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Geometric morphometrics throws light on evolution of the subterranean catfish *Rhamdiopsis krugi* (Teleostei: Siluriformes: Heptapteridae) in eastern Brazil

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Rhamdiopsis krugi is a highly specialized troglobitic (exclusively subterranean) catfish from phreatic water bodies of caves located within two separated metasedimentary basins in the region of Chapada Diamantina, Bahia state, Brazil. In order to test the hypothesis of isolation with differentiation of the groups from the Una-Utinga and Irecê metasedimentary basins, we compared five populations among themselves and with an epigeal species of *Rhamdiopsis*. This was accomplished using geometric morphometrics, a powerful tool for detecting differences in body shape at population and species levels. All studied samples differed significantly from each other, the epigeal sample being the most distinct and the Una Basin populations clustering together. Geological and hydrological barriers explain the differences among the subterranean populations. We discuss our results together with the autapomorphies found in *R. krugi*, which validate its monophyly. These results imply an old age for the *R. krugi* clade, more than 10 Myr; alternative hypotheses are also presented. We propose a two-step vertical colonization model of the subterranean habitat through the hyporheic zone by an epigeal ancestral, with a progressive acquisition of the autapomorphies characterizing *R. krugi*. For conservation purposes, the two differentiated sets of populations should be considered and referred to as *R. krugi* 'Una morphotype' and *R. krugi* 'Irecê morphotype'. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **114**, 136–151.

ADDITIONAL KEYWORDS: cave fish – colonization – phreatic – speciation – subterranean biology – troglobites.

INTRODUCTION

Brazil is distinguished worldwide because of its rich and diversified subterranean ichthyofauna, including 29 described and undescribed troglobitic (exclusively subterranean) species known thus far (Trajano & Bichuette, 2010; Cordeiro, Borghezán & Trajano, 2013; M. E. Bichuette, unpubl. data). In addition to

taxonomic richness, a high diversity in anatomical specialization is observed, ranging from species with slightly but significantly reduced eyes and pigmentation to those homogeneously depigmented and eyeless, with all degrees of variation in between (bilaterally asymmetrical eyes are common in some species). These character states frequently present a mosaic distribution, which may differ intra- and interspecifically, suggesting an independent regression of eyes and pigmentation. Brazilian troglobitic fishes occupy a large diversity of habitats, from

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typical lotic habitats to the phreatic zone filling networks of heterogeneous subterranean conduits from small fractures to large, voluminous spaces and even permanently perched aquifers in the epikarst (see review in Trajano & Bichuette, 2010).

Among these fishes, *Rhamdiopsis krugi* Bockmann & Castro, 2010 (Siluriformes: Heptapteridae) calls attention due to its high degree of specialization to the subterranean way of life. The 24 autapomorphies listed by Bockmann & Castro (2010) include classical troglomorphies (herein defined as character states that may be directly related to the subterranean selective regime), namely regression of eyes and melanistic pigmentation, which are extreme in this species. No individual of the 12 localities where it is known to occur has any traces of pigmentation, externally visible eyes, or pigmented visual cells seen by transparency. Among Brazilian troglobitic fishes, only another *Rhamdiopsis* species, still undescribed from Toca do Gonçalves Cave, northern Bahia state (cited as *Rhamdiopsis* sp. 2 in Mattox *et al.*, 2008; Trajano & Bichuette, 2010), the armoured catfish *Ancistrus formoso* Sabino & Trajano, 1997 (Siluriformes: Loricariidae), and *Stygichthys typhlops* Brittan & Böhlke, 1965, the only troglobitic characid (Characiformes) so far reported for South America, compare in degree of troglomorphism. It is noteworthy that these four species inhabit lentic waters typical of the phreatic zone.

Like the majority of the subterranean fishes from Brazil, *Rhamdiopsis krugi* is a siluriform (as are 27 out of 29 species so far known), belonging to the Neotropical family Heptapteridae (7 out of 27 species) (see Sullivan, Muriel-Cunha & Lundberg, 2013 for the exclusion of *Phreatobius* from Heptapteridae). This family (*sensu* Bockmann & Guazelli, 2003) is a monophyletic group, generally characterized by nocturnal activity, bottom-dwelling behaviour, with cryptobiotic (hiding) habits, and a carnivore-generalist diet (Sazima & Pombal, 1986; Sabino & Castro, 1990; Casatti & Castro, 1998; among others). These traits are considered to be exaptations (*sensu* Arnold, 1994) that increase the chances of successfully colonizing the subterranean environment. Fishes with pre-adaptations are already armed with the tools needed to survive in this new, particularly selective regime.

Rhamdiopsis krugi was discovered in 1991 in the Chapada Diamantina region, in the upper phreatic zone connecting to the surface through Poço Encantado Cave in the form of a huge lake, partially illuminated at the present time. Since then, specimens were found in several other localities in the region. Because of uncertainties regarding its phylogenetic affinities due to its many autapomorphies (Bockmann & Castro, 2010), it was frequently cited under different names for

about 17 years (*Rhamdella* sp., *Imparfinis* sp. n., Gen. n. sp.), when it was formally described in 2010.

Individuals of *Rhamdiopsis krugi* have been found in 12 caves distributed among two limestone metasedimentary basins, separated by sandstone rocks that may represent a barrier for catfish dispersal, at least at the present time (Bichuette *et al.*, 2008a). As part of a first approach to this biogeographic problem, Bichuette *et al.* (2008a) applied molecular tools using RAPD bands, and found evidence of separation between populations of those two different basins. Molecular analysis has been a complementary tool used in phylogenetic studies during the last 25 years, from those focusing on relationships among supraspecific taxa (although 'total evidence' is recognized as the most reliable method, many studies still rely solely on molecular evidence) to phylogeographic analyses that have been uncovering a great deal of cryptic diversity in terrestrial and aquatic species, including subterranean ones. To be widely applicable to other fields when it is necessary to identify a large number of specimens in limited time intervals or even when it is not possible to collect samples, groups defined by molecular data must be associated with their corresponding morphotypes. Barcoding has been proposed as an alternative to the time limitation problem, but the evident flaws of taxonomy without taxonomists – those who actually know organisms (i.e., phenotypes as real entities, the results of evolutionary processes) – have been convincingly pointed out in several recent papers (e.g. de Carvalho *et al.*, 2007, 2008, 2013; Boero, 2010; Ebach & de Carvalho, 2011).

Systematics is based mostly on morphology – for fishes, external morphology, osteology and, very recently and still incipiently, myology (e.g. Datovo & Bockmann, 2010). However, the characters traditionally used have relatively low discriminatory power at the infraspecific or population level, and therefore are not able to explain the hidden diversity revealed by molecular studies.

One of the main advantages of using landmark-based geometric morphometric analysis is the possibility of visualizing the differences in body shape through thin-plate spline diagrams, which can be associated with phylogenetic hypotheses to portray the morphological evolution of a given clade or group (Catalano, Goloboff & Giannini, 2010; Goloboff & Catalano, 2011).

However, the application of geometric morphometrics in systematic and evolutionary studies is rare for Brazilian troglobitic fishes (Reis, Trajano & Hingst-Zaher, 2006), which makes it difficult to comprehend aspects related to their colonization and evolution in the subterranean environment. The present study aims to use geometric morphometric tools to compare troglobitic *R. krugi* occurring in different

caves from Chapada Diamantina. We looked for differences in body shape and associated these with habitat characteristics. We have consequently proposed a hypothesis to explain the colonization of subterranean waters in the Chapada Diamantina by catfishes of the genus *Rhamdiopsis*. We compared the data with an epigeal congener from the same river basin.

