos (freático)
03	Furna Feia*	FFA	Baraúna/RN	05º 02' 12,76"	37º 33' 36,64"	Microbacias costeiras	Córrego da Mata	Furna Feia			11●		Córrego subterrâneo (freático)
04	Caverna Pedra Lisa*	PDL	Baraúna/RN	05º 02' 43,84"	37 º 31' 18,85"	Microbacias costeiras	Córrego do Virgínio	Pedra Lisa	10●	5●	5●		Córrego subterrâneo (freático)
05	Gruta do Olho d´Água do Cedro	ODC	Quixeré/CE	05º 12' 39,69"	37º 43' 35,87"	Rio Apodi-Mossoró	Córrego do Bom Sucesso	Cedro	2●		3●		Lago subterrâneo (freático)
06	Caverna da Aposta	APO	Quixeré/CE	05º 12' 39,69"	37º 43' 35,87"	Rio Apodi-Mossoró	Córrego do Bom Sucesso	Lajedo Grande	2●				Lago subterrâneo (freático)
07	Olho d´Água da Onça	ODO	Mossoró/RN	05º 28' 34,18"	37º 16' 48,73"	Rio Apodi-Mossoró	Rio do Carmo	Olho d´Água da Onça			5●		Nascente cárstica
08	Olho d´Água da Furna	ODF	GDS Rosado/RN	05º 28' 50,55"	37º 32' 29,94"	Rio Apodi-Mossoró	Riacho do Cote	Gangorrinha			7●		Ressurgência (freático)
09	Gruta Boca de Peixe	BPX	GDS Rosado/RN	05º 29' 04,56"	37º 33' 29,69"	Rio Apodi-Mossoró	Riacho do Cote	Bonito				8	Terrestre com raízes
10	Gruta do Poço Feio	PFE	GDS Rosado/RN	05º 29' 15,51"	37º 33' 33,40"	Rio Apodi-Mossoró	Riacho do Cote	Bonito			4	8●	Córrego subterrâneo e ressurgência (freático); terrestre, com raízes
11	Caverna do Marimbondo Caboclo/Água	CAG	GDS Rosado/RN	05º 29' 43,56"	37º 32' 43,46"	Rio Apodi-Mossoró	Riacho do Cote	Angicos	12		1		Lago subterrâneo (freático)
12	Caverna SN9	SN9	GDS Rosado/RN	05º 29' 41,33"	37º 32' 41,71"	Rio Apodi-Mossoró	Riacho do Cote	Angicos				1●	Terrestre, com raízes
13	Caverna Sombra de Ubaia	SUB	GDS Rosado/RN	05º 29' 41,64"	37º 32' 41,23"	Rio Apodi-Mossoró	Riacho do Cote	Angicos				1●	Terrestre, com raízes
14	Caverna da Bota	BOT	Felipe Guerra/RN	05º 31' 30,85"	37º 37' 05,29"	Rio Apodi-Mossoró	Riacho do Arapuá	Arapuá				7	Terrestre, com raízes
15	Caverna do Arapuá	ARA	Felipe Guerra/RN	05º 31' 48,25"	37º 36' 58,48"	Rio Apodi-Mossoró	Riacho do Arapuá	Arapuá				9	Terrestre, com raízes
16	Caverna da Seta	SET	Felipe Guerra/RN	05º 32' 40,23"	37º 38' 03,10"	Rio Apodi-Mossoró	Riacho do Arapuá	Arapuá				7	Terrestre, com raízes
17	Gruta da Rumana	RUM	Felipe Guerra/RN	05º 33' 54,12"	37º 39' 07,23"	Rio Apodi-Mossoró	Riacho do Saco	Rumana				10	Terrestre, com raízes

18	Gruta do Buraco Redondo	BRD	Felipe Guerra/RN	05º 34' 42,98"	37º 39' 04,99"	Rio Apodi-Mossoró	Riacho do Saco	Meio				9	Terrestre, com raízes
19	Gruta dos Troglóbios	TRO	Felipe Guerra/RN	05º 33' 24,27"	37º 39' 40,57"	Rio Apodi-Mossoró	Riacho do Arapuá	Rosário	11		5	12	Lago subterrâneo (freático); terrestre, com raízes
20	Caverna dos Crotes	CRO	Felipe Guerra/RN	05º 33' 37,92"	37º 39' 30,89"	Rio Apodi-Mossoró	Drenagem epicárstica / Riacho do Arapuá	Rosário		9	2	6	Lago subterrâneo (epicarste); terrestre, com raízes
21	Gruta da Descoberta	DBT	Felipe Guerra/RN	05º 33' 47,50"	37º 39' 55,77"	Rio Apodi-Mossoró	Riacho do Arapuá	Rosário				9	Terrestre, com raízes
22	Olho d´Água do Tetéu	ODT	Felipe Guerra/RN	05º 34' 02,63"	37º 40' 13,88"	Rio Apodi-Mossoró	Riacho do Abreu	Tetéu			4●		Nascente cárstica
23	Caverna dos Dois	CDS	Felipe Guerra/RN	05º 34' 06,51"	37º 40' 50,42"	Rio Apodi-Mossoró	Riacho do Abreu	Alfredo				7●	Terrestre, com raízes
24	Caverna Duas Urtigas	URT	Felipe Guerra/RN	05º 33' 35,48"	37º 41' 00,01"	Rio Apodi-Mossoró	Riacho do Abreu	Lapa				2●	Terrestre, com raízes
25	Caverna da Raposa	RAP	Felipe Guerra/RN	05º 33' 36,15"	37º 41' 03,77"	Rio Apodi-Mossoró	Riacho do Abreu	Lapa				2●	Terrestre, com raízes
26	Caverna Lapa I	LAI	Felipe Guerra/RN	05º 33' 35,50"	37º 41' 18,31"	Rio Apodi-Mossoró	Riacho do Abreu	Lapa				8●	Terrestre, com raízes
27	Lapa I/Engano	LIE	Felipe Guerra/RN	05º 33' 41,89"	37º 41' 42,25"	Rio Apodi-Mossoró	Riacho do Abreu	Lapa				6	Terrestre, com raízes
28	Caverna do Mosteiro	MOS	Felipe Guerra/RN	05º 33' 45,61"	37º 41' 44,19"	Rio Apodi-Mossoró	Riacho do Abreu	Lapa				6●	Terrestre, com raízes
29	Caverna Boa	CBO	Felipe Guerra/RN	05º 33' 59,50"	37º 41' 52,43"	Rio Apodi-Mossoró	Riacho do Abreu	Lapa	9●		3●	2●	Córrego e lagos subterrâneos (freático); terrestre, com raízes
30	Gruta dos Três Lagos	TLG	Felipe Guerra/RN	05º 35' 34,40"	37º 41' 12,70"	Rio Apodi-Mossoró	Riacho do Abreu	Brejo	9	4	14		Lago subterrâneo e ressurgência (freático)
31	Caverna dos Que Já Foram	QJF	Felipe Guerra/RN	05º 32' 47,68"	37º 39' 59,78"	Rio Apodi-Mossoró	Riacho do Arapuá	Toco				2●	Terrestre, com raízes
Total									67	17	64	122	

