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# Species limits and DNA barcodes in *Nematolebias*, a genus of seasonal killifishes threatened with extinction from the Atlantic Forest of south-eastern Brazil, with description of a new species (Teleostei: Rivulidae)

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*Nematolebias*, a genus of killifishes uniquely living in temporary pools of south-eastern Brazil, contains two nominal species, *N. whitei*, a popular aquarium fish, and *N. papilliferus*, both threatened with extinction and presently distinguishable by male colour patterns. Species limits previously established on the basis of morphological characters were tested using mt-DNA sequences comprising fragments of the mitochondrial genes cytochrome b and cytochrome c oxidase I, taken from 23 specimens representing six populations along the whole geographical distribution of the genus. The analysis supports the recognition of a third species, *N. catimbau*, new species, from the Saquarema lagoon basin, as an exclusive lineage sister to *N. papilliferus*, from the Maricá lagoon basin, and *N. whitei*, from the area encompassing the Araruama lagoon and lower São João river basins, as a basal lineage. The new species is distinguished from congeners by the colour pattern and the relative position of pelvic-fin base, besides 11 unique nucleotide substitutions. The distribution pattern derived from sister taxa inhabiting the Saquarema and Maricá basins is corroborated by a clade of the seasonal genus *Notholebias*, suggesting a common biogeographical history for the two genera.

## Introduction

Possibly the greatest present challenge for taxonomists is to catalogue the poorly known species diversity of tropical areas under intense process of environmental degradation (Brook et al., 2006; Costa et al., 2012). For example, the Atlantic Forest of eastern Brazil, the second largest forest of South America and one of the richest biodiversity centres in the world (Myers et al., 2000), concentrates a high number of species threatened with extinction (Tabarelli et al., 2005), many of them still poorly known. Aplocheiloid killifishes of the Neotropical family Rivulidae are particularly diversified in the Atlantic Forest, where they are represented by eight endemic genera and more than 40 endemic species (Costa, 2008, 2009, 2010). Most killifishes endemic to this biome are seasonal organisms, uniquely living in shallow temporary pools formed during the rainy seasons

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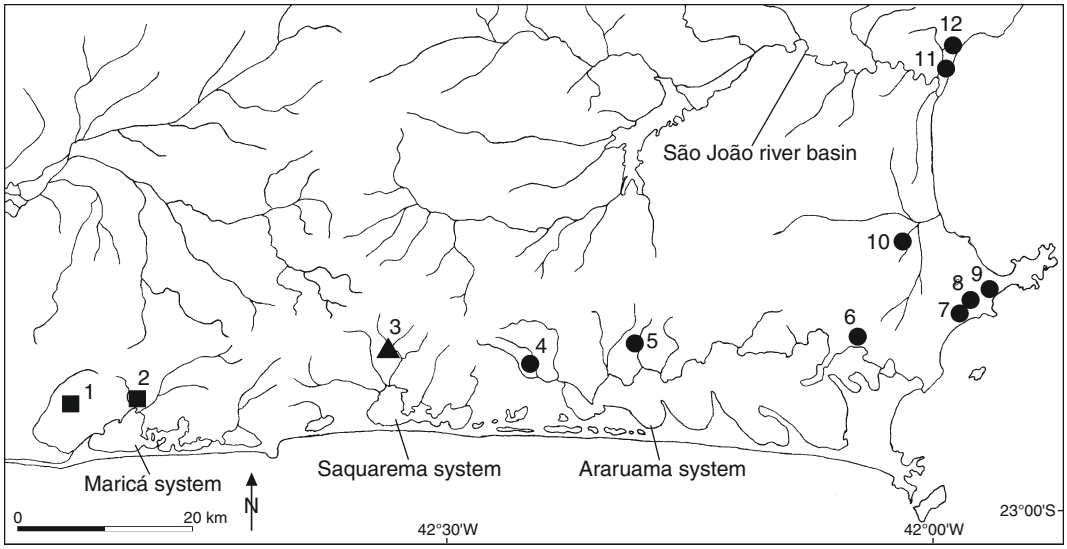


**Fig. 1.** Geographical distribution of *Nematolebias*: ●, *N. whitei*; ■, *N. papilliferus*; ▲, *N. catimbau*. Numbers indicate populations used in the analyses: **1**, Inoã; **2**, Maricá; **3**, Sampaio Correia; **4**, Bonsucesso; **5**, São Pedro da Aldeia; **6**, Caravelas; **7**, Botafogo; **8**, Barra de São João.

(Myers, 1942; Costa, 2002a, 2009), besides being restricted to small geographical areas and standing among the most endangered vertebrates of South America (Costa, 2002b, 2012).