MATERIAL AND METHODS

STUDY AREA

The distribution of *Rhamdiopsis krugi* is in an area geographically known as Chapada Diamantina, in the central portion of Bahia state, eastern Brazil. With an area of ca. 38 000 km², the Chapada Diamantina landscape is dominated by karst landforms formed by the dissolution of carbonatic and, to a lesser extent, siliciclastic rocks. Typical karst features include exokarst (karst in surface) landforms such as fluted rock outcrops and large springs that form waterfalls at vertical walls, enclosed depressions and sinking streams (Pereira, 1998), and endokarst, such as caves and networks of smaller spaces that define subterranean habitats (Juberthie, 2000).

Rhamdiopsis krugi specimens inhabit lentic waters in the upper phreatic zone of a large karstic area (over 300 km²), connected to the surface through caves (Bockmann & Castro, 2010). These catfish are found either in deep and large lakes with clean water or in isolated, soft-bottomed pools, showing very distinct physical conditions and behaviour between them. *Rhamdiopsis krugi* catfishes from Poço Encantado Cave are distributed from 0.3 until 15 m of depth, calmly swimming near the substrate and frequently, at the mid-water (average speed approximately 0.03 m.s⁻¹; Mendes, 1995), rarely are shown close the surface; populations from Gruta Moreno, Lapa do Bode, Gruta Natal and Canoa Quebrada caves are distributed from 0.3 to 1.8 m depth and show preference to swim close the substrate formed mainly by silt, limestone riffles and guano piles and, less frequently, at the mid-water and surface (M. E. Bichuette unpubl. data).

The five study caves are developed in exposed limestones of Neoproterozoic age, belonging to the Una Geological Group, Salitre Formation. They are situated in the Irecê (Canoa Quebrada Cave – 12°25'29.7"S 41°33'28.2"W; altitude 690 m) and Una-Utinga metasedimentary basins: Gruta do Moreno (12°48'32.7"S 41°09'53.1"W; altitude 310 m), Lapa do Bode (12°56'06.5"S 41°03'53.9"W; altitude 281 m), Poço Encantado (12°56'41.8"S 41°06'17.3"W; altitude 385 m) and Gruta Natal (12°59'32.4"S 41°05'32.8"W; altitude 290 m) (Fig. 1). These two basins are separated by much older, Mesoproterozoic exposed rocks of

the Chapada Diamantina Group, disposed in three layers, two of which are formed by sandstones (respectively, Morro do Chapéu and Tombador formations) intercalated by limestones of the Caboclo Formation (Schobbenhaus *et al.*, 1984) (Fig. 2A).

The presence of caves in the Chapada Diamantina sandstones, such as the 2000 m long Torras and Parede Vermelha caves (Gallão & Bichuette, 2012), indicates karstification of these rocks. According to Ford & Williams (2007), siliceous sandstones and quartzites can develop dissolutional karst landforms, but in a more limited range of forms and hydrogeological characteristics than calcareous ones. These authors also mention that the quartzose rocks of most caves are vadose features (karst features developed in the unsaturated zone, above the phreatic). This would be the case of caves in the Chapada Diamantina Formation, although the presence of scallops in some of them (R. Fraga pers. comm.) may indicate some karstic activity under phreatic conditions.

The distributional area of *Rhamdiopsis krugi* is within the range of the Rio Paraguaçu basin, that belongs to the eastern Brazilian region which comprises several parallel river basins running directly to the Atlantic Ocean (Ribeiro, 2006). Bockmann & Castro (2010) hypothesized that this species is derived from a clade distributed throughout a region comprising the Upper São Francisco, Upper Paraná basins and other eastern basins, which arrived in the Paraguaçu basin after an event of hydrological capture caused by neotectonism, when the Santo Antônio basin (located in the Irecê basin) became part of the Paraguaçu basin.

Specimens of *Rhamdiopsis krugi* have been found in 12 out of 19 caves with water bodies prospected by M. E. Bichuette in the Chapada Diamantina region (Fig. 1; Table 1), but with no records in sandstone caves of the Chapada Diamantina Formation, where other troglobitic catfishes occur, the Copionodontinae *Glaphyropoma spinosum* Bichuette, de Pinna & Trajano, 2008 and the undescribed *Copionodon* sp. (Trichomycteridae) (Bichuette, de Pinna & Trajano, 2008b; M. E. Bichuette in prep.). The main physical characteristics of the localities of the *R. krugi* localities are in Table 2. Specimens are deposited at Laboratório de Estudos Subterrâneos of Universidade Federal de São Carlos – Ichthyological collection (LESCI) and Laboratório de Ictiologia de Ribeirão Preto da Universidade de São Paulo (LIRP), both in Brazil.

SAMPLE COLLECTION

In total, 79 specimens were collected through hand-netting during several expeditions from 2004 to 2008: Gruta do Moreno Cave – GM (17 specimens), Poço Encantado Cave – PE (13 specimens), Lapa do Bode