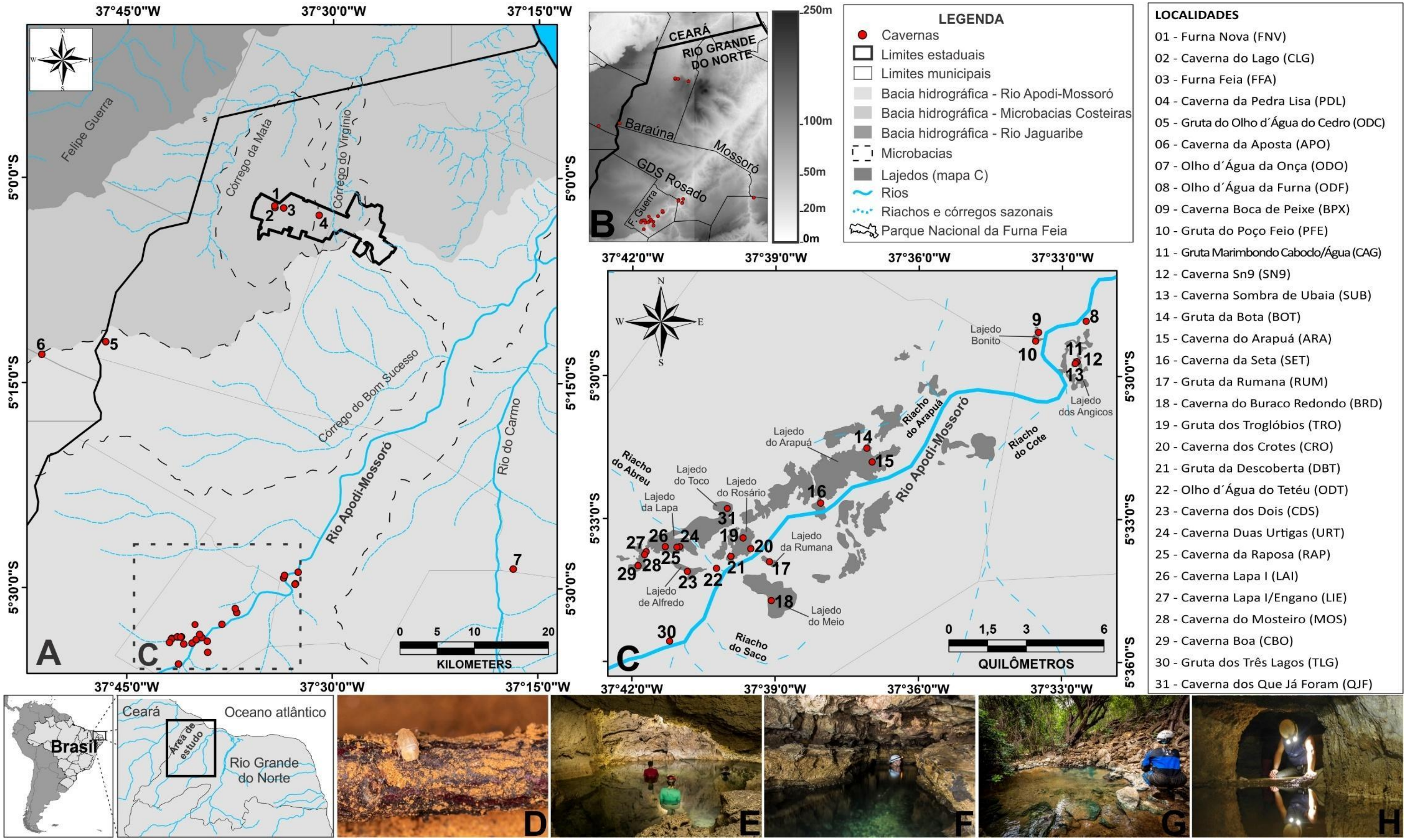

Figure 1. Caves and karstic springs sampled. (A) Location of the caves and karstic springs where individuals of the target groups of this study were collected; (B) relief; (C) enlargement of the area with a large concentration of localities in the municipalities of Felipe Guerra and GDS Rosado; and environments where the target species of the study are found: (D) terrestrial environment with roots (it is possible to see a nymph of *K. troglobia* on the root) in Arapuá cave, (E) underground phreatic lake in Três Lagos cave, (F) underground phreatic stream in Poço Feio cave,

(G) karstic spring in Olho d'Água do Tetéu cave, and (H) epikarstic lake in Crotes cave. Due to the impossibility of adequate visualization, because of the scale, the slabs are not shown in A.

Sample collection

In addition to the caves for which there were already records of the target groups (Ferreira et al., 2010; Bento et al., 2016; Bento et al., 2021), collections were conducted in other caves and karst springs with suitable habitats throughout the taxa area of occurrence.

All potential habitats (wet areas and areas with roots, in the case of *K. troglobia*; pools and underground streams, roots and submerged organic matter accumulations, for aquatic invertebrates) were visually inspected. Individuals of *K. troglobia* were manually collected with the use of tweezers and brushes, while aquatic invertebrates were collected using aquarium nets and cages with bovine liver pieces as baits (left in the water bodies for 24 hours). All the collected invertebrates were preserved in absolute ethanol (99.5% P.A) and stored under refrigeration (-20 ºC), sorted and identified to the lowest taxonomic level possible (using a stereomicroscope) and sent to specialists for confirmation of the taxa and possible description of new species.

DNA extraction, amplification and sequencing

Total DNA was extracted from tissues of the individuals preserved in ethanol using the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's recommendations. Partial sequences of the mitochondrial cytochrome C oxidase subunit I (cox1) gene were obtained and amplified by polymerase chain reaction (PCR) with the primers LCO-1490 (forward) and HCO-2198 (reverse) as described by Folmer et al. (1994).

