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ARTICLE

Is *Nematocharax* (Actinopterygii, Characiformes) a monotypic fish genus?

Silvia Britto Barreto, Lorena Andrade Nunes, André Teixeira da Silva, Ricardo Jucá-Chagas, Débora Diniz, Iracilda Sampaio, Horacio Schneider, and Paulo Roberto Antunes de Mello Affonso

Abstract: The combination of DNA barcodes and geometric morphometrics is useful to discriminate taxonomically controversial species, providing more precise estimates of biodiversity. Therefore, our goal was to assess the genetic and morphometric diversity in *Nematocharax*, a controversial monotypic and sexually dimorphic genus of Neotropical fish, based on sequencing of cytochrome *c* oxidase subunit I (COI) and morphometric analyses in seven populations of *N. venustus* from coastal rivers in Brazil. The average pairwise intrapopulation divergence in COI ranged from 0 to 2.2%, while the average pairwise interpopulation divergence varied from 0 to 7.5%. The neighbour-joining (NJ) tree resulted in five genetic groups (bootstrap ≥ 97%), which correspond to the five clusters delimited by the BIN System, GMYC, and bPTP, indicating that there might be at least five species (or OTUs) within *Nematocharax*. Morphometric differences among these genetic lineages were also identified. Apparently, sexual selection, restricted dispersal, and geographic isolation might have acted synergistically to cause the evolutionary split of populations. These data challenge the current view that *Nematocharax* is a monotypic genus inasmuch as evolutionarily significant units or even distinguished species were identified. Therefore, we recommend that the highly impacted coastal basins in northeastern Brazil should be prioritized in conservation plans.

Key words: COI, DNA barcoding, ichthyofauna, geometric morphometrics, species delimitation.

Résumé : La combinaison de codes à barres de l'ADN et de la morphométrie géométrique peut s'avérer utile pour distinguer des espèces controversées sur le plan taxonomique, ce qui permet de fournir des estimés plus précis de la biodiversité. Le but de ce travail était de mesurer la diversité génétique et morphométrique au sein du genre *Nematocharax*, un genre controversé de poisson néotropical monotypique à dimorphisme sexuel, par le biais du séquençage de la sous-unité I de la cytochrome *c* oxidase (COI) et d'analyses morphométriques chez sept populations du *N. venustus* présentes dans les rivières côtières du Brésil. La divergence intrapopulation moyenne au sein du gène COI variait entre 0 et 2,2 %, tandis que la divergence interpopulation moyenne variait entre 0 et 7,5 %. Un arbre neighbour-joining (NJ) a été produit et il est formé de cinq groupes génétiques (valeur de bootstrap ≥ 97 %), lesquels correspondent aux cinq groups définis par le système BIN, GYMC et bPTP. Cela suggère qu'il pourrait y avoir au moins cinq espèces (ou OTU) au sein du genre *Nematocharax*. Les différences morphométriques entre ces différents groupes ont aussi été identifiées. Apparemment, la sélection sexuelle, une dispersion limitée et un isolement géographique auraient agi de manière synergique pour causer une divergence évolutive de ces populations. Ces données remettent en doute la vision actuelle voulant que le *Nematocharax* est un genre monotypique du fait que des unités évolutives significatives, et peut-être même des espèces distinctes, ont été identifiées. Ainsi, les auteurs recommandent que les bassins côtiers fortement perturbés du nord-est du Brésil soient priorisés dans l'élaboration de plans de conservation. [Traduit par la Rédaction]

Mots-clés : COI, codage à barres de l'ADN, faune piscicole, morphométrie géométrique, délimitation d'espèces.

Introduction

Besides taxonomic implications, the recognition of species or evolutionary units is critical for ecology, biogeography, and conservation of biodiversity (Sites and Marshall 2004; Casciotta et al. 2013). However, discriminating species based only on morphological features is particularly difficult for some groups, such as Neotropical fish because of their richness (Lévêque et al. 2008),

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remarkable phenotypic plasticity (Wimberger 1992), and high number of cryptic species (Piggott et al. 2011). To solve such taxonomic uncertainties, different data should be analyzed simultaneously, to infer species boundaries through the complementarity among disciplines (Schlick-Steiner et al. 2010; Yeates et al. 2011). This approach, termed integrative taxonomy, has expanded over recent years, being particularly useful for conservation plans (Dayrat 2005), whose success requires a deeper knowledge of biodiversity (Mace 2004).

For instance, molecular markers and geometric morphometrics have been effective in identifying species and evolutionary units in Characidae (Ornelas-García et al. 2014; Gomes et al. 2015), one of the largest and most complex freshwater fish families (Nelson 2006). In this family, the genus *Nematocharax* stands out as a putative monotypic taxon, composed only of *Nematocharax venustus* (Weitzman et al. 1986), a rare situation in small characins. A distinctive feature of *N. venustus* is the sexual dimorphism, since males have elongated rays in dorsal, pelvic, and anal fins (Weitzman et al. 1986).

Nematocharax venustus was thought to be an endemic and potentially threatened species from Jequitinhonha River in southeastern Brazil (Weitzman et al. 1986). However, it was further recorded in coastal rivers in northeastern Brazil (state of Bahia) up to the Contas River basin, encompassing distinct biomes (Atlantic rainforest, caatinga or dry bushland, and transition zones) (Menezes and Lima 2008).

Recently, another species, *N. costai*, was described for this genus based on morphological studies in specimens from the Gongogi River sub-basin (Lower Contas River basin), Bahia, northeastern Brazil (Bragança et al. 2013). This species was recognized by the number of maxillary teeth in males, lack of hooks and spinules on dorsal and pelvic fins, reduced number of anal fin rays with spinules, presence of a long horizontal dark pink mark on caudal peduncle, number of supraneurals, and yellowish pelvic fins (Bragança et al. 2013). However, Menezes et al. (2015) reported no meristic, morphometric, osteological, or colouration differences to support the recognition of two species of *Nematocharax*, so that *N. costai* must be considered junior synonym of *N. venustus*. Nonetheless, these authors observed intraspecific variation of secondary sexual traits among geographically isolated populations of *N. venustus*.

Considering the lack of genetic information about the genus *Nematocharax* and its controversial taxonomy, we carried out DNA barcoding analyses based on COI sequences and geometric morphometrics in seven populations of *N. venustus* to establish their interpopulation differentiation and verify the actual species diversity of this freshwater fish genus.

Materials and methods

Sampling

A total of 212 specimens of *N. venustus* was collected in seven locations of the Contas (Gongogi 1, 2, and 3, and Upper Contas), Almada (Almada), and Jequitinhonha (Jequitinhonha 1 and 2) river basins (Fig. 1; Table 1), including the northern and southern range of this species. The license for collection of ichthyological material was granted by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; license number SISBIO 39728-1). All individuals were euthanized by immersion in iced water at 0–2 °C up to complete cessation of opercular movements, as described for tropical fishes (Blessing et al. 2010), and then photographed. Additionally, for each individual, a small fragment of muscle tissue (approximately 0.5 cm^2) was removed and preserved in 96% ethanol at –20 °C. The remaining whole bodies of the organisms were fixed in 10% formaldehyde.

For morphometric analysis, 198 specimens (juveniles and adults) were considered, since some of them were improper (slightly deformed) for morphological comparisons after fixation. In turn, 91 specimens (including both males and females in approximately equal proportion) were randomly chosen for barcode analysis, considering a minimum number of eight specimens per locality. The voucher specimens were deposited in the Zoology Museum at Universidade Federal da Bahia (MZUFBA), Brazil (UFBA 7953, 7954, 8016, 8017, 8018, 8019, and 8020).

Molecular analyses

DNA isolation, PCR, and sequencing

Total DNA was extracted from muscle tissue of each specimen using Wizard Genomic DNA Purification Kit (Promega, Madison, Wis., USA) according to manufacturer's instructions. Fragments of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene were amplified via PCR using the primers FishF2_t1 (5′-TGTAAAACGACGGCCAGT CGACTAATCATAAAGATATCGGCAC-3′) and FishR2_t1 (5′-CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGA ATCAGAA-3′), designed by Ward et al. (2005).

Each PCR reaction was performed with 0.2 mmol/L dNTPs (Invitrogen, São Paulo, Brazil), 1x buffer (Invitrogen, São Paulo, Brazil), 1.5 mmol/L $MgCl_2$ (Invitrogen, São Paulo, Brazil), 0.2 μmol/L of each primer, 1 U of platinum Taq DNA polymerase (Invitrogen, São Paulo, Brazil), 50 to 100 ng of DNA template, and ultrapure water to a final volume of 15 μL. The amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, Calif., USA), with the following program: 94 °C for 4 min; 40 cycles of 92 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and 30 s; and a final extension at 72 °C for 10 min.