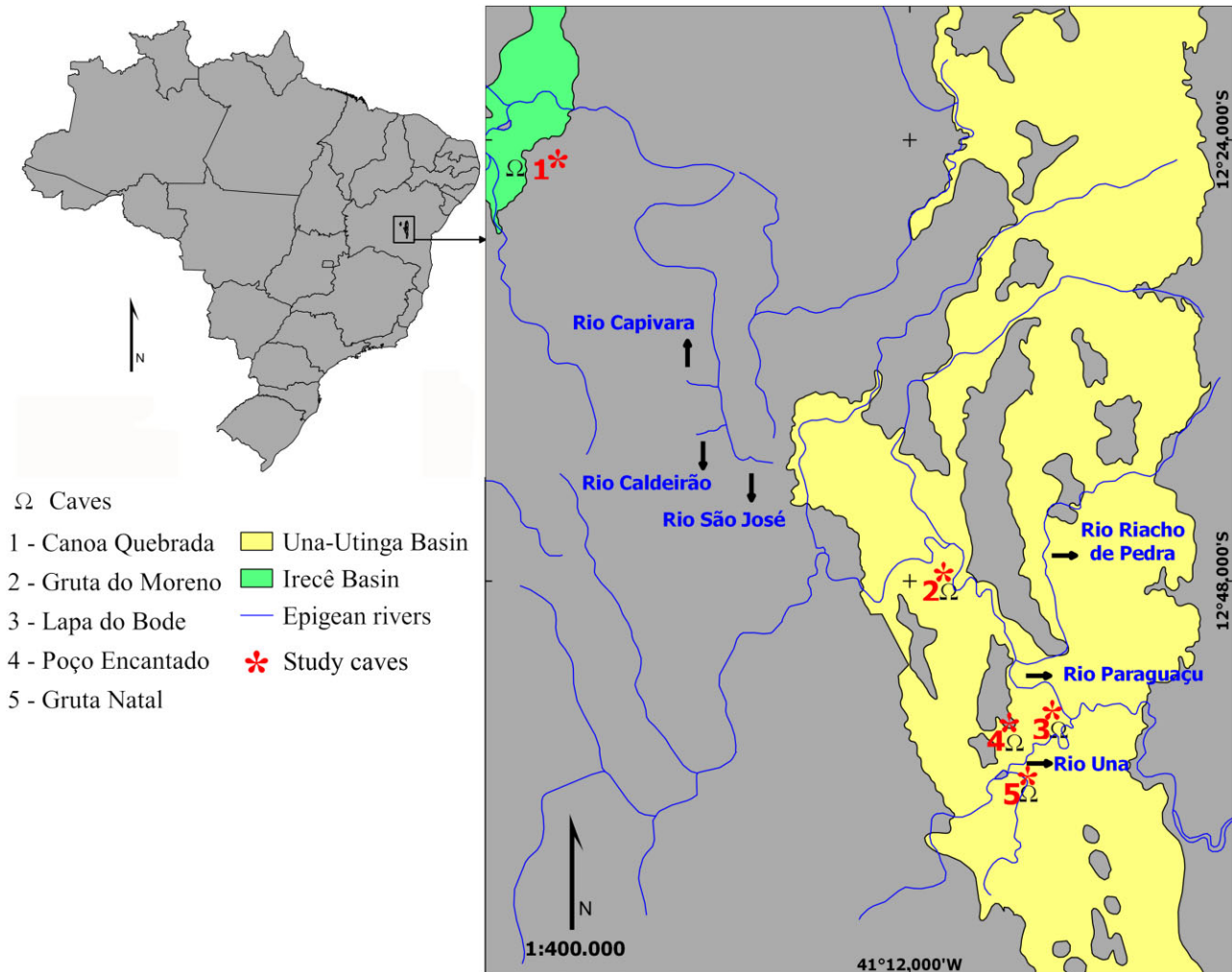


Figure 1. Map of Brazil showing Chapada Diamantina region (with municipalities), Rio Paraguaçu basin, Bahia state, eastern Brazil. In detail, the caves and epigeal rivers where *R. krugi* and *Rhamdiopsis* sp. occur. In detail, the two limestone metasedimentary basins, Una-Utinga and Irecê, separated by rocks of the Chapada Diamantina Group (see geological details at Fig. 2A).

Cave – LB (18 specimens), Gruta Natal Cave – GN (13 specimens) and Canoa Quebrada Cave – CQ (18 specimens). The specimens were killed by over-anesthesia in benzocaine solution, fixed with 10% formaldehyde and subsequently preserved in 70% ethanol. Because troglitic populations and subterranean habitats are generally fragile, we chose to collect the minimum number of specimens possible.

In order to test if there are differences in body shape, we used landmark-based geometric morphometric techniques to represent the shape of the catfishes, using the coordinates to test for significant differences in body size and shape (Monteiro & Reis, 1999).

For comparison, we included in the morphometric analyses seven specimens from an undescribed epigeal species of *Rhamdiopsis* (F. A. Bockmann

pers. comm.), collected in Rio Caldeirão (12°39'33"S 041°22'12"W; altitude 320 m) (six specimens), tributary of Rio São José, and in Rio Capivara near the dirt road Lençóis-Andaraí (12°37'26"S 041°22'35"W; altitude 341 m) (one specimen), both tributaries of the Rio Paraguaçu in Lençóis County (Fig. 1), which is basically a sandstone area (Fig. 2A).

Localities of troglitic *Rhamdiopsis* in the Irecê sedimentary basin were not included in the original description of *R. krugi* (see Bockmann & Castro, 2010). Therefore, in order to confirm the identification of catfish from Canoa Quebrada Cave, we checked the presence of the anatomical autapomorphies in two specimens from this cave, including a cleared and stained one (using the method of Taylor & van Dyke, 1985).

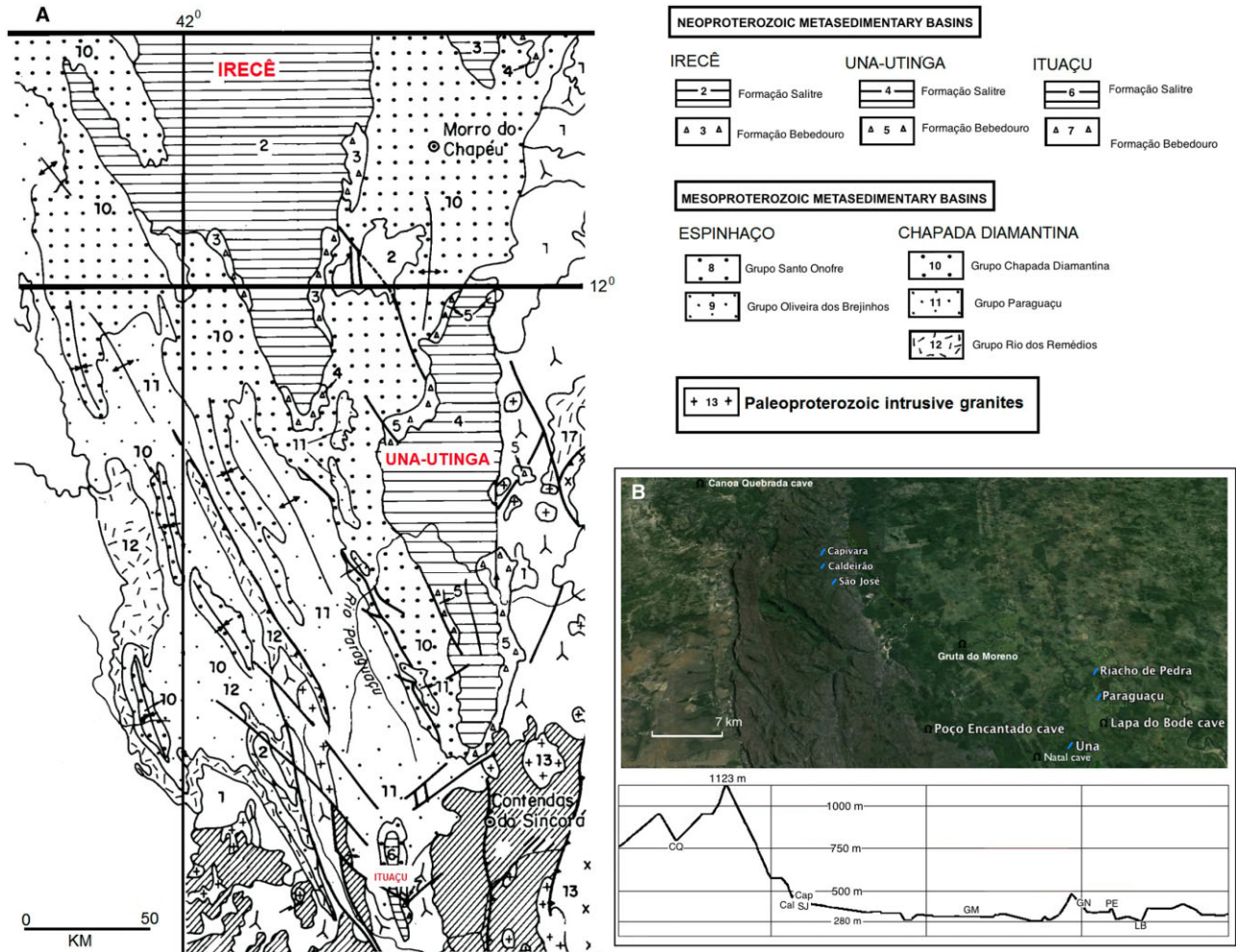


Figure 2. (A) Geology Modified from Schobbenhaus *et al.* (1984) and (B) elevation profile between Una-Utinga and Irecê metasedimentary basins; caves and epigeal rivers in detail. Chapada Diamantina region, Rio Paraguaçu basin, eastern Brazil. CQ – Canoa Quebrada Cave; GN – Gruta Natal; PE – Poço Encantado Cave; GM – Gruta do Moreno; LB – Lapa do Bode Cave; Cap – Rio Capivara; Cal, Rio Caldeirão; SJ – Rio São José.