PCR reactions were performed in 25µl volumes containing 5 µl of 5x GoTaq Reaction buffer (Promega), 2.4 µl of $MgCl_2$ (25mM), 2 µl of dNTP (2mM), 2 µl of DMSO, 0.5 µl of each primer (10mM), 0.2 µl of GoTaq® DNA Polymerase enzyme (Promega), and 4 µl of total genomic DNA. The thermocycling profile included 1 cycle of 4 minutes at 94°C; 35 cycles of 45 seconds at 94°C, 1 minute at 48°C, 1 minute at 72°C; and 1 cycle of 7 minutes at 72°C.

All PCR products were first visualized on a 1.2% agarose gel, purified and then used in a sequencing PCR reaction with the "Big DyeTM Terminator Cycle Sequencing Ready Reaction" kit (Applied Biosystems). The sequencing reactions, in both directions, were performed in 10 µl reaction volumes containing 0.5 µl of BigDye 2.5, 2 µl of 5x sequencing buffer (Applied Biosystems), 2 µl of the PCR products and 0.5 µl of primer. The thermocycling profile was: one 1-minute cycle at 96°C, 40 cycles of 15 seconds at 96°C, 15 seconds at 50°C, and 4 minutes at 60°C. The products of the sequencing reaction were analyzed on an automated DNA sequencer, model ABI 3730-Genetic Analyzer (Applied Biosystems).

Data Analysis

Consensus sequences were assembled using Geneious Prime software (https://www.geneious.com/prime/) and aligned using ClustalW (Thompson et al., 1994), implemented in the Mega X program (Kumar et al., 2018), followed by visual inspection for final adjustments. Additionally, DNA sequences were translated into amino acids to check for premature stop codons, indels, or nuclear DNA insertions. Saturation tests were performed in the DAMBE7 program (Xia, 2018).

For phylogenetic analyses, only single haplotypes were used, except when shared between different localities (in order to maintain geographic distribution information), and for that a haplotype distribution analysis was performed with the DNAsp v.5 program (Librado and Rozas, 2009). The nucleotide substitution model for each dataset was selected in the JmodelTest 2 program (Darriba et al., 2012) from the lowest BIC (Bayesian Information Criterion) and AICc (corrected Akaike Information Criterion) values. Using the selected evolutionary model (HKY+I+G for Cirolanidae sp.1, GTR+G for Cirolanidae sp.2, TrN+G for *Potiberaba* and HKY+I for *K. troglobia*), Bayesian inference (BI) was performed in Beast 2 (Bouckaert et al., 2019). Additionally, maximum likelihood phylogenetic reconstructions were performed (using GTR GAMMA I models for Cirolanidae sp.1 and *K. troglobia* and GTR GAMMA for Cirolanidae sp.2 and *Potiberaba*, with the -f to -x 1 options and 1000 bootstrap replicates) using RAxML 8 (Stamatakis, 2014) implemented in Geneious Prime. Sequences of Cirolanidae sp.2 were used as outgroups of Cirolanidae sp.1 (and vice versa), while *Latissus dilatatus* (Hemiptera: Issidae; GenBank accession: KX702947.1) and *Gammaracanthus loricatus* (Amphipoda: Gammaracanthidae; GenBank accession: JF965998.1) were used as outgroups for *K. troglobia* and *Potiberaba*, respectively.

BI and divergence timing analyses were performed using the "Relaxed Clock Log Normal" option after the likelihood ratio test, run in DAMBE7 (Xia, 2018), ruled out the strict molecular clock hypothesis for all taxa. Mutation rates of 1.25%/Ma (0.00625 subst./site/Ma) (subterranean Stenasellidae isopods; Yoshino et al. 2018) were used for Cirolanidae; 1.4%/Ma (0.007 subst./site/Ma) (Haustoriidae amphipods, Hancock et al, 2019) for *Potiberaba*; and 2.3%/Ma (0.0115 subst./site/Ma) (mitochondrial rate for insects; Wessel et al., 2013) for *K. troglobia*. In addition to coalescent model with constant population with four independent runs for each taxon with MCMC of 100 x 106 generations and sampling every 5000 generations. Convergence of the runs was checked in Tracer v1.7 (http://beast.bio.ed.ac.uk/Tracer), making sure that all ESS values were greater than 200. The consensus topology and posterior probability values were obtained in the TreeAnnotator program (applying 15% burnin), with subsequent graphical editing of the generated trees in FigTree (http://tree.bio.ed.ac.uk/).

In order to identify operational taxonomic units (OTUs, here interpreted as potential species), four different lineage delineation methods were used to verify the distribution of gene groups in the target taxa. Both exploratory approaches, such as ABGD (Automatic Barcode Gap Discovery, Puillandre et al., 2012) and mGMYC (multiple threshold Generalized Mixed Yule Coalescent, Fujisawa and Barraclough, 2013), and validation approaches such as BPP (Bayesian Phylogenetics and Phylogeography, Flouri et al., 2018), as well as bPTP (Bayesian Poisson Tree Process, Zhang et al., 2013) were used.

ABGD consists of the automatic clustering of sequences into potential groups (lineages or species) according to successive barcode gap values (the difference between the largest intraspecific and smallest interspecific distance). The analyses were performed on the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html), using the K2-P option, with intraspecific priors ranging from 0.001 to 0.25 in 10 steps, and relative gap width (X) of 1.5.

The other methods consist of analyses based on phylogenetic trees that incorporate population models, such as coalescence theory. They seek to determine the transition point between population and speciation processes based on the accumulation of substitutions between sequences (bPTP) or on the divergence time (mGMYC). Based on the trees generated in the Bayesian inference and Maximum Likelihood analyses, as detailed above, the mGMYC (https://species.h-its.org/gmyc/) and bPTP (https://species.h-its.org/ptp/) methods were run on the Species delimitation server (https://species.h-its.org/).

BPP, on the other hand, uses genetic datasets to adapt ancestral polymorphisms and coalescent processes using MCMC to calculate posterior probabilities of distinct species trees (Flouri et al., 2018). Four scenarios combining large and small ancestral populations and deep and recent divergences were used, which were run three times each, 5 x 105 generations and burnin of 5,000: (a) large ancestral population with deep divergences, $\theta \sim$ invgamma (2, 0.1) and $\tau \sim$ invgamma (2, 0. 1); (b) large ancestral population with recent divergences, $\theta \sim$ invgamma (2, 0.1) and $\tau \sim$ invgamma (3, 0.002); (c) small ancestral population with deep divergences, $\theta \sim$ invgamma (3, 0.002) and $\tau \sim$ invgamma (2, 0.1); and (d) small ancestral population with recent divergences, $\theta \sim$ invgamma (3, 0.002) and $\tau \sim$ invgamma (3, 0.002).