*Nematolebias* Costa, 1998 is a genus of seasonal killifishes endemic to the Atlantic Forest of the coastal plains of Rio de Janeiro state, south-eastern Brazil (Costa, 2002a). This region formerly comprised dense rain forests and broad swampy areas (Wied-Neuwied, 1820), but since the beginning of the 20th century it has been mainly occupied by open vegetation formations used as pasture for cattle, and more recently by a quick expansion of coastal urban centres. As a consequence of habitat loss, endemic killifish species are severely threatened with extinction (Costa, 2009, 2012).

*Nematolebias* has been considered the sister group to a speciose clade containing all other taxa of the tribe Cynolebiasini, easily diagnosed by the presence of hypertrophied papillae on the pectoral fin in males and the presence of a broad sub-distal orange stripe with overlapped golden lines on the anal fin in males (Costa, 2002a, 2006, 2010). Costa (2002a) revised *Nematolebias*, recognising two cryptic species (sensu Bickford et al., 2007), *N. whitei* (Myers, 1942), a popular aquarium fish and known from some populations in a long geographical area between the São João river basin and the Araruama lagoon basin, and *N. papilliferus* Costa, 2002, from two populations from the Maricá lagoon basin, and a single population from the Saquarema lagoon basin (Fig. 1). Both species were distinguished by characters of male colour patterns, including the presence of golden lines on the dorsal fin which is present in *N. papilliferus* but absent in *N. whitei*. More recent field work has revealed that the populations of *N. papilliferus* from the Maricá basin, including the type locality of the species, exhibit colour pattern slightly distinct from populations inhabiting the Saquarema basin, making clear the necessity of adding more data to test species limits. Thus, the objective of the present study is to combine revised data of morphology at the population level with mitochondrial DNA sequences obtained from six populations representing the whole geographic range of the genus.

## Material and methods

**Morphology.** Data on colour patterns were primarily based both on direct examination of live specimens during collections, and photographs of both sides of live individuals, at least five males and two females for each population, taken in aquaria between about 4 and 12 hours after col-

lection, between 1994 and 2012. Other morphological characters used in species description were obtained from specimens fixed in formalin just after collection, for a period of 10 days, and then transferred to 70 % ethanol. Material is deposited in the following institutions: BMNH, Natural History Museum, London; UFRJ, Instituto de Biologia, Universidade Federal do Rio de Janeiro; and ZFMK, Zoologisches Forschungsmuseum und Museum Alexander Koenig, Bonn. Morphometric and meristic data were taken following Costa (1995); measurements are presented as percent of standard length (SL), except for those related to head morphology, which are expressed as percent of head length. Fin-ray counts include all elements. Number of vertebrae and gill-rakers were recorded from cleared and stained specimens (c&s) prepared according to Taylor & Van Dyke (1985). Terminology for frontal squamation follows Hoedeman (1958) and for cephalic neuromast series Costa (2001).

**DNA extraction, amplification and sequencing.** Specimens were fixed in absolute ethanol immediately after collection and later preserved in the same solution; see Appendix 1 for list of specimens and respective GenBank accession numbers. Total genomic DNA was extracted from muscle tissue of the caudal peduncle using the DNeasy Blood & Tissue Kit (Qiagen), following manufacturer instructions. To amplify the fragments of the mitochondrial DNA were used the primers Cox1F and COIrev (Costa & Amorim, 2011; Sonnenberg et al., 2007) for the mitochondrial gene cytochrome c oxidase I (cox1) and primers L14724 and H15149 (Kocher et al., 1989; Meyer et al., 1990), for the mitochondrial gene cytochrome b (cytb). Polymerase chain reaction (PCR) was performed in 15 µl reaction mixtures containing 5 × Green GoTaq Reaction Buffer (Promega), 3.6 mM $MgCl_2$, 1 µM of each primer, 75 ng of total genomic DNA, 0.2 mM of each dNTP and 1U of Taq polymerase. The thermocycling profile was: (1) 1 cycle of 4–5 minutes at 94 °C; (2) 35 cycles of 1 minute at 92 °C, 1 minute at 48–54 °C and 1 minute at 72 °C; and (3) 1 cycle of 4 minutes at 72 °C. In all PCR reactions, negative controls without DNA were used to check contaminations. Amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing reactions were made using the BigDye Terminator Cycle Sequencing Mix (Applied Biosystems). Cycle sequencing reactions were performed in 10 µl reaction volumes containing 1 µl BigDye 2.5, 1.55 µl 5 × sequencing buffer (Applied Biosystems), 2 µl of the amplified products (10–40 ng), and 2 µl primer. The thermocycling profile was: (1) 35 cycles of 10 seconds at 96 °C, 5 seconds at 54 °C and 4 minutes at 60 °C. The sequencing reactions were purified and denatured and the samples were run on an ABI 3130 Genetic Analyzer. Sequences were edited using MEGA 5 (Tamura et al., 2011) and subsequently adjusted manually (total of 1130 bp).