The PCR products were stained with bromophenol blue and Gel Red™ (Biotium, USA) at a ratio of 3:1 and then visualized in 1% agarose gel after electrophoresis. The amplified products were purified in 20% polyethylene

Fig. 1. Map of South America highlighting the Eastern Atlantic Hydrographic Region (dark grey), in Brazil. In detail, the seven collection sites of *Nematocharax venustus*: Almada River basin (A, Almada), Contas River basin (B, Gongogi 1; C, Gongogi 2; D, Gongogi 3; G, Upper Contas), and Jequitinhonha River basin (E, Jequitinhonha 1; F, Jequitinhonha 2).

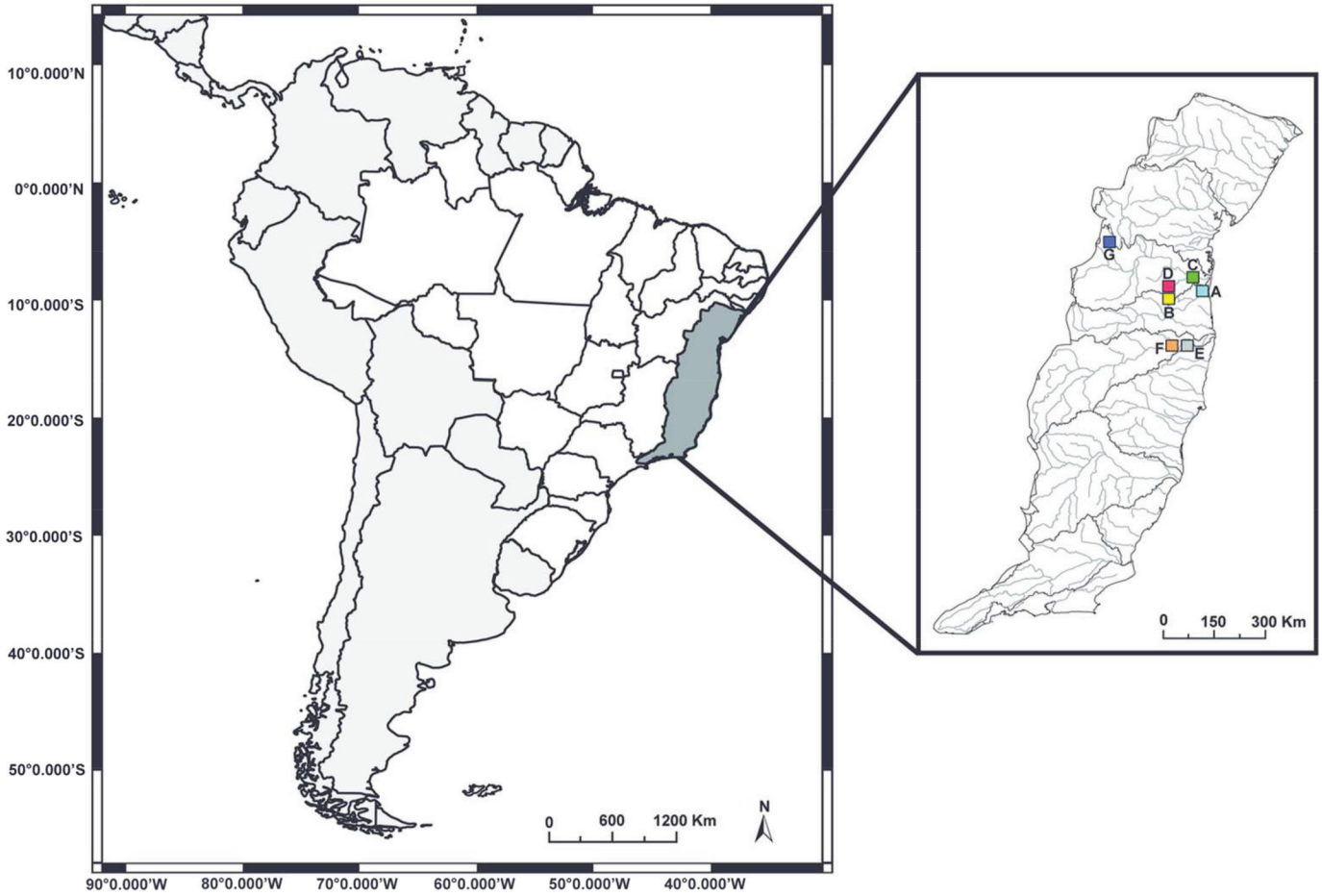


Table 1. Specimens of *Nematocharax venustus* analyzed in the present study, with information about collection site, basin, river/locality, coordinates, and sample size.

Collection site	Basin	River/locality	Latitude/longitude	Sample size (sex)
Almada	Almada River	Almada River/Uruçuca, BA	–14.658883/–39.223131	37 (4♂, 10♀, 23?)
Gongogi 1	Contas River	Tributary of Gongogi River/Nova Canaã, BA	–14.832417/–40.103459	17 (7♂, 9♀, 1?)
Gongogi 2	Contas River	Tributary of Gongogi River/Gongogi, BA	–14.342715/–39.462964	41 (19♂, 21♀, 1?)
Gongogi 3	Contas River	Cambiriba Stream/Iguaí, BA	–14.604444/–40.102222	24 (5♂, 7♀, 12?)
Upper Contas	Contas River	Água Suja River/Abaíra, BA	–13.409664/–41.633689	47 (15♂, 8♀, 24?)
Jequitinhonha 1	Jequitinhonha River	Limoeiro River/Itagimirim, BA	–16.085900/–39.619300	24 (8♂, 6♀, 10?)
Jequitinhonha 2	Jequitinhonha River	Jequitinhonha River/Salto da Divisa, MG	–16.094819/–40.001561	22 (13♂, 9♀, 0?)

Note: ♂, males; ♀, females; ?, undetermined or immature individuals.

glycol (PEG) and washed in 80% ethanol. The sequencing reactions were performed bidirectionally using the BigDyeTerminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA). After precipitation in 125 mmol/L EDTA, 100% and 70% ethanol, respectively, the sequences were read in an automatic sequencer ABI 3500 XL Genetic Analyser (Applied Biosystems, Foster City, Calif., USA).

Sequence analysis

First, the quality of individual sequences of each specimen (forward and reverse) was visualized using the BioEdit Sequence Alignment Editor 7.1.9 (Hall 1999). Then, the sequences were validated in MEGA 6 (Tamura et al. 2013) through translation into amino acids. Consensus sequences were obtained from the DNA Baser Sequence Assembler 4.16 (Heracle BioSoft SRL 2014), aligned with the ClustalW Multiple Alignment tool (Thompson et al. 1994) available in BioEdit 7.1.9, and manually edited.

All sequences were submitted to BLAST (Basic Alignment Search Tool) at the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) to confirm the sequencing of COI and that the sequences

matched to the expected taxonomic group (>90% match to other sequences in this fish genus; Ward 2009). The sequences were also compared with the BOLD database (Barcode of Life Data Systems, http://www.boldsystems.org/; Ratnasingham and Hebert 2007), using the Species Level Barcode Records option to check their similarities with other fish species. All sequences obtained during this study were deposited in the BOLD database under the project entitled "DNA barcoding of *Nematocharax* – PIABA" ("ProcessID" access numbers: PIABA001-14 to PIABA091-14). This procedure allowed examination of how the sequences were clustered into Barcode Index Numbers (BINs), as automatically provided by BOLD Systems after uploading the barcode sequences (see Ratnasingham and Hebert 2013). The BIN System indicates distinct operational taxonomic units (OTUs) that closely correspond to species by using the RESL (Refined Single Linkage) algorithm.

The number of variable sites and the nucleotide composition were obtained using the software MEGA 6, along with the NJ tree and the average pairwise distances (within and between collection sites, and within and between BINs), based on the Kimura-2-parameter (K2P) model (Kimura 1980) and complete deletion of missing data with 1000 bootstrap replicates (Felsenstein 1985). Haplotype (h) and nucleotide (π) diversities were calculated for samples per collection site using the DNA Sequence Polymorphism 5.10 (Rozas et al. 2010). A haplotype network was obtained with Haplotype Viewer (Ewing 2016) using the maximum likelihood method (Dnaml, PHYLIP 3.69) (Felsenstein 2009). Samples from each site were treated as populations based on the geographic distances (≥45 km), and to avoid a biased estimation of intrapopulation divergence.