OTUs were defined from the agreement between most lineage delimitation methods and geographic distribution. Subsequently, intra (between individuals of the same OTU) and interspecific (between individuals of different OTUs) mean genetic distances were estimated with the K2P substitution model, using MEGA X.

Analysis of molecular variance (AMOVA) was performed in Arlequin 3.5 software (Excoffier and Lischer, 2010) to test the hypotheses of genetic structuring of the target groups according to the distribution of localities in the different tributaries (microbasins) and limestone outcrops (*lajedos*).

RESULTS

Individuals were collected and sequenced from all target groups and in all localities for which occurrences were recorded. In addition, new occurrences were recorded that resulted in the extension of the distribution of all taxa (Table 1, Figure 1), so that this study covers all occurrence sites throughout the known range of the target groups.

Partial sequences of 593 bp cox1 were obtained from 67 individuals of Cirolanidae sp.1, with the identification of 32 haplotypes in nine caves, and from 18 individuals of Cirolanidae sp.2 (nine haplotypes) from three caves. For *Potiberaba*, partial sequences of 588 bp cox1 were obtained from 64 individuals and 23 haplotypes were identified in nine caves and two karst springs. Finally, 659 bp sequences were obtained from 122 individuals of *Kinnapotiguara* (20 haplotypes) from 20 caves.

Phylogenetic analysis and lineage delimitation

For Cirolanidae sp.1, the BI indicated three clades, two with high posterior probability values, even though this division is not supported by the ML nor by three of the four lineage delimitation methods. These methods indicate a single grouping for all localities regardless of the hydrographic division between basins and microbasins in the area of occurrence (Figure 2A). For Cirolanidae sp.2 the situation is completely different, and there is robust support in both BI and ML, as well as agreement among all four lineage delineation methods, for three monophyletic clades, each corresponding to a distinct cave and microbasin (Figure 2B). In addition to the lineage separation in Pedra Lisa cave (PDL) (which diverged approximately 7.66 Ma ago; 2.66-17.73 HPD), in a geographically distant and hydrologically distinct region, there are also different lineages in Três Lagos (TLG) and Crotes (CRO) caves (which diverged approximately 2.37 Ma ago; 0.53-9.97 HPD), which are nearby and located on the banks of the Apodi-Mossoró River. However, the drainage where the taxon occurs at CRO is epikarstic (Figure 1H) and without connection to the water table.

At Potiberaba, there is agreement between the observed clades and drainage patterns. There is robust support in BI and ML, corroborated by all four lineage delineation methods, for clades formed exclusively by the Olho d'Água da Onça (ODO) (which diverged approximately 5.88 Ma; 4.10-8.02 HPD) and PDL (which diverged approximately 2.71 Ma; 1.81-3.76 HPD) localities (Figure 2C). Such lineages are in distinct microbasins from each other and from the other localities, with the ODO located in the Rio do Carmo microbasin and the PDL in the Córrego do Virgínio microbasin. In spite of being geographically close to the latter, Furna Feia (FFA) is located in a different microbasin - Córrego da Mata. Although with less support in BI and ML, the clades formed exclusively by FFA and CRO (epikarstic drainage without connection to the water table, as already mentioned for Cirolanidae sp.2) are distinct lineages according to three (mGMYC, bPTP and BPP) of the four methods used, having diverged about 2.13 Ma [1.40-2.97 HPD] and 1.7 Ma [1.13-2.35 HPD], respectively. There is no agreement between BI and ML (low PP and/or bootstrap values), nor between the lineage delimitation methods, for the clades involving the remaining localities (including the type locality for *P. porakuara*, TLG) (Figure 2C).

