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# Molecular characterization of *Urocleidoides cuiabai* and *U. malabaricusi* (Monogenea: Dactylogyridae) from the trahira fish *Hoplias* aff. *malabaricus* in the Paraná River, Brazil

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**Abstract**

*Urocleidoides* ectoparasites are mainly found on fish of the neotropical regions. Although molecular research on monogeneans is available, no genetic data exist characterizing species in the *Urocleidoides* genus. Some DNA sequences have been efficacious in systematic studies and in the reconstruction of phylogenies of fish parasites. Relevant roles have been given to the sequence of the mitochondrial gene of cytochrome *c* oxidase I (COI). This study characterized COI sequences of the parasites *Urocleidoides malabaricusi* and *U. cuiabai* in trahira fish *Hoplias* aff. *malabaricus* of the flood plain of the Upper River Paraná, Brazil. The two species under analysis were distinguished by sequencing and analysing a 420-bp fragment of the COI gene, which suggested the existence of the cryptic species *U. malabaricusi*.

## Introduction

There is a great diversity of monogenetic parasite species in vertebrates, including sea and freshwater fish (Thatcher, 2006). Up to 300 monogenean species belonging to the Gyrodactylidae and Dactylogyridae have been described in Brazil, even though only a small percentage of species is known (Eiras *et al.*, 2010).

The genus *Urocleidoides* is one of the monogenetic genera found in freshwater fish in Brazil. Mizelle & Price (1964) originally characterized this genus by the presence of a sinistral vaginal sclerite, overlapping gonads, clockwise cirral rings, simple anchors, hooks with enlarged shanks and the reduction of the first and fifth pair in infected fish (Kritsky *et al.*, 1986). Based on the

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aforementioned features, the genus was reviewed by Kritsky *et al.* (1986), and only 8 out of 30 species listed by Kritsky & Thatcher (1983) were considered to belong to the genus.

Sixteen species of *Urocleidoides* are known in Brazil (Takemoto *et al.*, 2013; Moreira *et al.*, 2015), of which five are hosted by *Hoplias* aff. *malabaricus* (Bloch, 1794), namely *Urocleidoides eremitus* (Kritsky *et al.*, 1986), *U. malabaricusi, U. cuiabai, U. brasiliensis* and *U. naris* (Rosim *et al.*, 2011).

Morphological identification of *Urocleidoides* species is straightforward with well-established taxonomies. However, doubts exist with regard to the monophyletic origin of the genus because several species listed as *Urocleidoides* by Cohen *et al.* (2013) do not have the vaginal sclerite, which is an important criterion for the inclusion of the species within this genus (Kritsky *et al.*, 1986). Genetic data may be a great help in testing the hypothesis of the monophyletic origin for the species of *Urocleidoides*.

Although monogeneans are the most studied group of fish parasites in Brazil (Karling *et al.*, 2014), few studies have employed genetic data to investigate the phylogeny of these parasites in taxonomic studies (Gasques *et al.*, 2013). Recently, Mendoza-Palmero *et al.* (2015) conducted a phylogenetic study with 28S rRNA sequences and reported that there was no taxonomical support for the two subfamilies of the monogeneans in the neotropical region, and allocated them to other groups. The aforementioned work highlights the importance of such tools for taxonomists who aim at achieving greater precision in their studies.

Nucleotide sequence analysis of the barcode region of the mitochondrial cytochrome *c* oxidase gene (COI) has been very important in taxonomical studies. Sequence alignment of barcoded DNA of the gene, approximately 650 base pairs (bp) long, has been done to use the gene with a total size of 1590 bp in *Benedenia seriola* (Monogenea: Monopisthocotylea), a parasite whose mitochondrial genome has already been sequenced (Perkins *et al.*, 2010).

The degree of conservation of the COI gene makes it a good marker for species differentiation (Solé-Cava, 2008). Since it is a mitochondrial gene, it is haploid without any chance of genetic recombination, and may provide important information in the phylogenetic studies of closely related species (Perkins *et al.*, 2011).

Our study investigated the COI sequences of *U. malabaricusi* and *U. cuiabai* from the trahira fish, *H.* aff. *malabaricus*, of the flood plain of the Upper River Paraná, to collect the genetic information to be used in further phylogenetic studies on the genus.