**Species concept, species delimitation and diagnoses.** The unified species concept (de Queiroz, 2007) is herein adopted by expressing the conceptual definition shared by all traditional species concepts (i. e. species are lineages united through gene flow) when operational criterion elements to delimit taxa are excluded from concepts. Any of those criteria may separately provide evidence about species limits independently from the other criteria (de Queiroz, 2007), but evidence extracted from multiple operational criteria is considered to produce stronger hypotheses of lineage separation (de Queiroz, 2007), thus congruent to the proposal for an integrative taxonomy (Goldstein & DeSalle, 2010; Padial et al., 2010). Species are herein recognised when their limits are concordantly supported by three different operational criteria, two character-based and one tree-based (sensu Baum & Donoghue, 1995; Sites & Marshall, 2004), as described below.

The first method to delimit species used in this study was the Population Aggregation Analysis (Davis & Nixon, 1992), a character-based method (hereafter PPA, following Sites & Marshall, 2003), in which species are delimited by unique combination of morphological character states occurring in one or more populations. The analysis focused in diagnostic character states used by Costa (2002a), besides checking other characters commonly employed in killifish Systematics (e. g., Costa, 2006). Populations were seasonal pools or groups of neighbouring pools geographically isolated from other pools. PPA was applied to populations well represented in collections, with broad photo record and covering the whole geographical distribution of the genus, named according to the nearest locality: Barra de São João, Caravelas, Botafogo (type locality of *N. whitei*), São Pedro da Aldeia, Bonsucesso, Sampaio Correia, Maricá, Inoã (type locality of

*N. papilliferus*). Characters statements were formulated according to Sereno (2007).

The second method, proposed by DeSalle et al. (2005), is known as character-based DNA barcoding (e.g., Bergmann et al., 2009; hereafter CBB). It is similar to PAA, but directed to nucleotides as an alternative method for diagnosing taxa through DNA barcodes, since the original method was based on trees derived from the phenetic neighbour-joining algorithm and arbitrary genetic distance-based cutoffs (Hebert et al., 2005), which have been demonstrated to be inconsistent both in theoretical and practical aspects (e.g., DeSalle et al., 2005; Brower, 2006; Meier et al., 2006). According to this method, a unique combination of nucleotides of a site shared by different individuals of a same population or a group of populations supports species delimitation. This analysis included both cox1 and cytb sequences of 23 individuals representing the same populations used in PPA, except Rio das Ostras, São Pedro da Aldeia and Maricá, which were extinct in recent years. In addition, species were also diagnosed by unique nucleotide substitutions (hereafter UNS) shared by all analysed specimens (see 'diagnostic DNA-barcode loci' below). Optimization of nucleotide substitutions among lineages of *Nematolebias* were obtained from the MP tree described below, using TNT 1.1. Each unique substitution is represented by its relative numeric position determined through sequence alignment with the complete mitochondrial genome of *Kryptolebias marmoratus* (Poey, 1880) (Lee et al., 2001), followed by the specific nucleotide substitution in parentheses.

The third method used here was a tree-based method as proposed by Wiens & Penkrot (2002) (hereafter WP, following Sites & Marshall, 2003), which is based on the direct inspection of DNA trees generated from the phylogenetic analyses having as terminals at least two individuals of each focal species. When analysing tree topology, the term 'exclusive' is used instead of monophyletic, since the term monophyly is considered not applicable below the species level (e.g., de Queiroz & Donoghue, 1990). Clustered terminals with concordant geographic distribution forming mutual, well supported clades (exclusive lineages) are considered strong evidence for distinct species (absence of genetic flow with other terminal taxa), whereas failure of haplotypes from the same population to cluster together is considered as potential evidence for gene flow with other populations (Wiens & Penkrot, 2002; Sites & Marshall, 2003). Statistical support for clade is assessed by bootstrapping (Felsenstein, 1985), considering bootstrap values equal or higher 70 % as significant (Hillis & Bull, 1993). Terminal in-group taxa were the same described for the second method. Terminal out-group taxa were four species of other cynolebiasine genera, *Xenurolebias izecksohni* (Cruz), *Xenurolebias* cf. *myersi* (Carvalho), *Hypsolebias janaubensis* (Costa) and *Notholebias minimus* (Myers), and a basal rivulid taxon, *Kryptolebias marmoratus* (Poey). Phylogenetic analyses comprised both maximum parsimony (MP) and maximum likelihood (ML) methods. MP was performed with TNT 1.1 (Goloboff et al., 2008), using the 'traditional' search and setting random taxon-addition replicates to 10, tree bisection-reconnection branch swapping, multitrees in effect, collapsing branches of zero-length, characters equally weighted, and a maximum of 100 000 trees saved in each replicate. Branch support of the MP tree was assessed by bootstrap analysis, using a heuristic search with 1000 replicates and the same settings used in the MP search, but saving a maximum of 1000 trees in each random taxon-addition replicate. ML was run in MEGA 5, under the best nucleotide substitution model previously determined by MEGA; the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) was indicated as the best-fit model of sequence evolution. The ML analysis was performed with random-starting parameters and using a random-starting tree; branch support was calculated with 1000 nonparametric bootstrap replicates using the same settings.