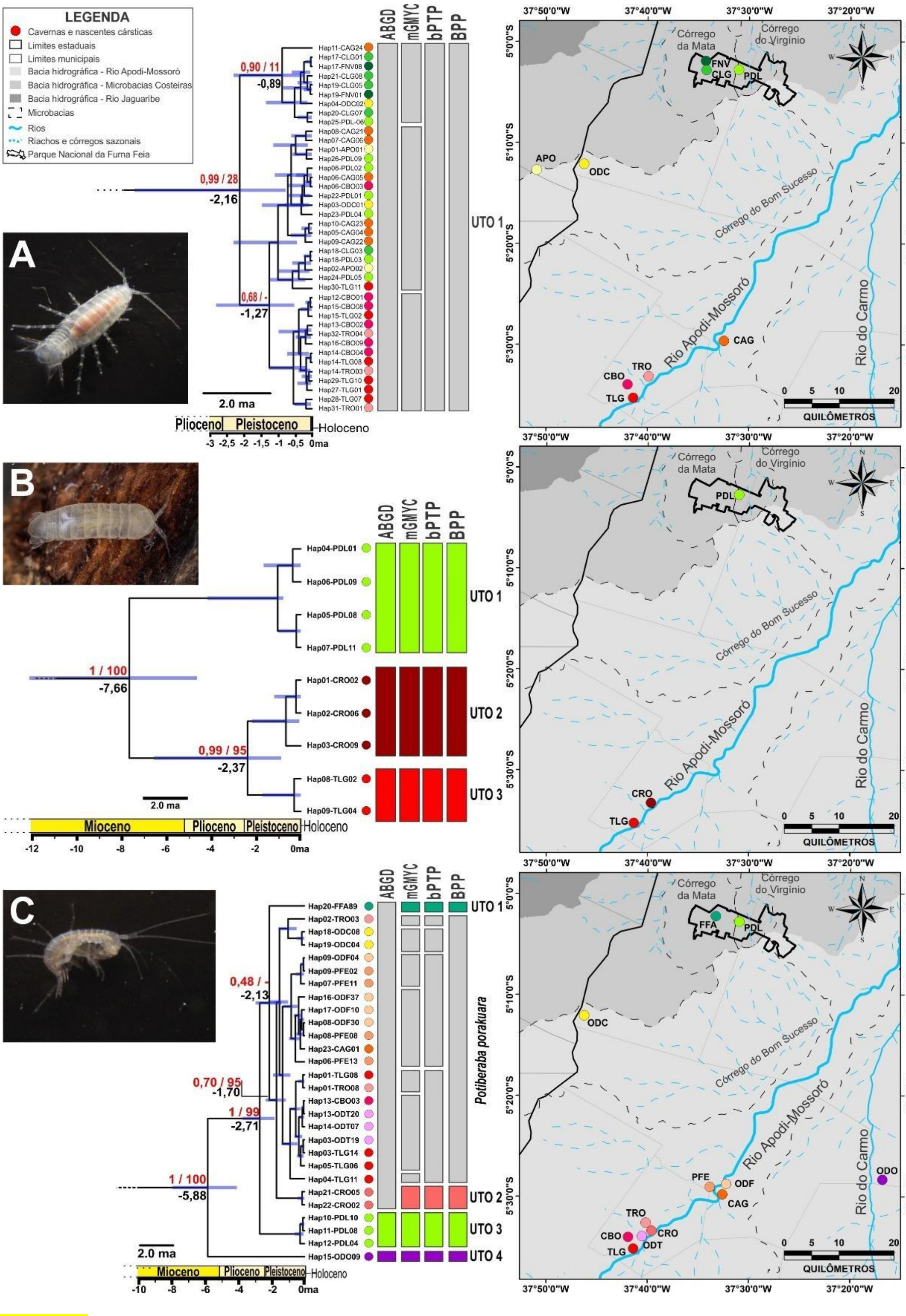

Figure 2. Phylogenetic consensus trees resulting from Bayesian inference with molecular clock, plus the results of lineage delimitation methods and location of the caves and karst springs with occurrence of the aquatic taxa, Cirolanidae sp.1 (A), Cirolanidae Sp.2 (B) and Potiberaba (C). At each node in the trees the posterior probabilities (above left), bootstrap values (resulting from the maximum likelihood phylogenetic reconstructions, above right), and the estimated mean age (below) are shown. The purple bars represent the 95% HPD intervals for the estimated ages. For purposes of graphical presentation, outgroups have been removed from the phylogeny.

At *Kinnapotiguara,* there is generally robust support, both in BI and ML, for the formation of eight monophyletic clades. They mostly correspond to the distribution of the caves according to limestone outcrops (*lajedos*), which is consistent with the results of lineage delimitation methods (Figure 3). According to the molecular clock BI estimates, the deepest divergence occurred between lineages on opposite margins of the Apodi-Mossoró River and would have occurred approximately 6.49 Ma [4.29-9.37 HPD] ago. The two main lineages on the left bank of the river would have diverged about 4.92 Ma [3.07-7.17 HPD] and those on the right bank 2.88 Ma [1.18-4.8 HPD] ago. The five lineages that correspond to the clades encompassing the localities on the left bank of the Apodi-Mossoró River diverged at about the same time (middle Pleistocene, around 1.5 Ma) (Figure 3).

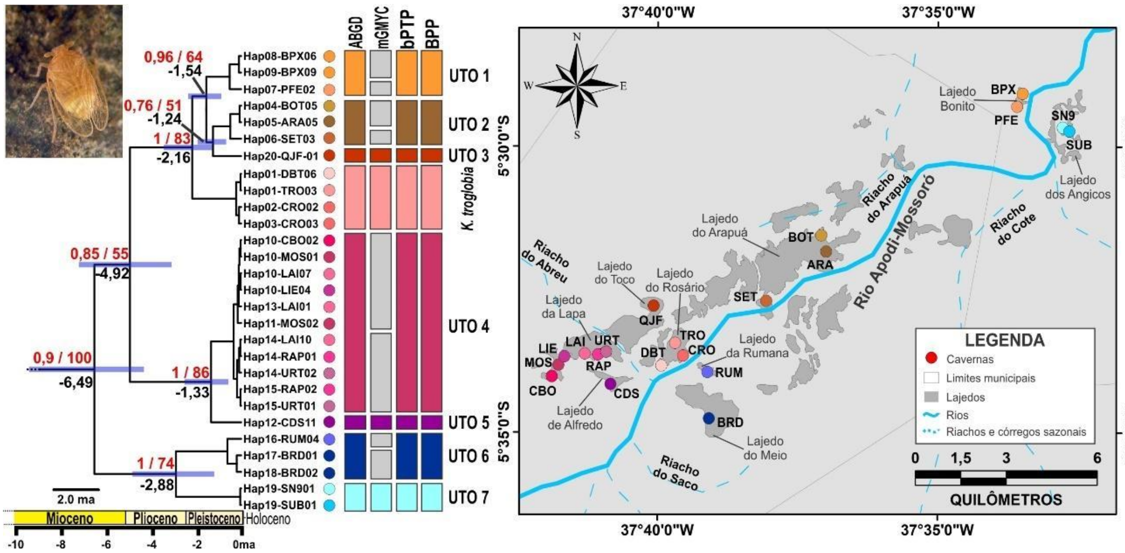

Figure 3. Phylogenetic consensus tree resulting from Bayesian inference with molecular clock, plus the results of lineage delimitation methods and location of the caves with occurrence of *Kinnapotiguara*. At each node of the tree, the posterior probabilities (above left), bootstrap values (resulting from the maximum likelihood phylogenetic reconstructions, above right), and the estimated mean age (below) are presented. The purple bars represent the 95% HPD intervals for the estimated ages. For purposes of graphical presentation, the outgroup has been removed from the phylogeny.

Genetic distances

Intraspecific genetic distances (between individuals of the same OTU) were low for all target taxa. They reached 0.74% for Cirolanidae sp.1 and ranged from 0.084% to 0.37% for Cirolanidae sp.2, from 0 to 0.93% for Potiberaba and from 0 to 0.8% for *Kinnapotiguara* (Tables 2, 3 and 4).

Aquatic taxa showed lower genetic distance values than terrestrial, although with different patterns among taxa. While Cirolanidae sp.1 showed low mean genetic distance values among all caves (resulting in a mean intraspecific distance of 0.74%), Cirolanidae sp.2 showed higher genetic distance values among lineages from different localities (2.1 % between TLG and CRO, which are geographically close, and ranging from 9.2% to 9.5% between these and PDL; Table 2, Figure 2B).

Table 2. Mean genetic distances (K2P) within-species (on the diagonal, in bold) and between the different OTU's identified for Cironalidae sp.2.

	UTO 1 (PDL)	UTO 2 (CRO)	UTO 3 (TLG)
UTO 1 (PDL)	**0,37%**		
UTO 2 (CRO)	9,5%	**0,16%**	
UTO 3 (TLG)	9,2%	2,1%	**0,08%**

Potiberaba lowest average genetic distances were found between *P. porakuara* and OTU 2 (lineage with occurrence in the epikarstic drainage in the CRO), 1.74% (Table 3). Higher values were found among the other OTUs - with emphasis on OTU 4 (karstic spring ODO), in the Rio do Carmo watershed, which presents genetic distances ranging from 6.1% to 7.6% in relation to the other localities. It is worth noting that OTUs 1 (FUF) and 3 (PDL), geographically close, but in distinct micro-basins, showed high genetic distances among themselves and among all other localities (Table 3, Figure 2C), indicating the role of barriers that delimit the drainages.