## Materials and methods

### *Collection and examination of monogeneans*

Sixteen specimens of the fish *H.* aff. *malabaricus* (trahiras) were caught in the River Paraná (Long Term Ecological Research – LTER) in April 2012, using nets of different mesh sizes, at locations near Porto Rico PR Brazil (22°43′S; 53°10′W) and the research centre Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia) of the Universidade Estadual de Maringá.

Processing of the specimens comprised thawing, removal of gills and analysis by stereoscopic microscopy to separate the parasites. Parasites were later identified by morphological analysis using an optical microscope and slides with water, according to Rosim *et al.* (2011). Each specimen was identified and stored individually in a microtube containing 50 µl of 96% ethanol. *Urocleidoides cuiabai* and *U. malabaricusi* were not found in all the fish analysed.

### *Molecular analysis*

DNA was extracted following the simplified protocol by Beltran *et al.* (2008). Sequencing of the partial COI gene was conducted using the amplification product of COI sequences obtained with primers COI_Mono_5′: 5′-TAATWGGTG-GKTTTGGTAA-3′, COI_Mono_3′: 5′-AA-TGCATMGGA-AAAAAACA-3′ and COI_Mono_int3′: 5′-ACATAATGA-AARTGAGC-3′ according to the protocol of Plaisance *et al.* (2008). Polymerase chain reaction (PCR) amplicons were sequenced using the primer COI_Mono_int3′ in MegaBACE 1000 (Amersham Biosciences, Little Chalfont, Bucks, UK).

The sequences were edited with BioEdit (Hall, 1999) and aligned using Clustal W (Thompson *et al.*, 1994). The determination of *p*-distance, the test for the best substitution model (ModelTest) and phylogeny reconstruction built by the maximum likelihood algorithm were carried out with MEGA 6 (Tamura *et al.*, 2013).

## Results

The continuous alignment of 420 bp from the nine sequences could be performed after manual editing. The mean nucleotide frequency reached 30% for A, 20% for T, 20% for C and 30% for G. The best sequences derived from *U. cuiabai* (Uc) and *U. malabaricusi* (Um) sequencing were taken into account for analysis. They were deposited in GenBank with the following accession numbers: Uc1 (KT625591), Uc2 (KT625592), Uc3 (KT625593), Uc4 (KT625594), Uc5 (KT625595), Um1 (KT625587), Um2 (KT625588), Um3 (KT625589) and Um4 (KT625590).

The alignment of sequences used as the basis for the preparation of the divergence index while taking *p*-distances into consideration is provided in table 1.

The mean *p*-distance between *U. cuiabai* and *U. malabaricusi* was 23.7% (±0.4%), i.e. 12.5 times higher than the intra-species mean of *U. cuiabai* (1.9%) (±0.73%). The mean intra-specific *p*-distance rate of *U. malabaricusi* was 14.4% (±7.0%), i.e. 7.4 times higher than the rate found in *U. cuiabai*.

The reconstruction of phylogeny was performed with the best substitution model indicated by ModelTest, which was the Hasegawa–Kishino–Yano test (Hasegawa *et al.*, 1985) with gamma distribution by maximum likelihood algorithm with 10,000 bootstrap re-samplings. Figure 1 demonstrates that the grouping of specimens of the species *U. cuiabai* is more concise, with lower bootstrap rates than *U. malabaricusi* ramifications.

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Table 1. Divergence matrix with *p*-distances between specimens of *Urocleidoides cuiabai* (from Uc1 to Uc5) and *Urocleidoides malabaricusi* (from Um1 to Um4).

| Specimen | Uc1 | Uc2 | Uc3 | Uc4 | Uc5 | Um1 | Um2 | Um3 | Um4 |
|---|---|---|---|---|---|---|---|---|---|
| Uc1 | | | | | | | | | |
| Uc2 | 0.019 | | | | | | | | |
| Uc3 | 0.012 | 0.012 | | | | | | | |
| Uc4 | 0.024 | 0.010 | 0.012 | | | | | | |
| Uc5 | 0.029 | 0.029 | 0.017 | 0.024 | | | | | |
| Um1 | 0.240 | 0.238 | 0.233 | 0.233 | 0.236 | | | | |
| Um2 | 0.243 | 0.240 | 0.236 | 0.236 | 0.240 | 0.162 | | | |
| Um3 | 0.240 | 0.231 | 0.231 | 0.226 | 0.238 | 0.171 | 0.195 | | |
| Um4 | 0.243 | 0.240 | 0.236 | 0.236 | 0.238 | 0.002 | 0.164 | 0.171 | |