The five sequences of *N. venustus* (HM562862—HM562866) from the Una River basin, previously available in BOLD, were incorporated into the alignments and analyses, as well as the sequences of related groups available in GenBank, including *Astyanax lacustris* (HM404906.1), *Hasemania nana* (FJ749062.1), *Hemigrammus marginatus* (HM906014.1), *Rachoviscus crassiceps* (HM562857.1), and *Hyphessobrycon bifasciatus* (HM064997.1). These sequences were selected based on their phylogenetic proximity (Thomaz et al. 2010; Oliveira et al. 2011), morphological similarity (Weitzman et al. 1986), and closest matching sequences of other characin species in the database.

Species delimitation methods

In addition to the BIN analysis, two approaches recognized for their robustness and accuracy were applied to molecular data to delimit independently evolving units (i.e., potential species): the General Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough 2013) and the Bayesian implementation of the PTP (bPTP) models (Zhang et al. 2013). For this, one individual per haplotype was selected. However, for the GMYC, two individuals were used in the lineages with a single haplotype (Upper Contas and Una) so that this analysis would be able to recover them as unique entities (Birky 2013). The best-fit substitution model was estimated using MrModeltest 2.2 (Nylander 2004), based on the Akaike information criterion (AIC).

Because the GMYC model requires an ultrametric tree, this was produced by BEAST 1.8.2 (Bayesian Evolutionary Analysis Sampling Trees; Drummond et al. 2012) using the following parameters: 50 million generations, with sampling every 5000 generations; HKY+I model; strict clock with an arbitrary mutation rate (1.0 substitution/site/Myr); and Yule prior. The program Tracer 1.6 (Rambaut et al. 2013) was used to check the analysis performance, verifying if the effective sample size (ESS) values were above 200. Subsequently, a single target tree was generated in TreeAnotator 1.8.2 (Rambaut and Drummond 2015), after a 10% burn-in. Using this tree as input, the GMYC analysis was performed on the GMYC web server (http://species.h-its.org/gmyc/; Zhang 2015) with the single threshold method.

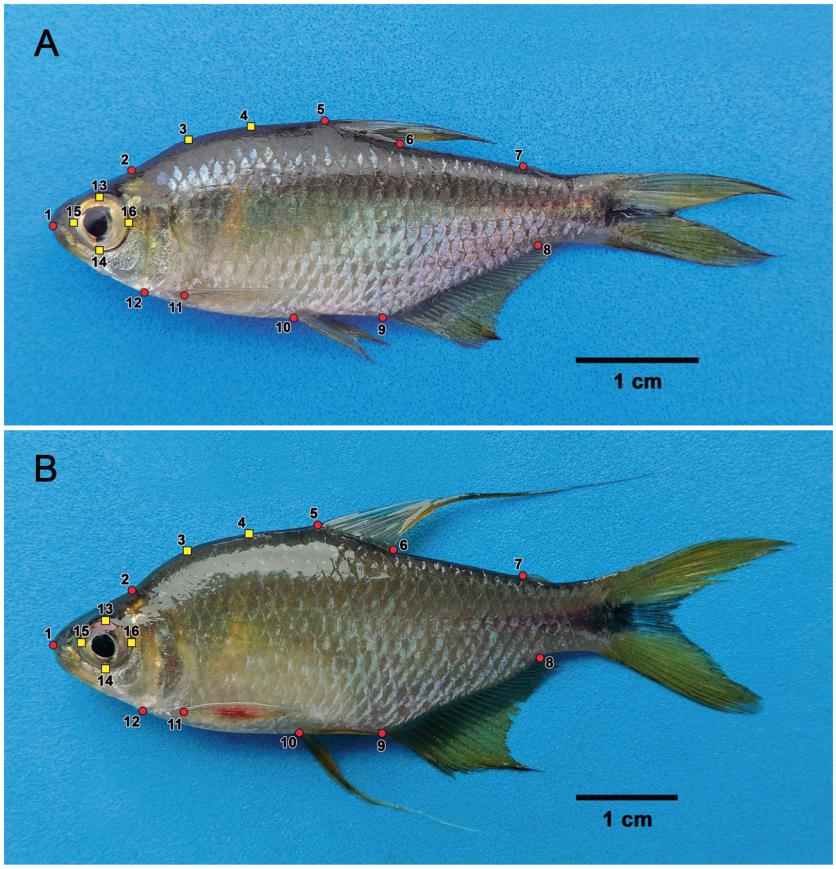
The bPTP model, in turn, adds Bayesian support values to delimited species on a given phylogenetic input tree (Zhang et al. 2013), which in this study was a maximum likelihood tree obtained in MEGA 6. The bPTP analysis was carried out on the bPTP web server (http://species.h-its.org/ptp/; Zhang 2015), with the following settings: rooted tree, due to the inclusion of an outgroup (*Hasemania nana*); 500 000 Markov Chain Monte Carlo (MCMC) generations (thin = 500); and 10% burn-in. On the same server, the MCMC convergence diagnostics were checked in the Likelihood Trace Plot option. In the resulting tree, whenever a node presented high posterior probabilities (>0.70) compared to the low probabilities of the outer and (or) inner nodes, we understood that it represents a unique OTU.

Morphometric analyses

The images for geometric morphometrics were taken from the left side of each specimen using the digital camera Nikon P510 (16.1 megapixels), with a metric scale. Then, the images were converted from the JPEG extension to the TPS format using tpsUtil 1.46 (Rohlf 2010*a*) to perform the measurements. From the images, 10 landmarks, located in distinctive points of tissue juxtaposition, and six semi-landmarks, were inserted (Fig. 2) using tpsDig2 2.16 (Rohlf 2010*b*). Afterwards, the points were aligned by Procrustes superimposition in MorphoJ version 2.0 (Klingenberg 2011). This method compares the landmark configurations by aligning the corresponding points inasmuch as the possible effects of rotation, translation, and scaling are removed, thereby extracting only information about body shape (Rohlf and Slice 1990).

To analyze the differences in body shape among populations, a principal component analysis (PCA) using the coordinates resulting from the Procrustes superimposition was performed. Since the body shape of males is highly variable depending on their ontogenetic development and reproductive season (Menezes et al. 2015), the

Fig. 2. Lateral view of (A) female and (B) male specimens of *Nematocharax venustus*, highlighting the 10 landmarks (red circle) and 6 semi-landmarks (yellow square) used in morphometric analyses. [Colour online.]



PCA included two datasets: (1) males + females and (2) only females. Thus, any bias was minimized by separating females from males in the quantification of intrapopulation shape variation by morphometric analyses.

As commonly used in geometric morphometrics, the generalized size of each individual was estimated using the centroid size, calculated by the square root of the summed squared distances between all landmarks and their centroid (i.e., the average landmark position) (Mitteroecker et al. 2013). The analysis of variance (ANOVA) was performed to verify whether the differences in body size among populations were significant or not, and the means were compared using the Tukey's test at 1%. In addition, a regression analysis (body size vs. PCA axis 1) was performed to evaluate the relationship between size and shape of individuals.

To ascertain the shape similarities among *N. venustus* females, cluster analysis using the unweighted pair-group method using arithmetic average (UPGMA) method with 10 000 permutations was performed in PAST 2.17c (Hammer et al. 2001). The cophenetic correlation was calculated to test the confidence of clusters. For those specimens with both molecular and morphometric data ($n = 73$), a multivariate analysis of variance (MANOVA) was performed to verify how the molecular groupings (BINs) segregate out by the body shape.

Table 2. Sample size (n) and average pairwise intrapopulation divergence (with minimum and maximum values in parentheses) in COI sequences of *Nematocharax venustus* based on the Kimura-2-parameter (K2P) model.

Population	Within group
Una ($n = 5$)	0.000 (0.000–0.000)
Almada ($n = 14$)	0.002 (0.000–0.005)
Upper Contas ($n = 13$)	0.000 (0.000–0.000)
Gongogi 1 ($n = 8$)	0.001 (0.000–0.002)
Gongogi 2 ($n = 14$)	0.003 (0.000–0.008)
Gongogi 3 ($n = 12$)	0.022 (0.000–0.042)
Jequitinhonha 1 ($n = 10$)	0.000 (0.000–0.000)
Jequitinhonha 2 ($n = 20$)	0.000 (0.000–0.002)

Results

DNA barcodes

COI sequences (652 base pairs, bp) were obtained from 91 specimens. The number of specimens per location ranged from 8 to 20, with an average of 13 sequences per population. The NJ tree comprised all obtained sequences plus five sequences of *N. venustus* from Una River basin available in BOLD, as well as five sequences of different species putatively related to *N. venustus*. These additional sequences were selected based on their integrity (few

Table 3. Average pairwise interpopulation divergence of *Nematocharax venustus* samples and average pairwise interspecific divergence with related species based on the Kimura-2-parameter model (K2P).