Table 3. Mean genetic distances (K2P) within-species (on the diagonal, in bold) and between the different OTUs identified for Potiberaba.

	P. porakuara	UTO 1 (FFA)	UTO 2 (CRO)	UTO 3 (PDL)	UTO 4 (ODO)
P. porakuara	**0,93%**				
UTO 1 (FFA)	2,6%	**0**			
UTO 2 (CRO)	1,7%	3,1%	**0,17%**		
UTO 3 (PDL)	2,9%	4,1%	3,7%	**0,17%**	
UTO 4 (ODO)	6,1%	6,8%	6,4%	7,6%	**0**

Kinnapotiguara cicadas showed the greatest variations in mean genetic distances between different OTUs (ranging from 3 to 14.7%), with the highest values between strains from opposite banks of the Apodi-Mossoró River (Table 4; Figure 3).

However, even among OTU's on the same bank the genetic distances were high and showed distinct patterns. While the lineages from the right bank of the river (OTU 6 - Rumana cave, RUM, and Buraco Redondo cave, BRD, and OTU 7 - lajedo dos Angicos) showed a genetic distance of 7.1%, *K. troglobia* and the OTUs 1 (lajedo Bonito), 2 (lajedo do Arapuá) and 3 (lajedo do Toco), on the left margin, present genetic distances varying from 3.4 to 5% regardless of the geographical distance. The distance between the *lajedo* do Rosário (where the caves with occurrence of *K. troglobia*) and the *lajedo* Bonito (ITU 1) is almost the same as the distance between the *lajedos* of Rumana and Meio (ITU 6) and the *lajedo* Angicos (ITU 7) (Table 4, Figure 3A). Still on the left margin, the genetic distances of OTUs 4 (lajedo da Lapa) and 5 (lajedo do Alfredo) (which is 3% between them) vary from 10.6 to 11.9% when compared to the other OTUs on the same margin (Table 4, Figure 3A), also independently of the geographical distance and compatible with a allopatric pattern.

Table 4. Mean genetic distances (K2P) within-species (on the diagonal, in bold) and between the different OTUs identified for *K. troglobia*.

	K. troglobia	UTO 1 Lajedo Bonito	UTO 2 Lajedo do Arapua	UTO 3 QJF	UTO 4 Lajedo da Lapa	UTO 5 CDS	UTO 6 RUM e BRD	UTO 7 Lajedo dos Angicos
K. troglobia	**0,08%**							
UTO 1 – Lajedo Bonito	3,4%	**0,59%**						
UTO 2 – Lajedo do Arapuá	4,1%	3,8%	**0,62%**					
UTO 3 – Lajedo do Toco (QJF)	4,7%	4,4%	5,0%	**0**				
UTO 4 – Lajedo da Lapa	10,6%	11,1%	11,9%	10,6%	**0,16%**			
UTO 5 – Lajedo de Alfredo (CDS)	10,8%	11,1%	11,8%	12,0%	3,0%	**0**		
UTO 6 - RUM e BRD	11,7%	12,1%	12,4%	12,7%	10,9%	11,3%	**0,8%**	
UTO 7 – Lajedo dos Angicos	13,9%	14,7%	14,5%	14,3%	12,2%	12,8%	7,1%	**0**

Genetic structuring

The AMOVA indicate that the distribution of localities according to tributaries (microbasins) explains 43.22 % (Φ_{CT} = 0.432, p = 0.049) of the genetic variation of cox1 observed for Cirolanidae sp.1 and 86.67% (Φ_{CT} = 0.867, $p<0.0001$) for *Potiberaba*. In view of the distribution and the small number of localities for Cirolanidae sp.2, the only possible distribution for performing AMOVA (in watersheds) did not show a significant result. There were no significant results for the distribution of localities with occurrence of aquatic taxa according to the different limestone outcrops (Table 5).

The AMOVA indicated that for *Kinnapotiguara*, the Apodi Mossoró River and its tributaries are biogeographic barriers explaining 78.91% of the observed genetic variation (Φ_{CT} = 0.79, $p < 0.0001$). However, the distribution of caves according to limestone outcrops (*lajedos*) better explains (94.72%) the observed variation (Φ_{CT} = 0.95, $p < 0.0001$) (Table 5).

Table 5. Analysis of molecular variance (AMOVA) test to assess how much of the genetic variation observed in the cox1 sequences of Cirolanidae sp.1, Cirolanidae sp.2, *Potiberaba* and *Kinnapotiguara* can be explained according to the location of the caves in relation to tributaries (microbasins) and in different limestone outcrops (*lajedos*). The highlighted lines correspond to the hypotheses that best explain the genetic variation.

Taxon	Hipótese	Entre grupos	Entre populações, dentro dos grupos	Dentro das populações	Φ_{CT}	p
Cirolanidae sp.1	***Tributários*** (ODC+APO / FNV+CLG / PDL / CAG / TRO / CBO+TLG)	43.22	3.67	53.11	0.432	0.049
	Lajedos (APO / ODC / CAG / CBO / TRO / TLG / FNV+CLG / PDL)	46.78	-0.88	54.11	0.468	0.06
Cirolanidae sp.2	***Bacias Hidrográficas*** (PDL / CRO+TLG)	75.77	22.15	2.08	0.758	0.34
Potiberaba	***Tributários*** (ODC / FFA / PDL / ODO / ODF+CAG+PFE / TRO / CRO / ODT+TLG+CBO)	86.67	3.18	10.15	0.867	<0.0001
	Lajedos (TRO+CRO / ODT / TLG / CBO / PFE / ODF / CAG / ODC / PDL / FFA / ODO)	40.32	48.77	10.91	0.40	0.42
Kinnapotiguara	***Tributários*** (CBO+MOS+LIE+LAI+RAP+URT+CDS / DBT+CRO+TRO+QJF+SET+ARA+BOT / BPX+PFE / RUM+BRD / SN9+SUB)	78.91	20.75	0.34	0.789	<0.0001
	Lajedos (TRO+CRO+DBT / CBO+MOS+LIE+LAI+RAP+URT / CDS / SET+ARA+BOT / QJF / BPX+PFE / RUM / BRD / SN9+SUB)	94.72	4.90	0.38	0.947	<0.0001

DISCUSSION

Lineage diversity

With the exception of Cirolanidae sp.1, all other groups showed extensive diversity of evolutionarily distinct lineages. Besides the identification of three OTU's for Cirolanidae sp.2 and four for *Potiberaba* (in addition to *P. porakuara*), *Kinnapotiguara* probably consists of a complex with seven other cryptic species besides *K. troglobia*. These results are supported by the clades recovered in phylogenetic analyses, lineage delimitation methods and genetic distances, as well as being in agreement with AMOVA.