DATA ANALYSES

To capture images, a digital camera with a resolution of 8.1 megapixels was used. Each specimen was photographed in dorsal view, horizontally aligned, and measured with a digital caliper with centesimal precision to obtain a size reference for the analyses.

Twelve landmarks were chosen in dorsal view to determine the general outline of the body and main features, such as relative size of head and anterior and posterior segments of the body, position of the nostrils, opercle openings and insertion of fins (Fig. 3): 1. Snout tip; 2. anterior margin of anterior nostril (right); 3. Same as 2 (left side); 4. anterior margin of posterior nostril (right side); 5. Same as 4 (left side); 6. posterior tip of supraoccipital bone process; 7. posterior tip of right opercle; 8. Same as 7 (left side); 9. origin of first ray of right pectoral fin; 10.

origin of first ray of left pectoral fin; 11. origin of first dorsal fin ray; 12. Anterior edge of caudal fin.

Morphometric data were acquired as homologous landmark coordinates of two dimensions, using the software TPSDig 2, version 2.10 (Rohlf, 2007).

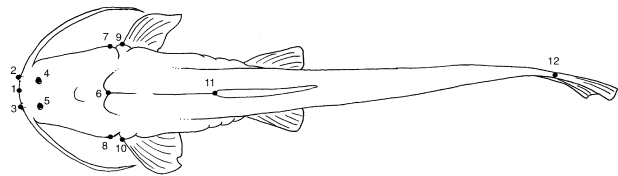
Size was computed as centroid size (Bookstein, 1991), and compared among epigeal and cave populations. To determine the degree of morphometric differentiation among species, the partial warp matrix, including the uniform component, was used in a multivariate analysis of variance (MANOVA). Canonical variates analysis was employed to verify the differences among groups and shape among different samples. It was explored by calculating least-squares Mahalanobis distances and employing those in a cluster analysis by the unweighted pair-group method, using arithmetic averages (UPGMA) to

Table 1. Caves explored searching for *Rhamdiopsis krugi* specimens in the Chapada Diamantina region, eastern Brazil. In bold, those with confirmed occurrence of the species. *Caves included in the present study

Cave	Locality	Altitude (m)
Irecê metasedimentary basin (limestones)		
Lapa Doce Cave	Iraquara	720
Pedra Furada, Lapa Doce I Cave	Iraquara	700
Poço do Guina, in Lapa Doce II Cave	Iraquara	690
Gruta da Artemizia Cave	Iraquara	710
Gruta Torrinha Cave	Iraquara	690
Gruta Azul Cave	Iraquara	620
Gruta Pratinha Cave	Iraquara	610
Gruta Fazenda Umburana Cave	Iraquara	718
Diva de Maura Cave	Iraquara	590
Gruta do Io Cave	Palmeiras	600
Canoa Quebrada* Cave	Palmeiras	690
Buraco do Cão Cave	Seabra	600
Chapada Diamantina Group (sandstones)		
Gruna do Criminoso Cave	Andaraí	826
Gruna dos Torras Cave	Andaraí	827
Una-Utinga metasedimentary basin (limestones)		
Gruta Natal* Cave	Itaetê, right margin of Rio de Una	290
Fazenda Rio Negro Cave	Itaetê, right margin of Rio de Una	280
Poço Encantado* Cave	Itaetê, left margin of Rio de Una	385
Lapa do Bode* Cave	Itaetê, left margin of Rio de Una	281
Gruta do Moreno* Cave	Nova Redenção	310
Poço Azul do Milu Cave	Nova Redenção	300

Table 2. Characterization of caves inhabited by *Rhamdiopsis krugi* populations

Cave	Habitat dimensions (type of water body)	Water current	Bottom
Canoa Quebrada Cave	Large conduits	Slow current	Silt and thin plates of calcite
Gruta Natal Cave	Large conduits	Slow current	Silt and thin plates of calcite
Poço Encantado Cave	Very large lake	Logged	Rocky walls, platforms with silt
Lapa do Bode Cave	Small, shallow pools	Logged	Silt and vampire bat guano
Gruta do Moreno Cave	Small conduits	Logged	Silt and vampire bat guano

**Figure 3.** Dorsal view of *R. krugi* showing the selected landmarks used for analyses. Explanation of each one is found in the text.

represent the similarities between configurations. All analyses were performed in the R programming environment (R Core Team, 2014) using the geomorph package for geometric morphometric analyses (Adams & Otarola-Castillo, 2013).

RESULTS

Results of analysis of variance (ANOVA) indicated significant differences in size among the populations studied ($F = 26.12$; $d.f. = 5$; $P < 0.001$), due to the larger size of the epigeal fish sampled (EP); the five cave populations did not differ significantly among themselves (Fig. 4).

Regarding body shape, the first five relative warps explained 95% of the observed variation, and were used in MANOVA, as well as in the canonical discriminant analysis. Results for the MANOVA revealed significant differences among populations (Pillai's trace = 1.43, approx. $F = 6.43$; num $d.f. = 25$; den $d.f. = 400$; $P < 0.001$). The scores of specimens in the first and second canonical variate axes (Fig. 5)

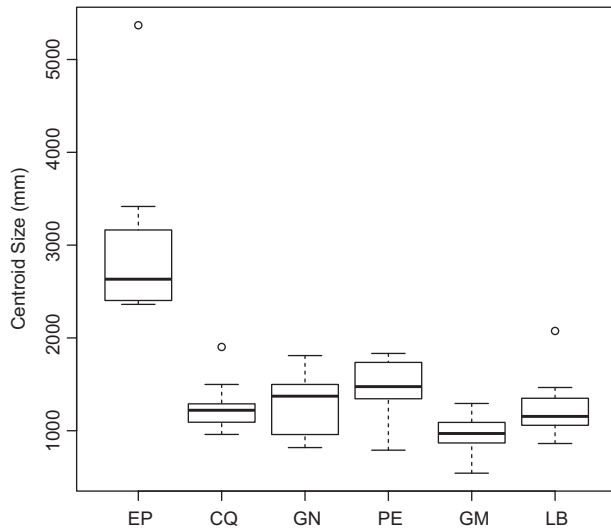


Figure 4. Box-plot of centroid size for each *R. krugi* studied population. The circles are the outliers. EP – epigean population CQ – Canoa Quebrada Cave; GN – Gruta Natal; PE – Poço Encantado Cave; GM – Gruta do Moreno; LB – Lapa do Bode Cave.