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Astyanax lacustris*		0.019	0.020	0.016	0.017	0.020	0.019	0.018	0.019	0.019	0.019	0.019	0.019
2. *Hasemania nana*	0.182		0.016	0.020	0.019	0.018	0.019	0.017	0.017	0.018	0.017	0.017	0.017
3. *Hemigrammus marginatus*	0.211	0.147		0.019	0.020	0.017	0.016	0.018	0.017	0.017	0.016	0.017	0.017
4. *Hyphessobrycon bifasciatus*	0.142	0.190	0.182		0.017	0.018	0.018	0.020	0.019	0.018	0.018	0.019	0.019
5. *Rachoviscus crassiceps*	0.159	0.191	0.200	0.175		0.020	0.019	0.020	0.018	0.018	0.018	0.018	0.018
6. Una	0.194	0.190	0.173	0.178	0.216		0.006	0.011	0.008	0.007	0.006	0.008	0.008
7. Almada	0.185	0.192	0.160	0.173	0.205	0.029		0.011	0.008	0.007	0.006	0.008	0.008
8. Upper Contas	0.174	0.157	0.167	0.186	0.201	0.070	0.075		0.011	0.011	0.010	0.011	0.011
9. Gongogi 1	0.184	0.167	0.164	0.187	0.199	0.043	0.041	0.074		0.007	0.005	0.002	0.002
10. Gongogi 2	0.188	0.183	0.174	0.179	0.193	0.035	0.029	0.074	0.036		0.004	0.007	0.007
11. Gongogi 3	0.187	0.177	0.169	0.185	0.199	0.040	0.035	0.074	0.025	0.019		0.005	0.005
12. Jequitinhonha 1	0.184	0.166	0.164	0.188	0.198	0.044	0.042	0.072	0.002	0.034	0.024		0.000
13. Jequitinhonha 2	0.184	0.167	0.165	0.188	0.198	0.044	0.042	0.072	0.002	0.034	0.024	0.000	

Note: Standard error values are shown above the diagonal.

undetermined nucleotides) and comparable length (bp) to those obtained in the present study. Thus, the total alignment comprised 101 sequences with 202 variable sites, no stop codons, and an average content of adenine (A), thymine (T), guanine (G), and cytosine (C) of 25.6%, 32.5%, 17.2%, and 24.7%, respectively.

The average pairwise intrapopulation divergence ranged from 0 to 2.2% (Table 2), with the highest mean value found in the population named as Gongogi 3 (2.2%, 12 individuals), whose the maximum pairwise divergence was 4.2%. The average pairwise interspecific divergence ranged from 0 to 21.6% (Table 3), and the highest value was found between specimens of *N. venustus* from Una River basin and *Rachoviscus crassiceps*. The average pairwise interpopulation divergence among *N. venustus* samples, in turn, ranged from 0 to 7.5%, with the highest value observed between samples from the Upper Contas sub-basin and the Almada River basin.

To construct the haplotype network (Fig. 3), only the COI sequences from the present study (n = 91) were used, resulting in 16 haplotypes. The number of haplotypes and the haplotype (h) and nucleotide (π) diversity values for each population are presented in Table 4. The highest haplotype diversity was found in Gongogi 2 (six haplotypes and seven polymorphic sites), while the highest nucleotide diversity was observed in Gongogi 3 (four haplotypes and 27 polymorphic sites).

The NJ tree (Fig. 4) revealed five genetic groups supported by bootstrap values equal to or higher than 97%, which are equivalent to the five BINs established by the BOLD System, as follows: (I) specimens from Jequitinhonha 1, Jequitinhonha 2, Gongogi 1, and Gongogi 3 (BOLD:ACR3999); (II) specimens from Almada (BOLD:ACR4000); (III) specimens from Una (BOLD:ACC0787); (IV) specimens from Gongogi 2 and Gongogi 3 (BOLD:ACR3998); and (V) specimens from Upper Contas (BOLD:ACR4542).

These same five potential species or independently evolving units were delimited by the GMYC, although with a non-significant p value at 5% level (p = 0.07). For the bPTP, we obtained a mean of 6.72 putative species, including the outgroup. However, using the criterion described above, we also found five OUT's for the *Nematocharax* samples (Fig. 4; see supplementary data, Table S1[1] and Fig. S1[1]).

The average pairwise divergence within BINs ranged from 0 to 0.4% (Table 5), with the highest mean value in BOLD:ACR3998 (n = 21) and maximum pairwise divergence equal to 0.9%. The average pairwise divergence between BINs ranged from 2.9% (between BOLD:ACR4000 and BOLD:ACC0787) to 7.5% (between BOLD:ACR4000 and BOLD:ACR4542) (Table 6).

Geometric morphometrics

The PCA indicated differences in body shape among the analyzed populations of *N. venustus*, considering both males and females, while the first two principal components explained 62.3% of the total variation. It was observed that the population from Upper Contas River (Fig. 5A) was clearly separated from the others, regardless of sex differences. The deformation grids show that most of the morphometric differentiation between the population from the Upper Contas River and the others is associated with head length and body height (Figs. 5B–5E). The ANOVA applied to the centroid size was significant ($p < 0.01$), and the Tukey's test revealed significant differences ($p < 0.01$) among mean values as well (Fig. 6). Thus, according to the variation of the centroid size, the population from Upper Contas River was composed of significantly smaller individuals than the others.

The PCA applied to the population of females from Gongogi, Almada, and Jequitinhonha rivers (Fig. 7A) demonstrated a greater morphological proximity between both populations from the Jequitinhonha River

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2015-0166.

Fig. 3. Haplotype network based on the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, demonstrating the relationships among the observed haplotypes of *Nematocharax venustus*. Each colour represents a location, and the size of the circles is proportional to the number of individuals (detailed within each circle) sharing the same haplotype. The numbers at the branches indicate the number of mutations among haplotypes.

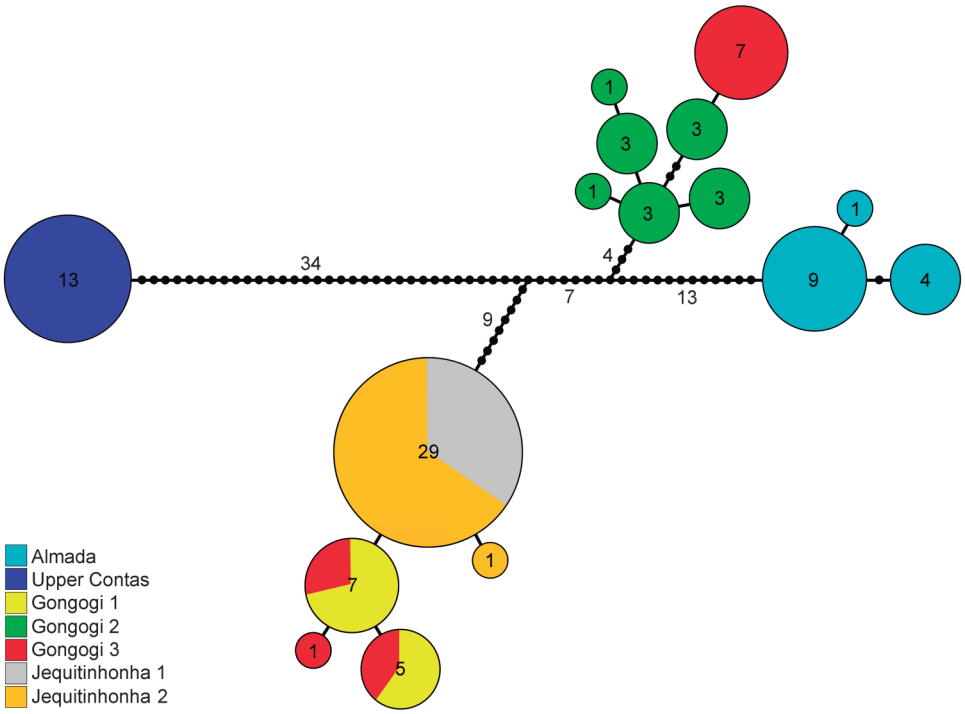


Table 4. Number of haplotypes, haplotype diversity, and nucleotide diversity in COI sequences of *Nematocharax venustus* populations.

Population	No. of haplotypes	Haplotype diversity	Nucleotide diversity
Almada	03	0.538	0.0015
Gongogi 1	02	0.536	0.0008
Gongogi 2	06	0.868	0.0033
Gongogi 3	04	0.652	0.0210
Upper Contas	01	0.000	0.0000
Jequitinhonha 1	01	0.000	0.0000
Jequitinhonha 2	02	0.100	0.0001

basin, while the populations from Gongogi and Almada had individuals scattered along both axes. In this case, the first four components were required to explain 67% of the total variation. Most of the variation in females was related to body height, head height, head length, and eye diameter (Fig. 7B–7E). Regression analysis revealed that the body shape of females varies with the size of the individuals ($p < 0.001$). Accordingly, the analysis of the centroid size using ANOVA was significant ($p < 0.01$) inasmuch as individuals sampled in Jequitinhonha 1 were bigger than those from Gongogi 2, with significantly different mean values (Fig. 8).