Multiple morphologically similar and genetically distinct species or lineages (often classified as a single species with a wide distribution) have been discovered in recent years, and cryptic diversity in subterranean organisms appears to be particularly common (Verovnik et al., 2003; Finston et al., 2007; Trontelj et al., 2009; Niemiller et al., 2012; 2013). In addition to the evolution of similar morphologies via convergent, or parallel, evolution in related groups (Culver and Pipan, 2009), the stability of subterranean habitats and their limited connectivity can lead to high levels of endemism (Gibert and Deharveng, 2002; Verovnik et al., 2003; Finston et al., 2007).

Aquatic subterranean organisms are assumed to have a wider distribution than terrestrial organisms, due to their presumed greater dispersal capacity and habitat connectivity (Barr and Holsinger, 1985; Lamoreux, 2004). However, studies have shown that stygobitic species can consist of several populations distributed according to hydrographic boundaries in karst terrain (Finston et al., 2007; Trontelj et al., 2009; Niemiller et al., 2012; 2013), and even consist of species complexes (Trontelj et al., 2009; Fišer et al., 2018). Since subsurface hydrological connectivity strongly affects gene flow and thus connectivity between populations in stygobitic species (Venarsky et al., 2009), the existence of different lineages of Cirolanidae sp.2 and *Potiberaba* according to the different microbasins as well as in the epikarstic drainage of Crotes Cave was expected.

In addition to Cirolanidae sp.2 and *Potiberaba* OTU 2, the epikarstic drainage in Crotes Cave also harbors other unique species. Such as a yet undescribed species of Oligochaeta troglobites, one of Calabozoidea (Isopoda), and the planaria *Hausera hauseri* (Tricladida: Dimarcusidae) (Leal-Zanchet et al., 2014; Bento et al., 2021).

Regarding the terrestrial subterranean environment, several recent molecular studies have sought to explain the phylogenetic relationships and distribution of cave invertebrates such as spiders (Zhang and Shuqiang, 2013; Mammola et al., 2015), scorpions (Bryson et al., 2014), amblypygi (Esposito et al., 2015), crickets (Allegrucci et al., 2017), beetles (Gómez et al., 2016), and leafhoppers (Wessel et al., 2013). Such studies often identify distribution patterns similar to those found for *Kinnapotiguara*, with distinct lineages unique to one or a few nearby caves. Furthermore, the role of the Apodi-Mossoró River and its tributaries as barriers to *Kinnapotiguara* dispersal expresses the role of rivers as barriers for terrestrial troglobites (Barr and Holsinger, 1985). Therefore, the results found here demonstrate the pattern of a disproportionately higher number of allopatric cryptic species in highly fragmented regions (Fišer et al., 2018), which is especially true for troglobitic species in karst areas (Bickford et al., 2007; Niemiller et al., 2012).

However, there were exceptions and the genetic distances in Cirolanidae sp.1 across its distribution are consistent with a low intraspecific variability for COI in Isopoda (0-1.8%, Brix et al., 2011; 0-1.6%, Rodcharoen et al., 2016). Similar pattern also occurs in other regions, such as in Europe, where there are macro-stygobionts with large distribution ranges, particularly among Isopods (Henry and Magniez, 2003).

Paleoclimate

It is difficult to draw precise conclusions about the events that determined the splits between the different lineages, because of the large confidence intervals around the estimates. Nevertheless, the divergence times are in line with paleoclimatic events (Werneck, 2011; Dupont et al., 2013; Herbert et al., 2016; Silva and Souza, 2018) that have shaped the current cave faunas in the tropics and around the world (White and Pipan, 2019).

Regionally and locally well-documented late Miocene oceanic regression (Rosseti et al., 2013) was the first major paleoclimatic event with effects on the isolation of subterranean lineages in the study area, which correlates with the global eustatic high level (Haq et al., 1987; Haq and Al-Qahtani, 2005). Sea level has since regressed to the current level, without any further large-scale transgression events (Bezerra et al., 2003; Haq et al., 1987; Haq and Al-Qahtani, 2005). Estimates indicate that the oldest lineages in Cirolanidae sp.2 (7.66 Ma; 2.66-17.73 HPD) and *Potiberaba* (5.88 Ma; 4.10-8.02 HPD) diverged at the end of the Miocene, which is in agreement with probable isolation as a function of allopatric events resulting from oceanic regression. Oceanic transgressions and regressions are also noted as explanations for the origin and distribution of many subterranean taxa in coastal regions (Alvarez et al., 2015; Humphreys, 2019; Delic et al., 2020), and the area of this study harbors an oceanic relict community composed of cirolanids (Ferreira et al., 2010), amphipods (Fisěr et al., 2013), and planaria (Souza et al., 2018).

After sea level decline, and subsequent oceanic regression, a period of aridification followed in the tropics due to global cooling at the end of the Miocene (Rosseti et al., 2013; Herbert et al., 2016). While glaciations are noted as primarily responsible for the isolation of ancestral lineages in the subterranean environment, and subsequent origin of troglobitic species in the Northern Hemisphere (White and Pipan, 2019), the establishment of arid climates played a similar role in tropical regions such as Australia (Finston et al., 2007; Guzik et al., 2011; Humphreys, 2019).

As with Cirolanidae sp.2 and some *Potiberaba* OTUs, most stygobitic species in Australia have distributions restricted to individual aquifers or drainages, as expected for taxa that are confined to subterranean aquatic habitats and have low dispersal capacity (Humphreys, 2019). Similar to what occurs in some caves in the study area, subterranean environments in arid regions in Australia harbor endemic troglobitic assemblages (Guzik et al., 2011; Bradford et al., 2013). In addition, comparable phylogeographic patterns among unrelated taxa in an ecological system are strong evidence of a shared spatio-temporal history (Carstens et al., 2005).