## Results

**PAA.** This analysis provided five informative characters, as listed below.

1. Flank in males, golden dots: (0) isolated in Barra de São João, Bonsucesso, Botafogo, Caravelas, Iguaba, São Pedro da Aldeia, and Tucuns populations, (1) connected by narrow light lines in Inoã and Maricá populations; both character states were recorded in Sampaio Correia population.
2. Dorsal fin in males, distal portion, golden marks: (0) rounded or slightly elongated small spots in Barra de São João, Bonsucesso, Botafogo, Caravelas, Iguaba, São Pedro da Aldeia, and Tucuns populations, (1) narrow

long lines in Inoã, Maricá, and Sampaio Correia populations.

3. Caudal fin in males, distal margin, distinctive row of white to light blue small spots: (0) absent in Inoã and Maricá populations, (1) present in Barra de São João, Bonsucesso, Botafogo, Caravelas, Iguaba, Sampaio Correia, São Pedro da Aldeia, and Tucuns populations.
4. Caudal fin in males, postero-dorsal portion, golden to metallic blue lines: (0) absent in Barra de São João, Bonsucesso, Botafogo, Caravelas, Iguaba, São Pedro da Aldeia, and Tucuns populations, (1) present in Inoã and Maricá populations; both character states were recorded in Sampaio Correia population.
5. Pelvic fins, medial position: (0) separated by a small interspace in Sampaio Correia population, (1) medially in contact in Barra de São João, Bonsucesso, Botafogo, Caravelas, Iguaba, Inoã, Maricá, Sampaio Correia, São Pedro da Aldeia, and Tucuns populations.

The distribution of the character states supports three species. The first one comprises the Inoã population, the type locality of *N. papilliferus*, and the Maricá population, differing from other congeners by the absence of a distinctive row of white to light blue small spots on the distal margin of the caudal fin in males (vs. presence).

The analyses of morphological characters did not provide informative variability to distinguish populations occurring between the Araruama lagoon and São João river basins, including the Barra de São João, Bonsucesso, Caravelas, Iguaba, and Tucuns populations, as well as the Botafogo population, the type locality of *N. whitei*, and the São Pedro da Aldeia, the type locality of *Pterolebias elegans* Ladiges, 1958, a synonym of *N. whitei* (Costa, 2002a). On the other hand, these populations together are morphologically diagnosable by the presence of rounded or slightly elongated small spots on the distal portion of the dorsal fin in males (vs. narrow long golden lines in the remaining populations).

PPA supports a third species corresponding to the Sampaio Correia population, for which no name is available. Individuals of this population combines light blue small spots on the distal margin of the caudal fin in males, absent in *N. papilliferus*, with narrow long golden lines on the dorsal fin in males, absent in *N. whitei*, besides having the pelvic-fin bases medially separated by a small interspace (vs. in contact in *N. papilliferus* and *N. whitei*).

The presence of golden dots vertically connected by narrow light lines on the flank in males (vs. isolated golden dots) and golden to metallic blue lines on the postero-dorsal portion of the caudal fin in males (vs. lines absent) are also useful to distinguish *N. papilliferus* and *N. whitei*, but do not distinguish these species from the third one, in which both characters are polymorphic.

**CBB.** *Nematolebias papilliferus*, as delimited by PAA above, is supported by the genetic variation found in individuals from two close localities in Inoã alone, since the Maricá population is presently extinct and thus no specimen from this population was sampled for genetic characters. The Inoã population is diagnosed by seven nucleotides: cox1.555 (C vs. T), cox1.591 (T vs. C), cox1.630 (A vs. T), cytb.41 (C vs. T), cytb.56 (G vs. A), cytb.222 (T vs. C), cytb.374 (G vs. A).

Similarly, *N. whitei* as delimited by PAA above is supported by the genetic variation found in individuals collected in the Barra de São João, Bonsucesso, Botafogo and Caravelas, since, according to recent field studies, Iguaba, São Pedro da Aldeia, and Tucuns populations seem to be extinct. *Nematolebias whitei* so delimited is diagnosable by 15 nucleotides: cox1.66 (C vs. T), cox1.106 (T vs. C), cox1.180 (A vs. G), cox1.231 (C vs. T), cox1.336 (G vs. A), cox1.387 (T vs. C), cox1.594 (C vs. T), cox1.618 (T vs. C), cox1.643 (C vs. T), cox1.702 (A vs. G), cytb.3 (G vs. A), cytb.68 (A vs. G), cytb.119 (C vs. T), cytb.170 (C vs. A), cytb.218 (C vs. T).

The third, still unnamed species indicated by PPA, corresponding to the Sampaio Correia population, is highly corroborated by CBB. It is diagnosable by eleven nucleotides: cox1.249 (T vs. C), cox1.252 (T vs. C), cox1.315 (T vs. C), cox1.351 (T vs. C), cox1.486 (C vs. T), cox1.516 (A vs. G), cox1.561 (T vs. C), cytb.167 (T vs. C), cytb.212 (A vs. G), cytb.221 (T vs. C), cytb.344 (A vs. G).