The UPGMA dendrogram based on morphometric differences (Fig. 9), with a reliability of 99%–100% in bootstrap and 96% of cophenetic correlation, showed three groups: one comprised the populations from the Jequitinhonha River basin and another with the three populations from the Gongogi and Almada rivers, while the population from Upper Contas remained isolated from all others. The MANOVA (Fig. 10) was statistically significant (Wilk's lambda = 0.05, $p < 0.01$), indicating that differences in body shape are also observed when comparing individuals belonging to different BINs. In this analysis, three BINs were discriminated with a reliability of 95% (ellipses), again showing the highest divergence of the Upper Contas BIN (BOLD:ACR4542). However, overlapped body shapes were observed among some lineages (BOLD:ACR3998, BOLD:ACR3999, and BOLD:ACR4000).

Discussion

The integration of COI sequences and geometric morphometrics were informative to assess the diversity in *N. venustus*, for which more than one evolutionary unit was verified. It is noteworthy that the results of the interpopulation genetic distances of *Nematocharax* samples were higher than the 2% divergence value commonly used to discriminate fish species (Hubert et al. 2008; Ward et al. 2009; Carvalho et al. 2011; Pereira et al. 2013).

The five clusters were consistently observed by NJ, BINs, GMYC, and bPTP analyses. Although these methods are based on distinct models, a growing number of studies confirm their reliability to infer putative species boundaries (Costa-Silva et al. 2015; Henriques et al. 2015; Lin et al. 2015), with particular efficiency for the new algorithm implemented in the BIN System (RESL). Ratnasingham and

Fig. 4. Neighbour-joining (NJ) tree based on the mitochondrial cytochrome *c* oxidase subunit I (COI) gene from populations of *Nematocharax venustus* and related species. The numbers indicate the bootstrap values. The Roman numbers indicate the five genetic groups supported by bootstrap ≥ 97%. The lateral bar represents the independently evolving units delimited by the Barcode Index Numbers (BINs) on Barcode of Life Data Systems (BOLD), and the General Mixed Yule Coalescent (GMYC) and Bayesian implementation of the PTP (bPTP) models.

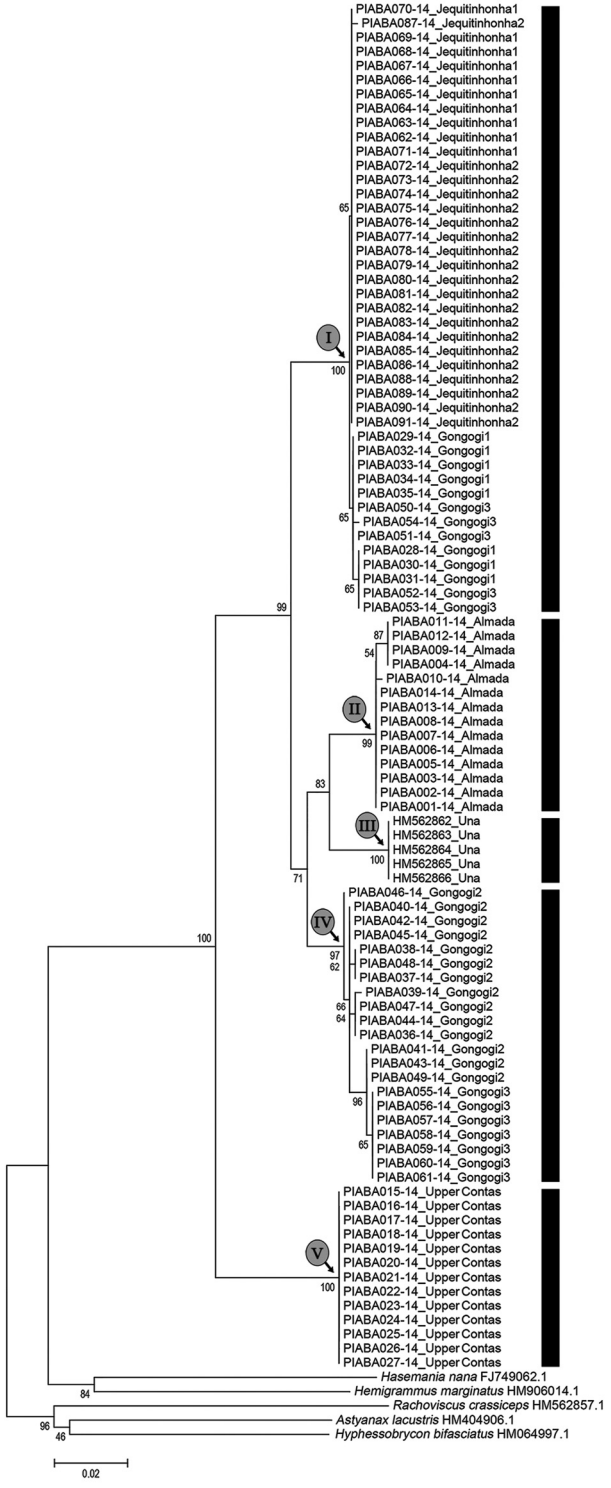


Table 5. Sample size (*n*) and average pairwise divergences within Barcode Index Numbers (BINs) of *Nematocharax venustus* (with minimum and maximum values in parentheses) based on the Kimura-2-parameter (K2P) model.

Barcode Index Number (BIN)	Within group
BOLD:ACR3999 (*n* = 43)	0.001 (0.000–0.005)
BOLD:ACR4000 (*n* = 14)	0.002 (0.000–0.005)
BOLD:ACC0787 (*n* = 5)	0.000 (0.000–0.000)
BOLD:ACR3998 (*n* = 21)	0.004 (0.000–0.009)
BOLD:ACR4542 (*n* = 13)	0.000 (0.000–0.000)

Table 6. Average pairwise divergences between Barcode Index Numbers (BINs) of *Nematocharax venustus* based on the Kimura-2-parameter (K2P) model.

	1	2	3	4	5
1. BOLD:ACR3999		0.008	0.008	0.007	0.011
2. BOLD:ACR4000	0.041		0.007	0.007	0.011
3. BOLD:ACC0787	0.044	0.029		0.007	0.011
4. BOLD:ACR3998	0.037	0.030	0.036		0.011
5. BOLD:ACR4542	0.072	0.075	0.070	0.074	

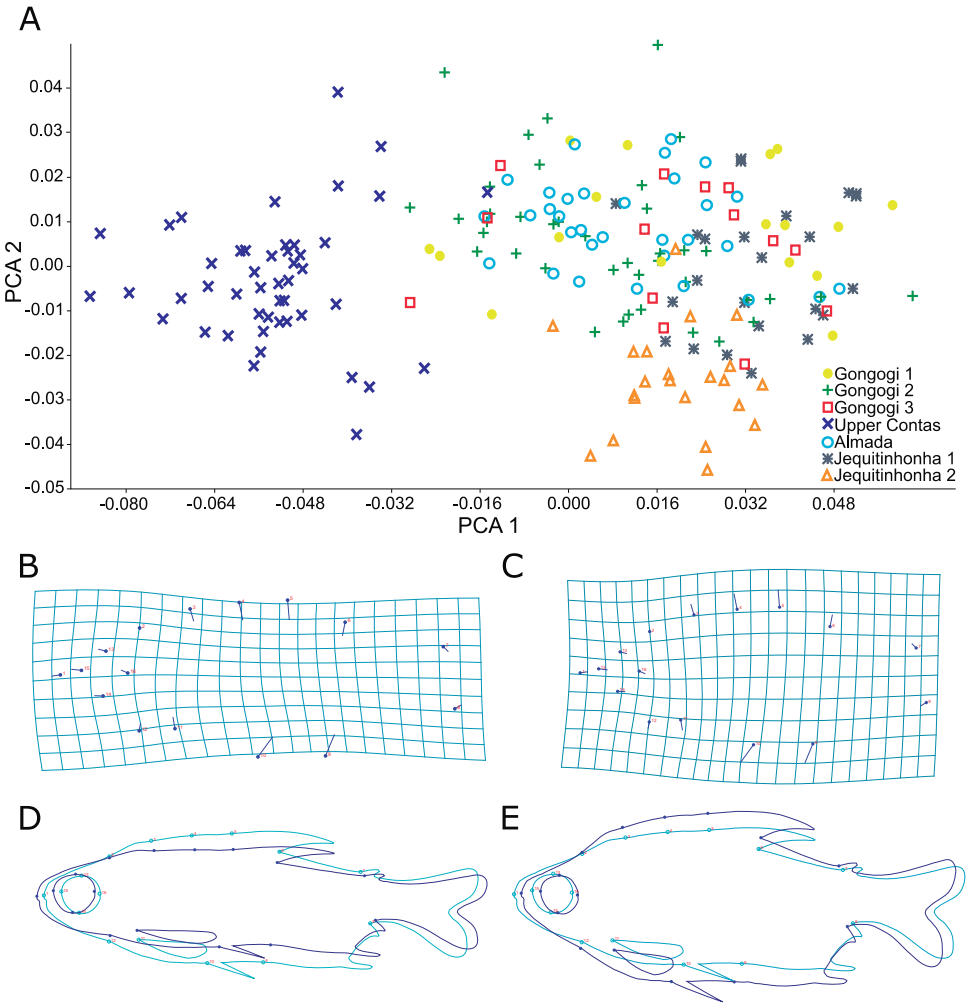
Note: Standard error values are shown above the diagonal.