Another important component of the intricate evolutionary history of the target groups of this study is the geological evolution of the Jandaíra Formation, whose late exhumation has been occurring since the Miocene. Consequently, an intense karstification process began, providing the development of large dissolved underground areas, resulting from the associated fracturing of these limestone and the concentration of meteoric water percolation zones (Bagni et al., 2020). The origin and expansion of these underground habitats occurred during the alternation between dry and wet periods (according to glacial and interglacial periods) that contributed to the expansion of semi-arid conditions and the dispersal and diversification of organisms adapted to seasonally dry tropical forests, such as the Caatinga (Werneck, 2011; Dupont et al., 2013; Costa et al., 2018). Therefore, the association between a large availability of subterranean environments and unfavorable external conditions must have played a key role in the diversification of troglobitic arthropods.

The analyses showed here, indicate that Cirolanidae sp.1 does not present the same pattern found for the other troglobitic taxa, and can be considered a single widely distributed species. This could be partly explained due to the larger body size of Cirolanidae sp.1 (>1cm) compared to Cirolanidae sp.2 (≈5mm) (unpublished data) and *Potiberaba* (≈ 3.5mm; Fiser et al. 2013), as active dispersal ability is positively related to body size (Jenkins et al., 2007; Shurin et al., 2009). Further studies are needed to explain whether such a condition may result from a higher dispersal capacity and/or a lower ecological specificity, allowing dispersal (and/or colonization) through aquifers not accessible to the other taxa and the maintenance of gene flow between different localities, even if only during wetter periods (Wang et al., 2004; Silva and Souza, 2018).

Two points related to the results involving *Kinnapotiguara* are worth noting. The first involves the estimates for lineage divergence based on the standard mitochondrial rate for insects (2.3%/Ma, or 0.0115 subst./site/Ma). Wessel et al. (2013) report that much faster mutation rates must have occurred in a complex of troglobitic cicada species living in lava tubes in Hawaii (whose geological evolution of terrain is much faster than in karst landscapes). A reliable estimate of the age of the Apodi-Mossoró River (probably responsible for the main divergence, estimated here at 6.49 Ma

[4.29-9.37 HPD]), could indicate a more appropriate mutation rate for *Kinnapotiguara*. However much higher rates compared to the one used here are not compatible with current estimates for the age of the geological fault that gave rise to the river channel - between the Miocene and Quaternary (Bagni et al., 2020).

The second point is related to the main divergence among the *Kinnapotiguara* lineages of the left margin of the Apodi-Mossoró River, which would have occurred around 4.92 Ma [3.07-7.17 HPD]. This divergence would be related to the Riacho do Abreu, and gave rise to the OTUs 4 (Lajedo da Lapa) and 5 (Lajedo do Alfredo) on the right bank of the drainage and to the other lineages on the left bank of this stream (*K. troglobia* and the OTU 1 - lajedo Bonito, OTU 2 - lajedo do Arapuá and OTU 3 - lajedo do Toco). The genetic distances between the OTU's in these two lineages do not vary according to geographical distances and are compatible with an allopatric pattern.

OTU´s Identification

There is a consensus that species are lineages of separately evolving populations or metapopulations (Padial et al., 2010), so they can be delimited using the criteria of genetic isolation (Fisěr et al., 2018). In addition to applying a wide range of delimitation analyses to the target taxa, we opted for a conservative approach defined from the agreement between most methods and the geographic distribution in order to delimit lineages (Carstens et al., 2013).

Although the increasing use of delimitation methods can lead to the genuine discovery of previously unknown evolutionary lineages, caution is needed. Small, recently isolated populations could already become reciprocally monophyletic through genetic drift and be diagnosable (Padial et al., 2010). Therefore, delimiting species associated with naturally fragmented and allopatric systems, such as caves, where similar selective pressures may promote morphological stasis or homoplasy, remains a challenging task (Bickford et al., 2007; Niemiller et al., 2012).

Therefore, inferences about species boundaries based only on genetic data should be considered hypotheses. The delimitation should be conducted taking into account other sources of information, such as life history, geographic distribution, morphology, behavior, among others. This constitutes the so-called integrative taxonomy (de Queiroz, 2007; Padial et al., 2010; Sukumaran and Knowles, 2017).

Analyses that include an estimate of species diversity from hypothetical lineages (even if using a single gene locus), in addition to distribution information, are an important starting point for conducting future research, especially for groups that are poorly studied and from extreme environments such as subterranean habitats (Fisĕr and Zagmajster, 2009; Carstens et al., 2013). Furthermore, several studies have found correspondence between cryptic lineages identified from mitochondrial DNA (usually a single marker, cox1) and groupings delineated from nuclear markers, including genomic data. Among the Arthropoda, some of these studies have involved insects (Janzen et al., 2017; Kozlov et al., 2017; Dincă et al., 2019), as well as Isopods (Guzik et al., 2019) and stygobitic amphipods (Zakšek et al., 2019).

In addition to genetic data, analyses were based on geographic distribution and evolutionary history to explain the isolation of lineages. Sampling across the known distribution of the target taxa, identified that microbasins are determinant for the differentiation of lineages in aquatic taxa that showed cryptic diversity (Cirolanidae sp.2 and Potiberaba) and also for *Kinnapotiguara*, in which case the *lajedos* determined an even greater structuring. Other barriers not identified here, possibly related to dispersal capacity and/or ecological specificities, and should explain the differences observed between Cirolanidae sp.1 and the other aquatic taxa. Thus, the OTUs delimited are presented as species hypotheses and further studies are recommended to test them. If confirmed, studies are needed to formally describe them based on an integrative taxonomy approach.

Additionally, only a small portion of the lineages identified here are officially protected in the PNFF (part of the distribution of Cironalidae sp.1, the OTU 1 of Cirolanidae sp.2, and the OTUs 1 and 3 of *Potiberaba*). Most of the remaining lineages are in areas exposed to several environmental threats, such as disorganized tourism, mining, oil exploration, deforestation and unsustainable exploitation of groundwater for irrigated fruit farming (Cruz et al., 2010; Ferreira et al., 2010). Therefore, urgent actions of control, planning and environmental education are needed for the conservation of the other lineages identified here, most with restricted distribution and possibly threatened with extinction.

CONCLUDING REMARKS

Comparative phylogeography can reveal rapid and robust information to access groups without expert taxonomists, or in the case of little morphological divergence. In addition, it can contribute to taxonomy by indicating the main groupings within each taxon. This initial information can be valuable in the case of little known and endemic groups, especially in drawing attention to possibly endangered lineages of conservation interest.

Additional research should be conducted with the inclusion of other molecular markers, as well as researches aimed at accessing ecological and life history information. Such information, coupled with the training of specialists in these taxa, is necessary to better understand the patterns identified here and design effective conservation strategies.

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