**WP.** The three species supported by PAA and corroborated by CBB, were also corroborated by the WP tree-based approach using the ML analysis (Fig. 2). Both *N. papilliferus* and the unnamed species appear as exclusive lineages supported by high bootstrap values (100 %). These two lineages form a well-corroborated clade, the sister group of *N. whitei*, which forms a broad exclusive lineage with high bootstrap value (97 %). The MP analysis, in which 701 characters were constant,

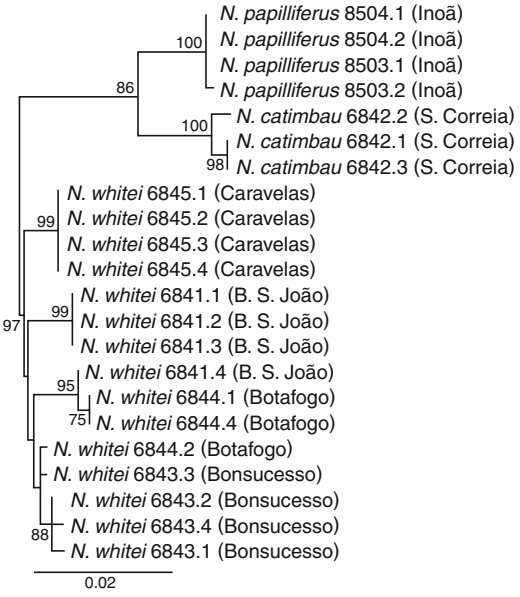


**Fig. 2.** Maximum Likelihood tree of phylogenetic relationships among species of *Nematolebias* inferred by using sequences of mitochondrial genes cytochrome c oxidase I and cytochrome b, total of 1130 positions. Out-groups not represented. Terminals include catalog numbers followed by nearest toponymy for population collecting site. Numbers are bootstrap values above 50 %.

140 variable but parsimony-uninformative, and 289 parsimony-informative, generated nine equally most parsimonious trees (not depicted; total length 741, consistency index 0.7800, retention index 0.7866, rescaled consistency index 0.6136). The consensus tree for the MP analysis was similar to the tree obtained from the ML analysis (Fig. 2), but *N. whitei* appeared as a basal non-exclusive lineage, as defined by Wiens & Penkrot (2002), with populations forming a polytomy. The unnamed species delimited by the different approaches is described below.

### *Nematolebias catimbau*, new species
(Figs. 3–4)

**Holotype.** UFRJ 8888, male, 45.7 mm SL; Brazil: Estado do Rio de Janeiro: Município de Saquarema: temporary pool in Catimbau river floodplain, about 1.5 km S of road RJ-106, Saquarema lagoon system, 22°51'53"S 42°33'15"W; W. J. E. M. Costa, P. F. Amorim, G. Aranha & F. Pereira, 12 July 2012.

**Paratypes.** All localities in Brazil: Estado do Rio de Janeiro: Município de Saquarema: Catimbau river floodplain, Saquarema lagoon system: UFRJ 5364, 27 males, 20.8–36.7 mm SL, 38 females, 16.9–34.4 mm SL; UFRJ 5365, 27.3–36.7 mm SL, 5 females, 23.4–26.8 mm SL (c&s); UFRJ 8893, 3 males, 26.2–30.3 mm SL, 3 females, 23.5–24.5 mm SL (c&s); temporary pool close to road RJ-106, 1.7 km from village of Sampaio Correia, 22°51'19"S 42°34'10"W; W. J. E. M. Costa, 1 June 2001. – UFRJ 6842, 3 females, 30.2–30.7 mm SL; same locality as UFRJ 5364; W. J. E. M. Costa et al., 24 June 2010. – UFRJ 8891, 3 males, 32.9–36.4 mm SL, 1 females, 24.1 mm SL; same locality as UFRJ 5364; W. J. E. M. Costa et al., 12 July 2012. UFRJ 8892, 1 male, 29.4 mm SL, 1 female, 24.1 mm SL; temporary canal close to road RJ-106, about 100 m E from locality of UFRJ 5364, 22°51'19"S 42°33'51"W; W. J. E. M. Costa et al., 12 July 2012. – UFRJ 8889, 1 male, 42.3 mm SL, 1 female, 33.6 mm SL; UFRJ 8890, 4 males, 36.9–43.8 mm SL, 7 females, 28.8–32.3 mm SL; BMHN 2013.6.23.13–14, 1 male, 44.8 mm SL, 1 female, 31.7 mm SL; ZFMK 56341-42, 1 male, 42.3 mm SL, 1 female, 28.8 mm SL; collected with holotype.