Hebert (2013) obtained a higher taxonomic performance in several groups, including fish, by using RESL than well-established algorithms (ABGD, CROP, and jMOTU). Moreover, these authors showed that the RESL approach was similar to GMYC, which currently stands out as one of the most robust and theoretically defensible methods. This last one, as well as bPTP, are tree-based methods used to discriminate species or independently evolving units, based on likelihood and Bayesian inference, respectively (Fujita et al. 2012). Thus, the convergent result of these analyses greatly reinforces the hypothesis of new candidate species, indicating that there might be at least five independently evolving species (or OTUs) within *Nematocharax*. In addition, considering that this study encompassed four out of six basins where the genus is known to occur, it is possible that other lineages are yet to be found.

Although the GMYC has provided the same well-supported pattern of species delimitation when compared to the other approaches, the result showed marginally non-significant *p* values ($p = 0.07$), which may be due to an insufficient divergence time among the lineages. Therefore, further studies using additional genetic markers may be needed before describing new species, particularly for those lineages not readily distinguishable by morphology.

The morphometric analysis also showed that body shape usually segregates along with genetic lineages. However, especially for the BIN with the lowest bootstrap value (97%) in the NJ tree (BOLD:ACR3998), the morphometric traits overlap with individuals from other BINs (Fig. 10). This result might be related to the highest average pairwise divergence within this BIN, which might be reflected in the body shape variation.

Fig. 5. (A) Principal component analysis (PCA) of the body shape in *Nematocharax venustus* specimens. In the graph, each location is represented by a symbol; (B) and (C) Deformation grids representing morphological extremes in the first principal component (PCA1). The negative score is disposed at the left side, and the positive at the right. The vectors indicate the direction of the variation of each landmark and semilandmark; (D) and (E) Outlines display the variation in body shape among the specimens. The dark blue lines represent the deformation, and the light blue lines represent the average shape of individuals.

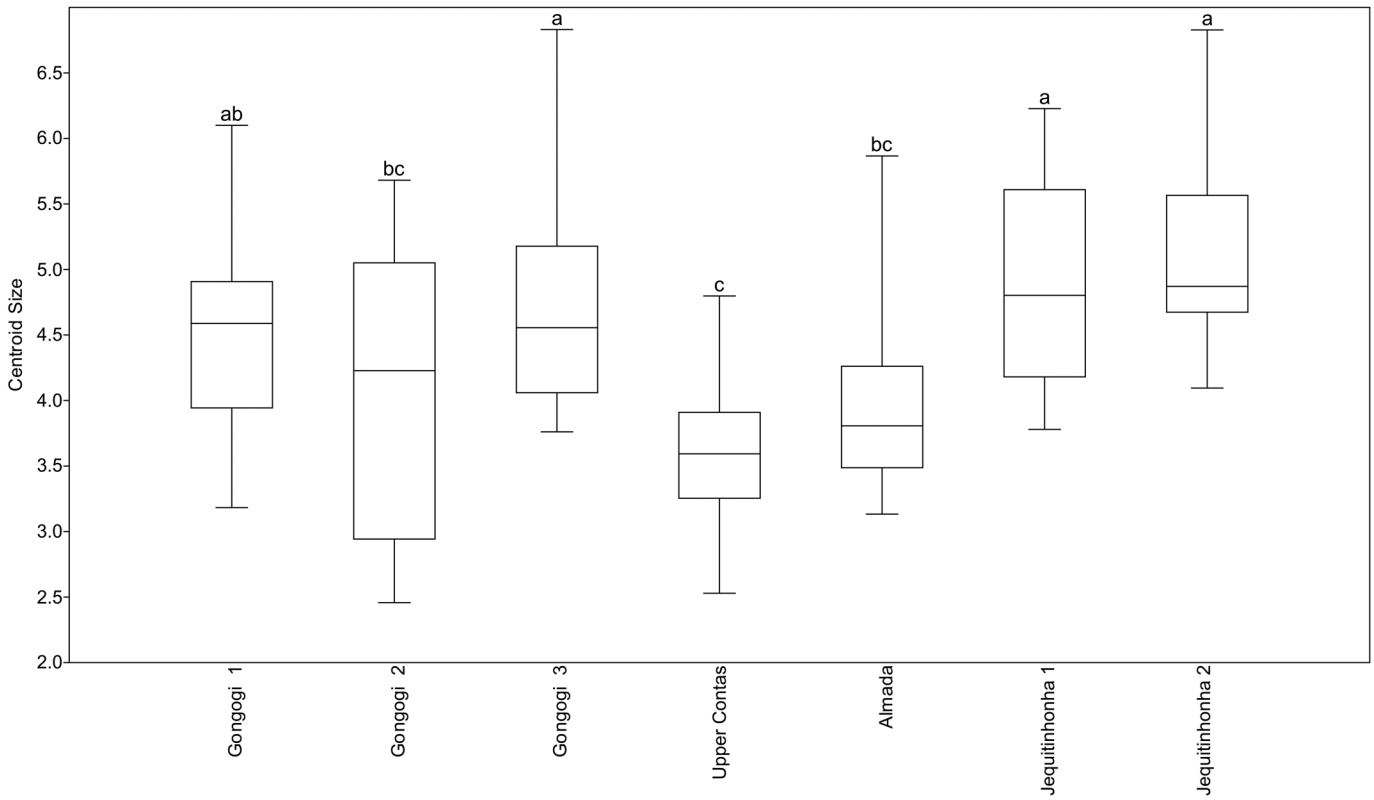


Based on both morphometric and genetic data, the population from Upper Contas was the most divergent, suggesting the presence of a new species of *Nematocharax* in the headwaters from the Diamantina Plateau, in Bahia, Brazil. Indeed, the divergence value above 7% of this population when compared to the other samples of *Nematocharax* is compatible with that reported for distinct genera from the same family (Ward et al. 2009). Previous reports by Zanata and Camelier (2010), Zanata and Serra (2010), and Barbosa and Costa (2011) suggest that the ichthyofauna of this overlooked region probably encompasses a high number of endemic species, reinforcing the importance of this region as a biodiversity hotspot.

The geological processes related to the mountainous landscapes surrounding the Upper Contas River subbasin have probably driven the evolutionary history of this candidate new species of *Nematocharax*. Indeed, the landscapes of basins along the Eastern Atlantic hydrographic region, where the Contas River basin is located, are characterized by hills from the eastern border of the Brazilian crystalline shield, such as Diamantina Plateau and Espinhaço Hills (Ribeiro 2006). This region has undergone remarkable tectonic instability during the Mio-Pliocene (Saadi 1995), thus favouring the complete isolation of populations in Upper Contas. This process would account for their high divergence in COI sequences (~7%) when compared to other populations and their morphological singularity (lower body depth and short rays in pelvic fins). Additionally, the identification of a single haplotype shared by specimens from Upper Contas reinforces this hypothesis since the isolation of a few individuals in headwaters would result in reduced intrapopulation diversity by genetic drift.

The results from geometric morphometrics and DNA barcoding in populations from Jequitinhonha River basin were also concordant, revealing their close relationship by both methods and the lack of genetic variation in

Fig. 6. Box-plot demonstrating the variation of the centroid size in *Nematocharax venustus* specimens by location. The bars show the mean values ± standard deviation. Different letters represent significant differences in mean values in Tukey's test at 1%.



COI sequences. *Nematocharax venustus* is commonly found in lentic and shallow habitats covered by marginal vegetation (Menezes and Lima 2008). However, the Jequitinhonha River basin has been severely affected by run-offs related to the loss of riparian vegetation, as well as pollution, impounding, and introduction of exotic species (Menezes and Lima 2008). These changes in natural habitats have probably caused a bottleneck effect in the populations from this river basin, eventually determining low levels of haplotype diversity.