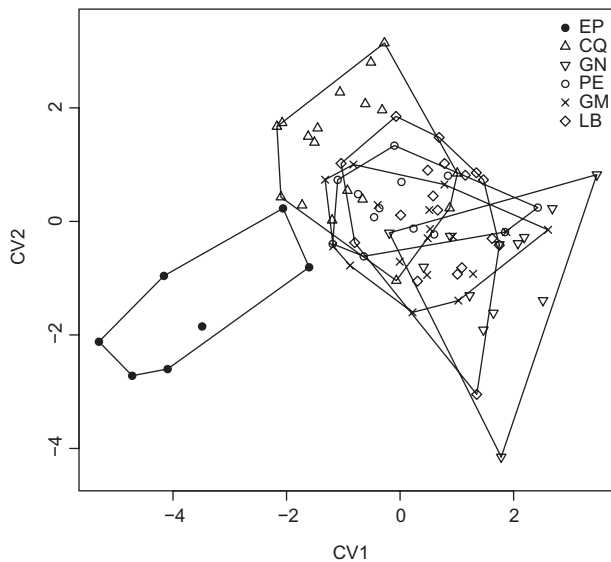


Figure 5. Ordination of all *R. krugi* specimens along the first two canonical axes based on partial warps plus the uniform component for each population. EP – epigean population CQ – Canoa Quebrada Cave; GN – Gruta Natal; PE – Poço Encantado Cave; GM – Gruta do Moreno; LB – Lapa do Bode Cave.

confirmed this trend. Body shape was observed for size, and separated the epigean sample from the five cave populations in the first canonical axis, but not in the second. Among the cave populations, the only sample from Irecê sedimentary basin (Canoa

Quebrada Cave – CQ) differed from the one from Gruta Natal (GN) in the Una Basin, both in the first and second axes. The three remaining studied populations from the Una Basin – Gruta do Moreno (GM), Lapa do Bode (LB) and Poço Encantado (PE) – did not show a marked tendency towards separation based on body shape, when considering the scores from the canonical analysis, except for the positioning of the Lapa do Bode and Canoa Quebrada populations in the extremes of the group formed by the cave samples.

The phenogram in Figure 6 confirms that the epigean sample was the most distinct in body shape, and that the population from CQ in the Irecê basin is slightly different in shape from the other four cave populations, located in the Una Basin. Among these, the GN sample on the right margin of the Una River is separated from the other three, located on the opposite side of this river.

The splines in Figure 6 show a modification in body proportions, due to an elongation of the anterior portion of the trunk in the five subterranean populations when compared to the epigean sample. This is represented by an increase in the distance between the posterior edge of the supraoccipital process (landmark 6) and the origin of the dorsal fin (landmark 11), as well as the posterior edges of the operculae (landmarks 7 and 8) and the origin of the first rays of the pectoral fins (landmarks 9 and 10). Conversely, we could not recognize any trend regarding the posterior portion of the body (distance between the landmark 11 and the basis of the caudal fin – landmark 12) in the subterranean fishes as a unit, since a slight diminishing of this distance may be observed in the GM sample. Yet the shape of CQ specimens is very similar to the epigean ones. A widening of the snout in the subterranean populations is also visible by the separation of the anterior edges of the nostrils (landmarks 2 and 3).

The landmarks responsible for most of the shape differences among the populations from the Una and Irecê basins were those related to snout and body width and relative position of the origin of the dorsal fin. Shape changes associated with the first canonical axis correspond to a general change in body shape, with the population from CQ presenting a slender body closer to the epigean sample. The second axis, which separates mainly GM from PE populations, both from Una Basin, corresponds to a larger spacing between the origins of the dorsal and caudal fins, and also to a subtle widening of the operculum.

Regarding body width, the epigean, and especially the CQ fishes, are more slender than the mean shape (represented by dashed lines in the spline plates), whereas a tendency towards the broadening of the body is present in the Una Basin populations, with GM fish being more robust (Fig. 6 – at right).

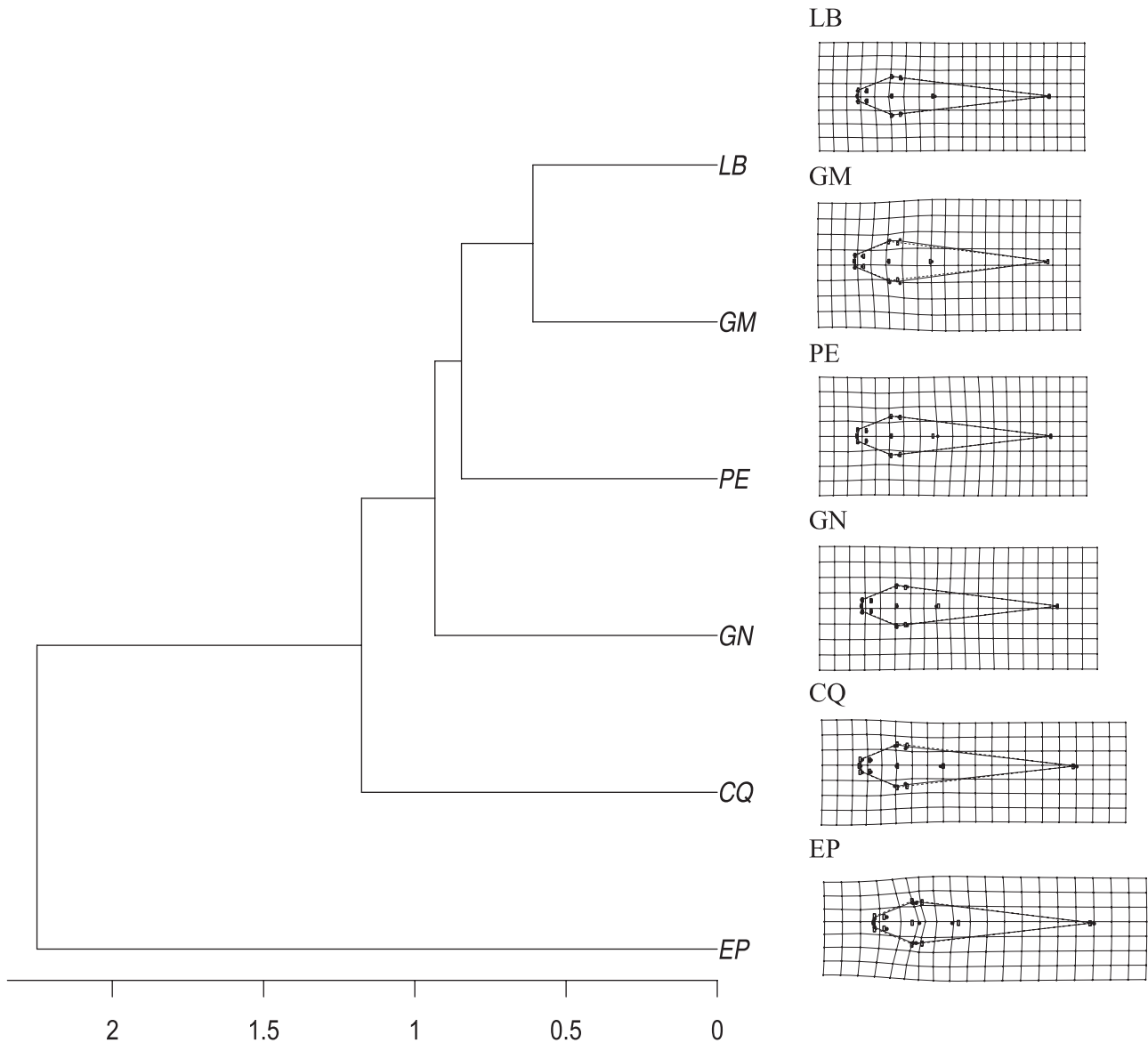


Figure 6. Morphological relationships among the six populations of *R. krugi* represented by the UPGMA clustering of the Mahalanobis D2 values; and body shape represented by thin-plate splines, associated with each population (continuous line) and superimposed over the mean shape of the whole sample of *Rhamdiopsis*, shown at right in the figure. EP – epigean population; Irecê basin: CQ – Canoa Quebrada Cave; Una-Utinga Basin: GN – Gruta Natal; PE – Poço Encantado Cave; GM – Gruta do Moreno; LB – Lapa do Bode Cave.