**Diagnostic morphological character states.** *Nematolebias catimbau* is similar to *N. papilliferus* and distinguished from *N. whitei* by the presence of long narrow golden lines on the distal portion of the dorsal fin in males (vs. dots); it is distinguished from *N. papilliferus* in possessing a distinctive row of small iridescent spots on the distal margin of the caudal fin in males (vs. row absent). It is distinguished both from *N. papilliferus* and *N. whitei* by having the pelvic-fin bases medially separated by a small interspace (vs. in contact).

**Diagnostic DNA-barcode loci.** *Nematolebias catimbau* is distinguished from all congeners by the following eleven UNS: cox1.249 (C > T), cox1.252 (C > T), cox1.315 (C > T), cox1.351 (C > T), cox1.486 (T > C), cox1.516 (G > A), cox1.561 (C > T), cytb.167 (C > T), cytb.212 (G > A), cytb.221 (C > T), cytb.344 (G > A). It is similar to *N. papilliferus*, with which it shares the following eleven UNS: cox1.66 (C > T), cox1.106 (T > C), cox1.180 (A > G), cox1.231 (C > T), cox1.594 (C > T), cox1.618 (T > C), cox1.656 (A > G), cytb.3 (G > A), cytb.68 (A > G), cytb.119 (C > T), cytb.170 (C > A). It is distinguished from *N. papilliferus* by the latter having the following seven UNS: cox1.555 (T > C), cox1.591 (C > T), cox1.630 (T > A), cytb.41 (T > C), cytb.56 (A > G), cytb.222


**Fig. 3.** Live individuals of *Nematolebias catimbau*, Brazil: Rio de Janeiro: Sampaio Correia. **a,** UFRJ 8888, holotype, male, 45.7 mm SL; **b,** UFRJ 8889, paratype, female, 33.6 mm SL.

(C > T), cytb.374 (A > G), and from *N. whitei* by the latter having the following four UNS: cox1.336 (A > G), cox1.387 (C > T), cox1.643 (T > C), cytb.218 (T > C).

**Description.** Morphometric data are in Table 1. Largest male examined 45.7 mm SL; largest female examined 33.6 mm SL. Dorsal and ventral profiles gently convex from snout to end of dorsal and anal-fin bases, nearly straight on caudal peduncle. Body slender, subcylindrical anteriorly, slightly deeper than wide, to compressed posteriorly; greatest body depth at level of pelvic-fin base. Eye small, positioned on dorsolateral portion of head side. Snout short. Vomerine teeth 6–7.

Extremity of dorsal and anal fins pointed in males, rounded in females; single filamentous ray on tip of dorsal fin reaching vertical through posterior portion of caudal-fin base. Caudal fin rounded. Pectoral fin long, elliptical, posterior margin in vertical through base of 7th or 8th anal-fin ray in males, between anus and urogenital papilla in females. Tip of pelvic fin reaching base of 3rd or 4th anal-fin ray in males, reaching urogenital papilla in females. Pelvic-fin bases medially separated by small interspace about one

fourth of pelvic-fin width. Dorsal-fin origin through vertical between base of 8th and 10th anal-fin rays in both sexes. Dorsal-fin rays 16–18 in males, 13–15 in females; anal-fin rays 23–24 in males, 20–22 in females; caudal-fin rays 28–30 in males, 27–28 in females; pectoral-fin rays 14–15 in males, 13–14 in females; pelvic-fin rays 6 in both sexes. Total vertebrae 31–32.

Frontal squamation E-patterned. Longitudinal series of scales 29–31; transverse series of scales 7–8; scale rows around caudal peduncle 16. No contact organs on scales. Papillate contact organs on first seven rays of pectoral fin in males. Cephalic neuromasts: supraorbital 15–17, parietal 1–2, anterior rostral 1, posterior rostral 1, infraorbital 1–2 + 21–22, preorbital 3, otic 3, post-otic 3–4, supratemporal 1, median opercular 1, ventral opercular 4–6, preopercular plus mandibular 35–40, lateral mandibular 7–8, paramandibular 1. One neuromast on each scale of lateral line. Two neuromasts on caudal-fin base.

**Colouration.** Males. Side of body light pinkish brown with vertical rows of golden dots, often in close proximity or united on abdominal region and caudal peduncle to form narrow bars. Dorsum pale brown. Venter pale pink. Opercular and infraorbital region light greenish golden with dark reddish brown bars. Iris light yellow with dark reddish brown bar on middle part. Dorsal reddish brown, with golden lines on membrane between rays on distal two thirds of fin and dots on basal portion. Anal fin reddish brown with golden dots on basal region; sub-marginal orange band overlapped by series of transverse golden to metallic blue lines often interconnected by ventral extensions; dark grey to black zone dorsally adjacent to anterior part of sub-distal orange band; distal margin dark red. Caudal fin reddish brown with light blue dots on middle and dorsal portion, gradually larger dorsally; series of united light blue small spots close to postero-dorsal and middle margins of fin. Pectoral fin reddish hyaline with golden dots and short lines. Pelvic fin reddish brown with golden short lines.