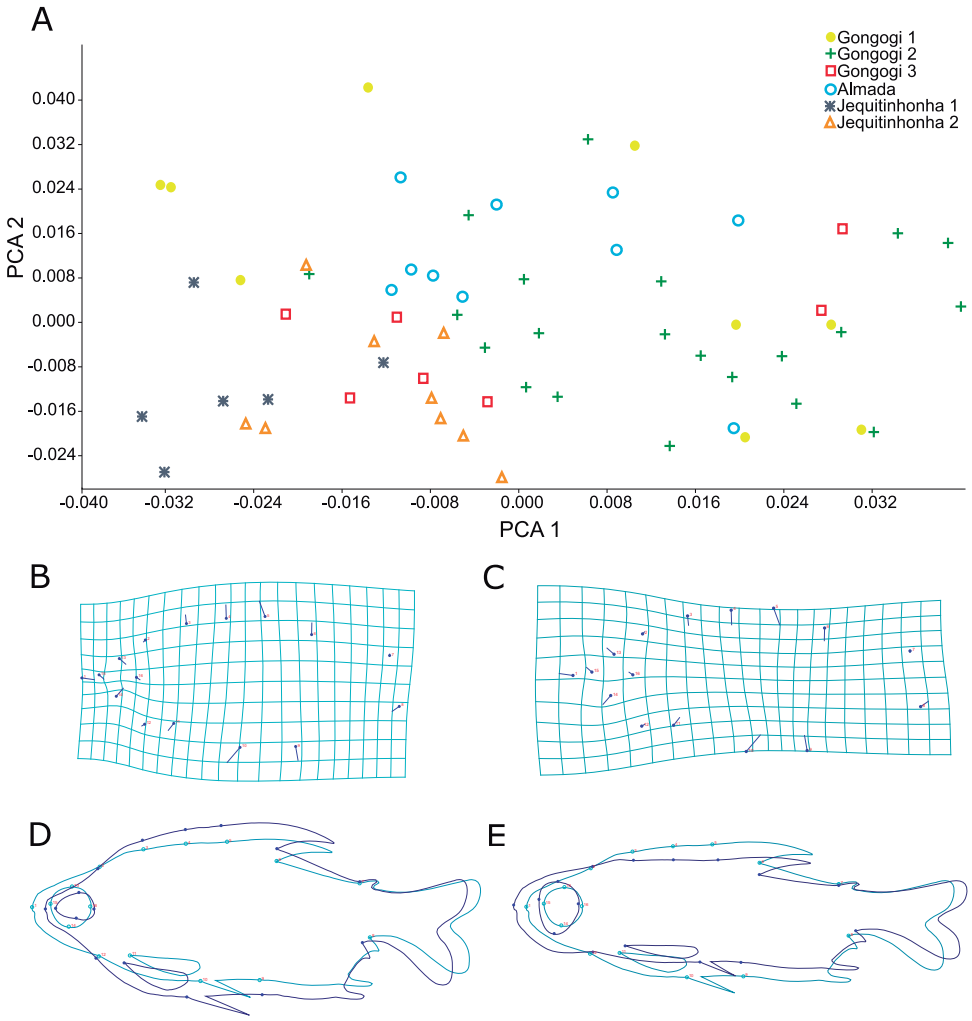
It should be pointed out that *N. venustus* was recently removed from the Red List of Threatened Species after the identification of large populations in Cachoeira, Almada, and Contas river basins. Nonetheless, according to the present data, these populations are genetically divergent from that observed in Jequitinhonha River basin, which is probably endangered due to the abovementioned habitat degradation. To confirm this inference, the utilization of other markers, such as microsatellites, is recommended to verify the extent of this apparent intrapopulation genetic homogeneity in samples from both Upper Contas and Jequitinhonha rivers.

Despite the differences in body shape of females between the populations from Jequitinhonha and Gongogi rivers, the DNA barcodes revealed no consistent differentiation between *N. costai* and *N. venustus*, as observed by Menezes et al. (2015) based on morphological analyses. Nonetheless, some specimens from Gongogi 3 (previously considered the type locality of *N. costai*) and all individuals from Gongogi 2 formed a distinct cluster with bootstrap value of 97%. Thus, marked genetic differences were detected within the population named as Gongogi 3 (four haplotypes), indicating the presence of highly divergent lineages. This result also demonstrates the importance of a broader sampling of specimens to portray the actual diversity of COI sequences in a taxon besides being restricted to five specimens as often used in DNA barcoding (Ward et al. 2009).

In fact, the ichthyofauna of the Gongogi River subbasin seems to be differentiated from other tributaries in the Contas River basin since new species have been reported for this sub-basin (e.g., Vari et al. 2010). Again, just like the rivers from the Diamantina Plateau, Gongogi River represents a priority area for the conservation of Brazilian aquatic biota in spite of its high degree of environmental degradation. Moreover, the identification of distinct evolutionary units, including sympatric and syntopic forms, within a group traditionally regarded as monotypic such as *Nematocharax* is surprising. Yet, some biological features of this genus can provide insights to its rapid evolutionary divergence.

Accordingly to the simulation provided by Turner and Burrows (1995), speciation can be readily established in sympatric conditions when sexual selection is involved. In this model, the reversal of the preference of a female over a single male trait can lead to full reproductive

Fig. 7. (A) Principal component analysis (PCA) of the body shape in *Nematocharax venustus* females. In the graph, each location is represented by a symbol; (B) and (C) Deformation grids representing morphological extremes in the first principal component (PCA1). The negative score is disposed at the left side, and the positive at the right. The vectors indicate the direction of the variation of each landmark and semilandmark; (D) and (E) Outlines display the variation in body shape among the specimens. The dark blue lines represent the deformation, and the light blue lines represent the average shape of individuals.



isolation and speciation in a small population over a short period of time. Since sexual dimorphism is conspicuous in the studied species (e.g., elongated fins and attractive colouration of males), as typically observed in species under sexual selection (Andersson 1994), the same process could be inferred to explain the differentiation of *Nematocharax*. Alternatively, it is also possible that some divergence can occur in allopatry, followed by dispersal and cohabitation again in sympatry, which might be occurring in Gongogi 3, a contact zone between distinct evolutionary units found in this study.

Nematocharax is not the only case in which sex dimorphism and geographic isolation can be associated with deep genetic divergence of fish species. For instance, *Phalloceros caudimaculatus* Hensel, 1868 (Cyprinodontiformes, Poeciliidae) is another Neotropical fish species with sex dimorphism that was further split into 21 new species by Lucinda (2008). Although most of these species seem to have evolved in allopatry, some sympatric species are also described. Since poecilids are strongly marked by differences in external anatomy of both sexes and females show a clear preference for some traits in males such as size of genitalia (Langerhans et al. 2005), distinct patterns of sexual selection might have influenced speciation as well. Nonetheless, the potential role of sexual selection on the divergence of *Nematocharax* lineages requires further investigation.

Likewise, the speciation process in small characins like *Nematocharax* is also favoured by their low dispersal, determining parapatric or allopatric isolation and divergence. In addition, the typical short life cycles of these fish ensure reduced generation intervals in which short-term genetic differences can be easily established. Similarly, genetic differences were observed in populations of *Astyanax* aff. *bimaculatus* (yellow-tailed characin)

Fig. 8. Box-plot demonstrating the variation of the centroid size in *Nematocharax venustus* females by location. The bars show the mean values ± standard deviation. Different letters represent significant differences in mean values in Tukey's test at 1%.