## Discussion

Two sequences, namely the internal transcribed spacers (ITS1 and ITS2) of rDNA and the mitochondrial cytochrome *c* oxidase I (COI), have been widely used in the molecular analysis of parasites of the phylum Platyhelminthes. The mutation rates were about 1% for ITS among the researched congener species in the phylum and were about 10% for the COI gene (Vilas *et al.*, 2005). Locke *et al.* (2010) reported similar results in parasites of the genus *Diplostomum* of the subclass Digenea.

Although molecular analysis for the establishment of phylogenetic relationships of some monogenetic species, such as *Gyrodactylus*, was successful, others did not have similar success because of the high variability of the regions under analysis. This was the case for *Lamellodiscus* in the molecular analysis using COI and ITS. Poisot *et al.* (2011) registered high degrees of variability within the group, with a low co-relationship between morphological and molecular characteristics.

Vilas *et al.* (2005) investigated variability rates in Platyhelminthes and reported distances of approximately 10% divergence for congener species (for gene COI). The authors recommended that rates above 5% divergence should be investigated. Ratnasingham & Hebert (2007) suggested that species should be investigated when the COI nucleotide sequence divergence of a group is higher than 2%. In our study, the mean divergence rate among the four *U. malabaricusi* specimens is much higher than suggested.

Additionally, the inter-species *p*-distance rates were higher than those obtained for other Monopisthocotylea species (Poisot *et al.*, 2011). However, it must be noted that our study contained a small number of specimens; an increase in sampling size will probably decrease the inter-species *p*-distance rates. Despite the aforementioned statement, a diversity greater than expected in *U. malabaricusi* may be verified when the mean rate of intra-species *p*-distance (14.4 ± 7.0%) is compared with that of other monogenetic species, which varied between 0.32% in *Euryhaliotrema grandis* and 8.6% in *Protopolystoma símplices* (Poisot *et al.*, 2011).

Furthermore, the topology in the phylogeny of *U. malabaricusi* reinforces the hypothesis of cryptic species, due to the high bootstrap rates occurring in these branches. Conversely, *U. cuiabai* shows much lower rates in a more concise clade. The situation is repeated when the tree is elaborated with amino acid sequences from the analysed sequences, indicating a complex with three different species in *U. malabaricusi*.

Because the number of cryptic species is directly related to the number of individual sequences of parasites analysed (Poulin, 2011), the COI sequences are adequate for characterization of cryptic species (Criscione *et al.*, 2005;

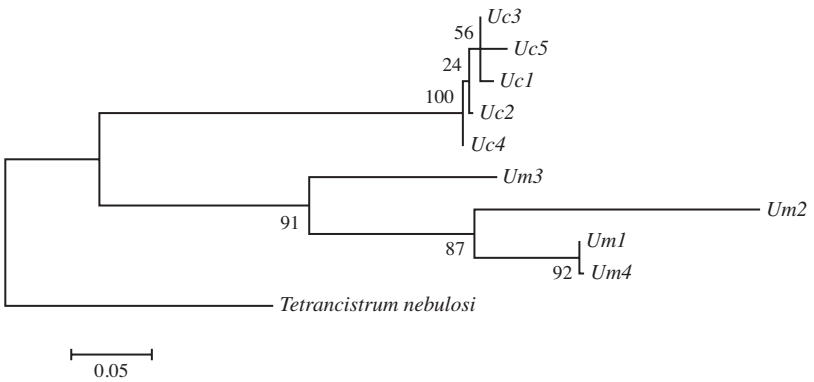


Fig. 1. Phylogenetic relationships of *Urocleidoides cuiabai* (Uc) and *Urocleidoides malabaricusi* (Um), with the maximum likelihood algorithm by the Hasegawa–Kishino–Yano method, with gamma distribution and 10,000 bootstrap re-sampling. The barcode sequence of *Tetrancistrum nebulosi* (KJ0001340) was used as the outgroup.