Females. Side of body light brown, with 8–12 dark grey bars; 1–3 black spots on anterocentral portion of flank, 1–4 on posterior portion of caudal peduncle. Dorsum pale brown. Venter light grey. Opercular region pale green. Iris light yellow, with dark brownish grey bar. Unpaired fins hyaline with dark brownish grey small spots; anterodistal part of anal fin pale blue, spots pinkish brown. Paired fins hyaline.

**Distribution and ecology notes.** *Nematolebias catimbau* is known only from the floodplains of a small river draining into the Saquarema lagoon (Fig. 1), the Catimbau river, which is crossed by the road RJ-106 at its middle section. It was not

**Table 1.** Morphometric data of *Nematolebias catimbau*.

| | holotype | paratypes | |
|---|---|---|---|
| | male | males (10) | females (8) |
| Standard length (mm) | 45.7 | 34.1–44.8 | 30.4–33.6 |
| **Percent of standard length** | | | |
| Body depth | 27.0 | 25.4–28.6 | 26.3–28.2 |
| Caudal peduncle depth | 16.1 | 13.8–16.2 | 13.0–14.5 |
| Predorsal length | 57.1 | 56.5–60.9 | 65.5–67.8 |
| Prepelvic length | 42.9 | 42.0–47.0 | 50.0–54.1 |
| Length of dorsal-fin base | 28.1 | 25.4–29.6 | 17.6–20.3 |
| Length of anal-fin base | 39.0 | 36.4–39.6 | 24.6–27.7 |
| Caudal-fin length | 42.0 | 42.2–44.9 | 40.4–44.6 |
| Pectoral-fin length | 27.3 | 26.6–28.7 | 24.0–27.6 |
| Pelvic-fin length | 9.0 | 8.2–9.6 | 8.8–10.2 |
| Head length | 26.0 | 25.7–27.9 | 28.7–30.2 |
| **Percent of head length** | | | |
| Head depth | 93 | 86–94 | 78–82 |
| Head width | 80 | 76–82 | 76–85 |
| Snout length | 16 | 14–17 | 14–16 |
| Lower jaw length | 26 | 22–26 | 19–23 |
| Eye diameter | 25 | 25–30 | 26–30 |

found in other drainages connected to the Saquarema lagoon, including the Mato Grosso river, the largest of the Saquarema lagoon basin. *Nematolebias catimbau* is always found in shallow temporary pools, about 30–50 cm deep, formed during the rainy seasons, usually between March and May, and between October and December. All the pools were in open vegetation area. Pools adjacent to the road RJ-106 have lost original vegetation in recent years and were in part drained, whereas the whole region north to that road have been drained for agriculture and the original vegetation substituted by plantations, not resting temporary pools. During field studies conducted in July 2012, we found the species in pools between 1 and 2.5 km south of that road. It is estimated that the area occupied by the species in 2002 was about 15 km$^2$, but in July 2012, it had been reduced to about 5 km$^2$.

**Etymology.** The name *catimbau* has its origin in the Tupi-Guarani language and is an allusion to the occurrence of the species in the floodplains of the Catimbau river.

## Discussion

The integration of morphology and sequences of mt-DNA supports recognition of three species in *Nematolebias*. This delimitation is consistent with their geographic distribution: *N. papilliferus* is endemic to the Maricá lagoon system, occupying the western-most part of the geographic distribution of the genus; *N. catimbau* is endemic to the Saquarema lagoon system, and *N. whitei* occurs in a broader eastern area between the São João river and the whole Araruama lagoon system (Fig. 1). All these three areas are physically separated by mountain ranges, including the Mato Grosso range, between the Maricá and Saquarema lagoon systems, with altitudes between 100 and 890 m, and a small unnamed mountain range, about 50–330 m of altitude, between the Saquarema and Araruama lagoon systems, whereas the area between the Araruama lagoon system and the lower São João river basin are connected by broad plain areas just above the sea level. The sister group relationship between *N. papilliferus* and *N. catimbau* supported in the phylogenetic analyses (Fig. 2) indicates a biogeographical pattern involving the Maricá and Saquarema lagoon systems, which is corroborated by a sister species pair of another seasonal killifish genus. *Notholebias fractifasciatus* Costa, endemic to the Maricá system is considered the sister group of *N. vermiculatus* Costa & Amorim, endemic to the Saquarema lagoon system (Costa & Amorim, 2013).

A popular biological identification programme, known as DNA barcoding (Hebert et al., 2003), has been proposed on the basis of the universal use of a small fragment of the cytochrome oxidase subunit 1 gene (cox1) of mitochondrial DNA. The choice of cox1 as a universal molecular tool for biological identification was justified by it combining a rare occurrence of insertions and deletions, availability of robust universal primers, great range of phylogenetic signal and its broad applicability among animal taxa (Hebert et al., 2003). However, among the several criticisms against such a proposal, particularly the use of that fragment has been seen with scepticism by some researchers (e. g., Hurst & Jiggins, 2003; Meier et al., 2006), besides subsequent problems recorded in obtaining cox1 barcodes for some animal groups (e. g., Vences et al., 2005; Whitworth et al., 2007). For *Nematolebias*, the present study indicate that both a fragment of cox1 and cytb are informative to diagnose and to identify species consistently to the results independently provided by morphological characters.