DISCUSSION

MONOPHYLY OF *RHAMDIOPSIS KRUGI*: COLONIZATION OF THE SUBTERRANEAN HABITAT AND SPECIATION

Rhamdiopsis krugi was described on the basis of samples from Poço Encantado (the type-locality), Lapa do Bode and Natal Caves (Bockmann & Castro, 2010). The osteological and external autapomorphies described by these authors are also present in specimens from Canoa Quebrada Cave (M. E. Bichuette pers. obs.), as well as the behaviours observed in

habitat – life in lentic habitat, non-cryptobiotic and non-photophobic behaviours.

Among the 24 autapomorphies listed by these authors to define the species, eight clearly represent troglomorphisms: eyes completely absent and correlated atrophied optic foramen; body coloration absent (regressive, neutral character states; Wilkens, 2010); fatty tissue broadly spread throughout the body, probably as an adaptation to starvation (Weber, 1996; Hüppop, 2012); adults of small to medium body size, reaching 53.5 mm of standard length (SL) (most

heptapterids are larger than 5 cm SL – Bockmann & Guazelli, 2003), probably as an adaptation to life in small spaces; non-photophobic behaviour and poorly developed circadian rhythmicity, probably regressive characters (Trajano *et al.*, 2009); non-cryptobiotic behaviour (*idem*), associated to marked mid-water activity, possibly correlated to another autapomorphy, the deep and rounded anal fin; life in lentic habitats (most catfishes, including heptapterids, inhabit water bodies with medium to fast velocity).

Troglobitic organisms in general are highly homoplastic, widely known for reduced eyes and melanic pigmentation, a phenomenon also observed in the behavioural traits mentioned, which are common traits of troglobitic heptapterids (Trajano & Bockmann, 1999) and of several other siluriforms (Parzefall & Trajano, 2010). Such is not the case with the several osteological and external morphological character states unrelated to the subterranean way of life, which, when combined, encompass a strong phylogenetic signal (e.g. subpreopercle absent; posterior limb of the transverse process 4 undivided, with spatulated shape; posterolateral corner of the posterior portion of the posterior branch of the transverse process of vertebra 4 extending approximately to mid-length of the transverse process of vertebra 5; posterior lobe of the adipose fin straight; hypural 5 usually co-ossified to hypural 4 at its distal portion; dorsal hypural plate typically with seven rays – respectively, autapomorphies 5, 7, 8, 10, 12 and 13 in Bockmann & Castro, 2010).

Emphasis must be given to the peculiar, widely exposed pseudotympanum. This is a most remarkable autapomorphy of *R. krugi*, probably involved in the enhancement of sound sensitivity (Bockmann & Castro, 2010) with low probability of homoplasy. These observations also support the presumed monophyly for the presently studied taxon. Therefore, there is little doubt that all studied populations diversified as a result of a single colonization and subsequent speciation event.

In addition to the smaller size, a slender and elongate body (due to the elongation of the anterior portion of the trunk), a broad head and snout, and dorsal and pectoral fins displaced backwards with a large space between the opercular opening and the origin of the pectoral fin, as shown by the morphometrics results, may be added as putative autapomorphies for *R. krugi*, pending on further confirmation, based on examination of specimens of other epigeic species, such as *R. microcephala* and *R. moreirai*. A broadened head in comparison with epigeic relatives is relatively frequent among specialized troglobitic vertebrates, including representatives of diverse fish families such as amblyopsids, bythitids, synbranchids, characids, ictalurids and, among heptapterids, *Rhamdia* spp.,

and salamanders (Durand, 2005; Christiansen, 2012). Its adaptive value may be related to the expansion of the cephalic surface allowing for the increase in number of sensorial structures, such as taste buds, free neuromasts and electroreceptors (described for the intensively studied Mexican tetra characin, *Astyanax jordani* (Hubbs & Innes, 1936), cave amblyopsids, and the iconic European salamander, *Proteus anguinus* Laurenti, 1768, among others (e.g. Boudriot & Reutter, 2001; Niemiller & Poulson, 2010); the enlargement of the mouth allowing for capture of larger prey, an important adaptation in the typical food-poor subterranean environment; and/or an enhanced hydrodynamic effects in front of the head while swimming (Wilkins, 2005). All of these explanations may apply to the broadened snout of *R. krugi*.

The monophyly of the taxon composed of the populations from both Una and Irecê metasedimentary basins poses an initial problem: how could these populations, currently living in, at least partially, isolated metasedimentary basins have originated from a single ancestral population? As a first approach to this question, it is useful to examine available data about: (1) the geology of the region and rates of karstification; (2) the state-of-art of the systematics and distribution of the genus; and (3) evolution of the major South American river basins.

The genus *Rhamdiopsis* is distributed in two major river basins, the Paraná (running to the south, in direction of Mar del Plata) and the São Francisco, and a set of several parallel rivers running toward the east coast of eastern Brazil (Bockmann & Guazelli, 2003; Bockmann & Castro, 2010). Ancient dates have been proposed for the formation of the South American basins and the diversification of the freshwater SA ichthyofauna. For instance, according to Lundberg *et al.* (1998) the Paraná and São Francisco drainages were established, in configurations similar to the present-day ones, by the late Oligocene–early Miocene (from 30–20 Ma). Ribeiro (2006), analyzing Brazilian coastal basin histories, which includes Rio Paraguaçu basin, proposed a pattern where Brazilian eastern coastal rivers are inhabited by remaining taxa of an ancient biogeographic history, such as the Copionodontinae basal catfishes (Trichomycteridae), and consider a Mesozoic age (late Cretaceous) for the initial phase of diversification of this endemic ichthyofauna.

Rhamdiopsis krugi is likely a basal species within the genus (F. A. Bockmann pers. comm.), and its high degree of troglomorphy makes it reasonable to suppose that it was isolated in the subterranean habitat very early in its evolutionary history. Low denudation rates were estimated from the São Francisco Craton, around 30 ± 10 m/Myr, within the range

recorded for Padre Cave, another karst area in Bahia (Auler *et al.*, 2009). Associating these rates with the ca. 300 m difference in altitude between the top of Chapada Diamantina and the base level of the Irecê basin (Fig. 2B) and based on the history of phreatic habitats formation, the latest underground connection between Irecê and Una-Utinga limestones across the Chapada would have occurred approximately 10 Mya. In this scenario, this would be the minimum age for the origin of the *R. krugi* lineage; a seemingly old age, but consistent with its high degree of morphological and behavioural specialization, as well as with its basal position within the genus. This is because *Rhamdiopsis* catfishes, with 17 species (most of them undescribed yet), are distributed in several rivers of the eastern and Paraná basins (Bockmann & Castro, 2010), its origin consequently predating the separation of these basins in the late Miocene. Old ages have been proposed for other South American freshwater fishes, such as the armoured catfish, genus *Hypostomus* (up to 11.8 Myr – Montoya-Burgos, 2003). Langecker & Longley (1993) proposed older ages, in the late Early Miocene, for the highly specialized phreatobitic ictalurids, *Satan euryostomus* Hubbs & Bailey, 1947 and *Trogloglanis pattersoni* Eigenmann, 1919.