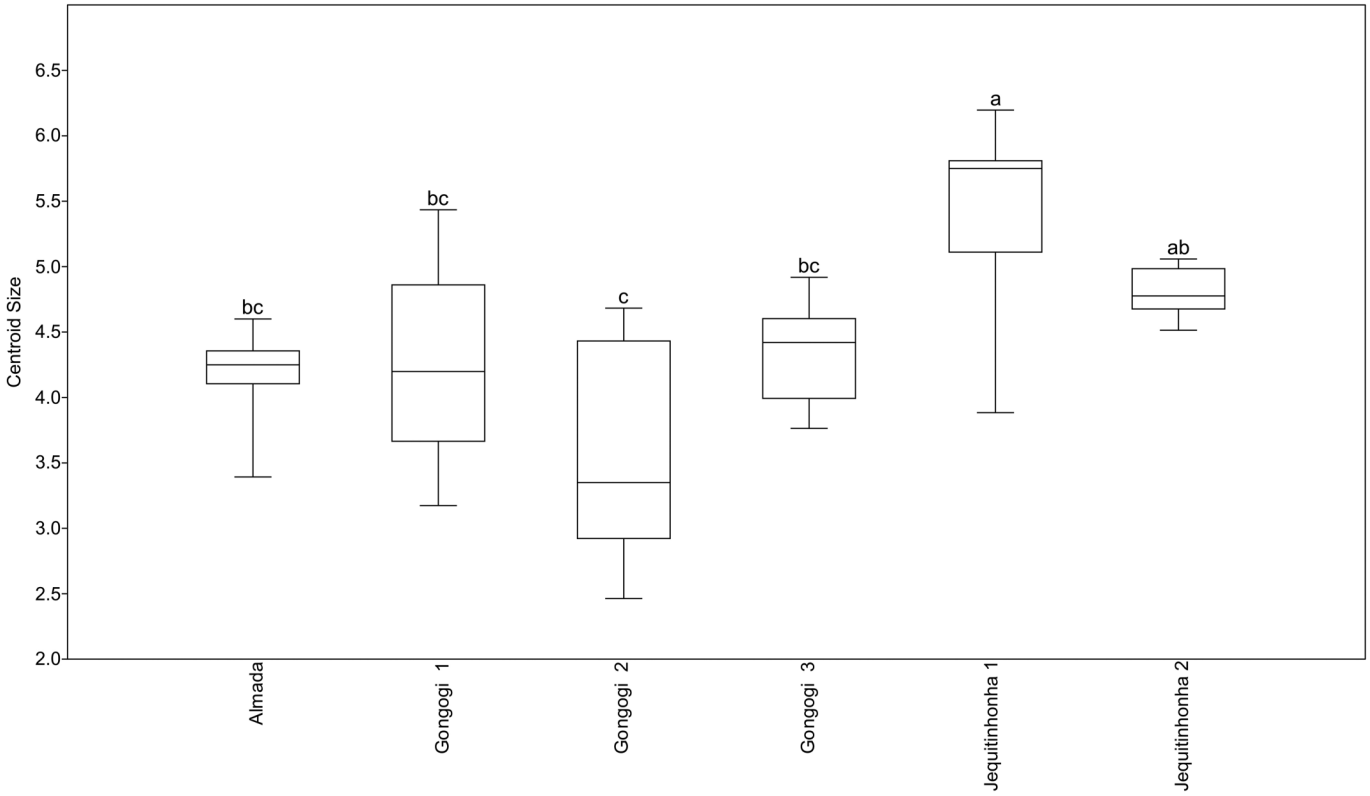
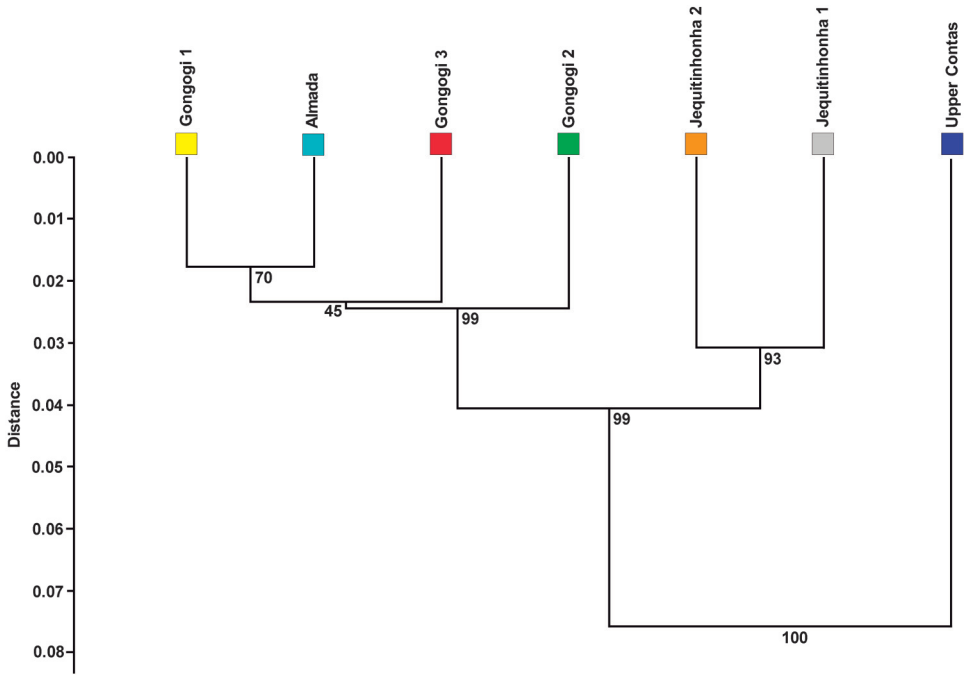
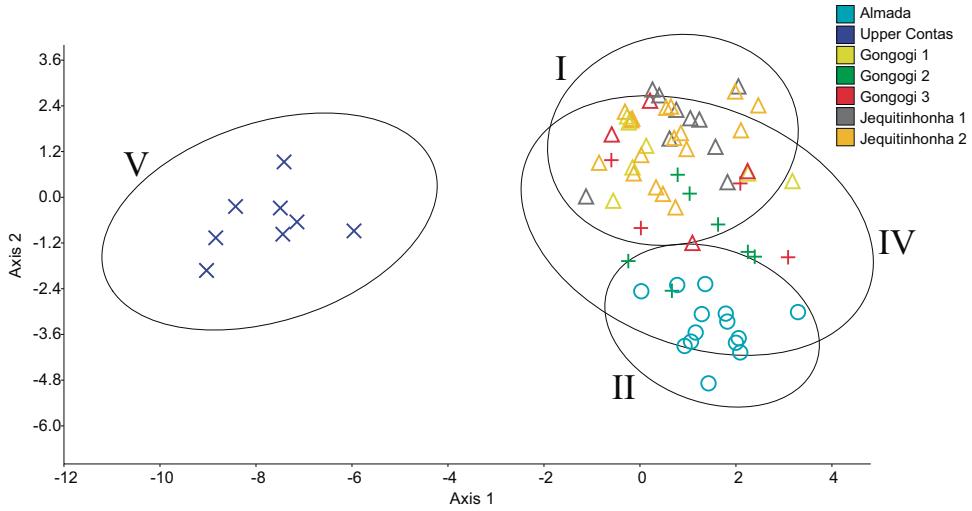


Fig. 9. UPGMA dendrogram showing the average morphometric distances among the body shape of *Nematocharax venustus* females from different locations in Atlantic eastern Brazilian basins.



isolated for nearly 40 years by dams along Middle Contas River (Pamponet et al. 2008). Furthermore, the Middle Contas River basin and the Gongogi River sub-basin are located within a major glacial Pleistocene refugium (Carnaval and Moritz 2008). As a result, these areas are characterized by high genetic diversity and deep evolutionary divergence in different animal groups (Carnaval et al. 2009; Martins 2011), including fishes (Almeida et al. 2013).

Fig. 10. Multivariate analysis of variance (MANOVA) showing the variation in body shape among the Barcode Index Numbers (BINs) of *Nematocharax venustus*. The Roman numbers indicate the BINs: I (BOLD:ACR3999); II (BOLD:ACR4000); IV (BOLD:ACR3998); and V (BOLD:ACR4542), each of which is represented by a symbol. The colours represent the collection sites. BIN III was not included because it refers to the COI sequences previously available in BOLD, lacking morphometric information.



A relationship between morphometric and geographic distance was observed only for the populations from Almada and Gongogi rivers. However, the population from Almada River formed an isolated cluster with 99% bootstrap in the NJ tree. The same result was observed in the population from the Una River basin (100% of bootstrap) (Fig. 4). Therefore, DNA barcoding has likely pinpointed evolutionary units even when external morphological differences were absent or subtle. On the other hand, the differences in the body shape of females (Fig. 9) are not necessarily related to genetic differences, since phenotypic plasticity is widely recognized in fish, being dependent on environmental conditions and different selective pressures (Klingenberg et al. 2003; Clabaut et al. 2007; Arechavala-Lopez et al. 2011).

In summary, both DNA barcode and geometric morphometric data supported the presence of evolutionarily significant units in *Nematocharax*. This conclusion highlights the importance of integrative taxonomy (Dayrat 2005) and suggests a systematic revision in this fish genus, particularly of populations from the Contas River basin. Finally, we show that the coastal basins of eastern Brazil represent priority areas for biodiversity conservation, which contrasts with the increasing threats to such aquatic systems, including bioinvasions and contamination of biota by toxic substances (e.g., Jesus et al. 2014). Therefore, we expect that this study raises awareness among the environmental agencies responsible for the preservation and restoration of water resources in the region before species are lost even before being formally described.

Acknowledgements

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