Continuous field studies during the last 18 years have shown that seasonal killifishes from the coastal plains of the Rio de Janeiro state are severely threatened with extinction (Costa, 2002a, 2009). Most recent studies concluded that *N. whitei* is an endangered species and *N. papilliferus* is on the edge of survival, with habitat loss reaching over 95 % (Costa, 2012). The present study indicates that species diversity in the genus *Nematolebias* is greater than supposed before, detecting the occurrence of a new cryptic species, *N. catimbau*, endemic to a small geographically isolated area, which is under recent and impacting process of habitat loss, making it endangered.

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**Appendix 1.** List of specimens, and respective catalogue numbers, localities, coordinates and GenBank accession numbers

| | catalog number | locality | | GenBank | |
|---|---|---|---|---|---|
| In-groups | | | | | |
| *Nematolebias catimbau* | UFRJ 6842.1 | Sampaio Correia | 22°51'53"S 42°33'15"W | KF311334 | KF311310 |
| | UFRJ 6842.2 | Sampaio Correia | 22°51'53"S 42°33'15"W | KF311335 | KF311311 |
| | UFRJ 6842.3 | Sampaio Correia | 22°51'53"S 42°33'15"W | KF311336 | KF311312 |
| *Nematolebias papilliferus* | UFRJ 8503.1 | Inoã | 22°55'21"S 42°55'42"W | KF311337 | KF311313 |
| | UFRJ 8503.2 | Inoã | 22°55'21"S 42°55'42"W | KF311338 | KF311314 |
| | UFRJ 8504.1 | Inoã | 22°55'22"S 42°55'55"W | KF311339 | KF311315 |
| | UFRJ 8504.2 | Inoã | 22°55'22"S 42°55'55"W | KF311340 | KF311316 |
| *Nematolebias whitei* | UFRJ 6845.1 | Caravelas | 22°48'10"S 41°57'50"W | KF311341 | KF311317 |
| | UFRJ 6845.2 | Caravelas | 22°48'10"S 41°57'50"W | KF311342 | KF311318 |
| | UFRJ 6845.3 | Caravelas | 22°48'10"S 41°57'50"W | KF311343 | KF311319 |
| | UFRJ 6845.4 | Caravelas | 22°48'10"S 41°57'50"W | KF311344 | KF311320 |
| | UFRJ 6844.1 | Botafogo | 22°43'59"S 42°02'29"W | KC990496 | KF311321 |
| | UFRJ 6844.2 | Botafogo | 22°43'59"S 42°02'29"W | KF311345 | KF311322 |
| | UFRJ 6844.3 | Botafogo | 22°43'59"S 42°02'29"W | KF311346 | KF311323 |
| | UFRJ 6844.4 | Botafogo | 22°43'59"S 42°02'29"W | KF311347 | KF311324 |
| | UFRJ 6843.1 | Bonsucesso | 22°52'31"S 42°25'30"W | KF311348 | KF311325 |
| | UFRJ 6843.2 | Bonsucesso | 22°52'31"S 42°25'30"W | KF311349 | KF311326 |
| | UFRJ 6843.3 | Bonsucesso | 22°52'31"S 42°25'30"W | KF311350 | KF311327 |
| | UFRJ 6843.4 | Bonsucesso | 22°52'31"S 42°25'30"W | KF311351 | KF311328 |
| | UFRJ 6841.1 | Barra de São João | 22°34'34"S 41°59'10"W | KF311352 | KF311329 |
| | UFRJ 6841.2 | Barra de São João | 22°34'34"S 41°59'10"W | KF311353 | KF311330 |
| | UFRJ 6841.3 | Barra de São João | 22°34'34"S 41°59'10"W | KF311354 | KF311331 |
| | UFRJ 6841.4 | Barra de São João | 22°34'34"S 41°59'10"W | KF311355 | KF311332 |
| Out-groups | | | | | |
| *Hypsolebias janaubensis* | UFRJ 6787.3 | Janaúba | 15°47'57"S 43°19'18"W | HQ833489 | JQ612772 |
| *Notholebias minimus* | UFRJ 8270.2 | Campo Grande | 22°57'00"S 43°36'45"W | KC990493 | KF311333 |
| *Xenurolebias izecksohni* | UFRJ 8204.3 | Linhares | 19°12'54"S 39°57'57"W | KF311357 | |
| *Xenurolebias* cf. *myersi* | UFRJ 8200.1 | Conceição da Barra | 18°34'01"S 39°44'36"W | KF311356 | |