Following the progressive erosion of limestones over the Chapada Diamantina sandstones, the limestones of the Una and Irecê basins became separated, causing the isolation of two sets of *R. krugi* populations. Nevertheless, it must be noted that because the axis of the folding sedimentary layers declines in a south to north direction, the dissection of these limestones progresses from south to north. Therefore, more recent connections may have occurred through the north in the lower areas around the Chapada Diamantina, where limestones of the Bebedouro Formation presently surface (see Fig. 2B). Even a present-day connection is possible through subterranean aquifers in subsurface limestones. According to this hypothesis, the presence of *R. krugi* or closely related species, besides Itaetê, Iraquara, Palmeiras and Nova Redenção, is predicted for Souto Soares, Utinga, Wagner, Ruy Barbosa and Lajedinho regions (Fig. 1, see municipality boundaries).

An alternative hypothesis, less likely but still possible, is a current connection through the Chapada Diamantina sandstones. It is widely accepted that karst processes may develop in siliciclastic rocks. However, cited cases of speleogenesis in sandstones and quartzites refer to vadose caves. Although the authors do not disclose the possibility of conduit aquifers in such rocks (e.g. Ford & Williams, 2007: 389–390 – ‘most caves in quartzose rocks are vadose features’), no actual example is presently known. Nevertheless, as already mentioned, the presence of

scallops in some sandstone caves of the Chapada Diamantina may indicate some karstic activity under phreatic conditions. Thus this hypothesis deserves attention. Currently, the Irecê base level is at least 300 m higher than the Una base level (Fig. 2B). Under these conditions, and considering the small size of *R. krugi* and the large distances between these basins, there is a higher probability of dispersion from Irecê to Una than *vice-versa*. This is consistent with the greater similarity between specimens of EP and CQ than in relation to specimens from Una.

Based on the set of autapomorphies of *R. krugi* herein discussed, we propose that the colonization of the subterranean environment by the predecessors of present-day *R. krugi* occurred in two steps:

1. Colonization by typically stream-dwelling *Rhamdiopsis*-like specimens of the hyporheic zone (interstitial spaces of sediments of the streambed, considered a transition zone between surface water and groundwater; Gibert, Danielopol & Stanford, 1994). This could be achieved by miniaturization through pedomorphosis, a process proposed for miniaturized fish species in headwaters and also in the subterranean habitats (Trajano & Bockmann, 1999; Bichuette & Trajano, 2004). If the specimens were small enough to move in the interstices and able to live from the resources provided by this restrictive environment, and if their number was sufficient to form an effective population, then the hyporheic habitat could be colonized. These two conditions are quite difficult to be achieved, however, with chance playing a non-negligible part. This explains why only representatives of this genus, among several others observed in surface waters (M. E. Bichuette unpubl. data), have subterranean representatives in the region. The selective regime in the hyporheic environment also favors a slender, elongated and flexible body. Additionally, the food limitation typical of subsurface habitats would select for the presence of fatty cells spread throughout the body (autapomorphy 18 in Bockmann & Castro, 2010). Another autapomorphy probably appearing at this phase is the reduced, very short lateral line in the trunk, a trait observed in other phreatobitic catfishes such as the heptapterids *Rhamdiopsis* (undescribed species from Toca do Gonçalves, northern Bahia state), the phreatobiids *Phreatobius* spp., and the ictalurids *S. euryostomus* and *T. pattersoni* (Bockmann & Castro, 2010), as well as in the characiform *Stygichthys typhlops* (Moreira *et al.*, 2010). On the other hand, troglobitic fishes living in open channel subterranean streams, such as the heptapterids *Pimelodella kronei* (Ribeiro, 1907), *P. spelaea*

Trajano, Reis & Bichuette, 2004 and *Rhamdia* spp., have complete lateral lines (Bockmann & Castro op. cit.). These observations indicate that the reduction of the lateral line is related to life in narrow spaces, where lateral sensitivity would be impaired. In this case, it is interesting to note that the main selection force might be the confined environment (i.e., in tight spaces). This hypothesis is reinforced by examples gathered among other heptapterids: species living in confining environments such as litter, tend to present characteristics such as miniaturization, slender body, reduced lateral line. Examples are *Gladioglanis* species, *Myoglanis koepckeii* Chang, 1999, *Acentronichthys* and *Phreatobius* species. Epigeal species of *Rhamdiopsis* – *R. microcephala* (Lütken, 1874) and *R. moreirai* Haseman, 1911 – also live in confined spaces, interspersed in the marginal vegetation.

- Colonization through the hyporheic zone of the hypogean habitat beneath it. Subterranean spaces are more heterogeneous and larger than in the hyporheic zone, requiring adaptations that contrast with those selected in the latter. Therefore, those tiny fishes with reduced lateral lines face a new problem: navigating and finding food and mates in large spaces of a lentic environment. At this phase, selection for improvement of sensory abilities may account for the extreme development of the pseudotympanum and broadening of the head and snout. In addition to offering an increased surface for free neuromasts, taste buds, and other sensory structures, a broadened head, associated with the repositioning of the dorsal and pectoral fins, would reduce pitching and rolling during swimming in mid-water. Enhanced stability is pivotal for energy economy in food-limited habitats, as observed for those inhabited by *R. krugi* (the presence of large colonies of vampire bats, *Desmodus rotundus* (Geoffrey, 1810), observed in Lapa do Bode and Gruta do Moreno – Table 2 – is a very recent occurrence, associated with livestock brought to the Neotropical region by Europeans as a food source). Furthermore, a stabilizing selection for maintenance of the small size of *R. krugi* is expected under these conditions. Langecker & Longley (1993) hypothesize that the small size of *Satan eurystomus* and *Trogloglanis pattersoni*, compared to epigeal ictalurids and in other troglobitic fishes as well, is an adaptation for energy economy in the deep phreatic zone.

Like all of South America, Bahia was subject to an alternation of dry and wet periods, well documented over the last 350 000 years (Wang *et al.*, 2004; Auler *et al.*, 2006). It is reasonable to suppose that climatic

fluctuations have occurred during the Miocene as well. Thus the paleoclimatic model (Barr, 1968; Trajano, 1995) may apply to the presently studied catfish. According to this model, during dry periods with the disruption of surface drainage, the fauna in subsurface habitats (hyporheic and/or subterranean) could survive and, once isolated, differentiate. Accelerated divergence rates are expected because subsurface selective regimes highly contrast with surface ones, characterized mainly by absence of photoautotrophy and food scarcity (sometimes extreme), as well as the functional impossibility of visual orientation. Thus, colonizing populations would be relatively small, prone to genetic drift by founder effects and other factors.

The hypothesis of vertical colonization of the subterranean environment through the hyporheic zone is also compatible with models of parapatric speciation. In any case, the high degree of morphological, ecological and behavioural specialization of *R. krugi*, characterized by an elevated number of autapomorphies, well above the usual for epigeal species, indicate a long period of evolution in the phreatic habitat. Bockmann & Castro (2010) stated that these autapomorphies could be functionally related, appearing in ‘packages’ due to paedomorphosis.

DIVERGENCE WITHIN *RHAMDIOPSIS KRUGI*:

DIFFERENTIATION OF (SEMI)ISOLATED POPULATIONS BASED ON GEOMETRIC MORPHOMETRIC ANALYSIS

Geometric morphometric analysis shows that there are significant differences in body shape between CQ, GN caves and the group formed by PE, LB and GM caves. Such differences may be due to the geographic isolation provided by geological and hydrological barriers. These barriers are represented by insoluble rocks (Irecê versus Una) and running waters (GN versus PE + LB + GM). Hydrological barriers have also been proposed to explain divergence between two molecular clades of troglobitic amblyopsid fish, *Amblyopsis spelaea* Dekay, 1842, separated by the Ohio River, USA (Niemiller *et al.*, 2013). Based on multilocus genetic data, up to 15 putative cryptic species were identified by Niemiller, Near & Fitzpatrick (2012) within the nominal species *Typhlichthys subterraneus* Girard, 1859 (Amblyopsidae), showing how the subterranean biodiversity may be underestimated.

The water bodies in which *R. krugi* lives inside PE, LB and GM caves belong to the same aquifer, since the variations in water level follow those observed in the Rio de Una (Karmann, Fraga & Mendes, 2002). The divergence between GM (broader body), GN and PE caves (shorter posterior segment of the body) may

be explained by underground isolation by depositional processes, since several connecting ducts are colmated by sediment (R. Fraga and F. Laureano pers. comm.).

Biomolecular analysis based on RAPD bands (Bichuette *et al.*, 2008a) mostly agreed with our results: a bootstrapped Maximum Likelihood tree using Contml and Seqboot (1000 iterations; Phylip 3.5) arbitrarily rooted with two heptapterid species (*P. kronei* and an epigean species of *Rhamdiopsis*) showed that individuals from the same basin (Una or Irecê) generally clustered together – Moreno Cave population clustered with Natal cave population and then Moreno/Natal cave populations clustered with PE and LB cave populations. Interestingly these populations are in the same limestones of Una Basin. These four clusters were quite different from Canoa Quebrada/Torrinha cave populations, caves located in the Irecê basin (Bichuette *et al.*, 2008a). This topology, similar to the obtained in our study (Fig. 6), corroborates the hypothesis of separation between populations from different sedimentary basins.

The differences detected among the five studied populations may be related to habitat characteristics selecting for different body shapes (Table 2, Fig. 7A, B). In fact, the main differences in shape were related to body width and relative proportion of body segments, with the repositioning of the pectoral and dorsal fins. We hypothesize that these characteristics are related to the stabilization of the body as an adaptation for the activity in the water column, more advanced in the PE cave (Fig. 7A) and less in CQ cave (Table 2, Fig. 7B), for example.

Genetic drift might also exert influence over this differentiation. Leinonen *et al.* (2006) analyzed body differences in marine and isolated lacustrine populations of threespine sticklebacks. These authors suggest that genetic drift played a major role in phenotypic divergence among lake populations, given that these are small-sized fish, and that natural selection may become ineffective in such cases.

It is important to stress that, as with any phenogram based on similarities like those generated by molecular data, the phenogram depicted in Figure 6 is not a cladogram (i.e., a hypothesis of phylogenetic relationships resulting from the application of the comparative method). For instance, the grouping of GMA and LB caves samples may be due to the retention of a plesiomorphic body shape in relation to specimens from PE cave. The huge lake in the latter cave is the most specialized among the habitats of *R. krugi* populations, requiring morphological and behavioural adaptations for swimming in spaces that are very large for these tiny fish that originated from a typical bottom-dweller living in lotic epigean habitats.



Figure 7. Habitat characteristics. (A) the large dimension lake of Poço Encantado Cave, Itaetê, Bahia state; one of the localities of *R. krugi* at Una-Utinga metasedimentary basin. (B) typical conduits at Canoa Quebrada Cave with thin plates of calcite in the surface; one of the localities of *R. krugi* at Irecê metasedimentary basin. Photographies: A. Gambarini.

Because no other population from Irecê basin was included in our analysis, one cannot state that the CQ cave population is basal in relation to the Una populations. Nevertheless, greater similarity to the epigean sample indicates that these catfish retained a more plesiomorphic body shape, with the set of Una populations presenting a more advanced condition. Further studies are needed, including additional populations from Irecê basin and a larger number of characters (e.g. myology, brain morphology). Additionally, cave localities to the north of Chapada Diamantina should be prospected in order to test the hypothesis of connection through the limestone outcrops of the Salitre Formation in the Iraquara, Souto Soares and Barra do Mendes regions.

Morphometric methods have been for the last half-century the basic toolkit for the study of organismal

shape and its covariation with size, time, environment, geography, ecology, development and other factors (MacLeod, 2008). Over the last 10 years the collection of techniques denominated geometric morphometrics, grounded in a strong theoretical and mathematical framework, has provided the ability to robustly interpret, through multivariate axes and deformation grids, the differences in shape between organisms. As noted by MacLeod (2008), it is surprising that both traditional (*sensu* Marcus, 1990) and geometric morphometrics never explored in an appropriate way the correlation between form and taxonomy, even though the recognition of taxonomic entities is strongly based on morphology, while the use of molecular approaches yield limited results in some cases. This, combined with the need to develop new methods of identifying taxonomic entities, and also taking into account the taxonomic impediment (de Carvalho *et al.*, 2007, 2008), suggests that morphometric approaches may play an important role, not yet properly explored in systematics.

CONSERVATION RECOMMENDATIONS

Because the distributional range of *R. krugi* is located outside protected areas such as the Chapada Diamantina National Park, these populations are not protected by law. Therefore, they are subject to local environmental impacts, including: habitat conversion due mainly to limestone exploration and garbage and debris accumulation (due to low water circulation; Trajano & Bichuette, 2006); uncontrolled exploration of the subterranean waters in that region; pollution of the aquifers by pesticides and poorly regulated speleotourism. The maintenance of genetic diversity at all levels is essential for the preservation of biodiversity; hence conservation policies should be directed at each of the *R. krugi* populations because, as we have shown, they differ significantly in morphology. However, conservation regulations are extremely bureaucratic and only formally named species may be included in Brazilian Red Lists. Consequently, instead of being concerned with protecting real biological units, the environmental authorities only focus on strict taxonomic determinations. Because the taxonomic impediment is especially serious in countries with mega-diversity, many biological units in Brazil are extinct before they can be officially protected, with considerable loss of such diversity. Therefore, for conservation purposes, we propose a referral to the two sets of differentiated populations of *R. krugi* as *R. krugi* 'Una morphotype' and *R. krugi* 'Irecê morphotype'. We also recommend special attention to each population, whose protection should be prioritized.

CONCLUSIONS

Our study, based on a geometric morphometric approach, uncovered robust evidence of isolation among the monophyletic subterranean catfish species, *R. krugi*. Geological and hydrological barriers explain the differences among the subterranean populations. A parapatric model is proposed, with a hypothesis of vertical colonization of the subterranean environment through the hyporheic zone. These barriers are represented, respectively, by insoluble rocks (Irecê versus Una) and running waters (GN versus PE + LB + GM). The high degree of morphological, ecological and behavioural specialization of *R. krugi*, characterized by an elevated number of morphological autapomorphies, indicates a long period of evolution in the phreatic habitat – more than 10 Myr. The correlation between form and taxonomy, our focus herein, shows that morphometric approaches may play an important role in systematics, and should be widely applied in many evolutionary studies. Considering conservation strategies, we must protect the two differentiated sets of *R. krugi* populations, referred to as the *R. krugi* 'Una morphotype' and *R. krugi* 'Irecê morphotype'.

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