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Vilas *et al.*, 2005; Nadler & De León, 2011). Additionally, because only four *U. malabaricusi* specimens were investigated in this study, other cryptic species may occur in the complex.

Analysis of COI sequences seems to be promising for the differentiation of the species in this genus. The region's sequence analysis is thus an identification tool for new species by reducing the role of phenotypic plasticity as interference in the process. Molecular data will make the elaboration of clearer phylogenetic reconstructions among the species of *Urocleidoides* possible and enable clarification of their origin.

Finally, further investigations on the genus should be conducted, with more representative samples and associated with a specific region of the nuclear genome, for the better understanding of cryptic species.

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## Conflict of interest

None.

## Ethical standards

The project 21944/2012 was approved by the Ethical Committee for Animal Research of UNIPAR on 22 September 2011.

## References

**Beltran, S., Galinier, R., Allienne, J.F. & Boissier, J.** (2008) Cheap, rapid and efficient DNA extraction method to perform multilocus microsatelite genotyping on all *Schistosoma mansoni* stages. *Memórias do Instituto Oswaldo Cruz* **103**, 501–503.

**Bloch, M.E.** (1794) Der malabarische hecht. pp. 149–150 *in* Bloch, M.E. (*Ed.*) *Naturgeschichte des Auslandische Fische*. Berlin, Achter Theil. J. Moreno.

**Cohen, S.C., Justo, M.C.N. & Kohn, A.** (2013) *South American Monogenoidea parasites of fishes, amphibians and reptiles*. 663 pp. Rio de Janeiro, Oficina de Livros.

**Criscione, C.D., Poulin, R. & Blouin, M.S.** (2005) Molecular ecology of parasites: elucidating ecological and microevolutionary processes. *Molecular Ecology* **14**, 2247–2257.

**Eiras, J.C., Takemoto, R.M. & Pavanelli, G.C.** (2010) *Diversidade dos parasitos de peixes de água doce do Brasil*. 333 pp. Maringá, Clichetec.

**Gasques, L.S., Souza, G.T.R. & Graça, R.J.** (2013) Das moléculas aos parasitos: a aplicação da biologia molecular nos estudos de Ictioparasitologia. pp. 149–167 *in* Pavanelli, G.C., Takemoto, R.M. & Eiras, J.C. (*Eds*) *Parasitologia, Peixes de água doce do Brasil*. Maringá, Eduem.

**Hall, T.A. (**1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

**Hasegawa, M., Kishino, H. & Yano, T.** (1985) Dating the human–ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**, 160–174.

**Karling, L.C., Ueda, B.H., Takemoto, R.M. & Pavanelli, G.C.** (2014) Scientometric study on the parasitology of freshwater fish in Brazil. *Neotropical Helminthology* **8**, 131–140.

**Kritsky, D.C. & Thatcher, V.E.** (1983) Neotropical Monogenea. V: Five new species from the Aruana, *Osteoglossum bicirrosum* Vandelli, a freshwater teleost from Brazil, with the proposal of *Gonocleithrum* n. gen. (Dactylogyridae: Ancyrocephalinae). *Proceedings of the Biological Society of Washington* **96**, 581–597.

**Kritsky, D.C., Thatcher, V.E. & Boeger, W.A.** (1986) Neotropical Monogenea. Revision of *Urocleidoides* (Dactylogyridae, Ancyrocephalinae). *Proceedings of the Helminthological Society of Washington* **53**, 1–37.

**Locke, S.A., Mclaughlin, J.D., Dayanandan, S. & Marcogliese, D.J.** (2010) Diversity and specificity in *Diplostomum* spp. metacercariae in freshwater fishes revealed by cytochrome *c* oxidase I and internal transcribed spacer sequences. *International Journal for Parasitology* **40**, 333–343.

**Mendoza-Palmero, C.A., Blasco-Costa, I. & Scholz, T.** (2015) Molecular phylogeny of neotropical monogeneans (Platyhelminthes: Monogenea) from catfishes (Siluriformes). *Parasites & Vectors* **8**, 164.

**Mizelle, J.D. & Price, C.E.** (1964) Studies on monogenetic trematodes. XXVII. Dactylogyrid species with the proposal of *Urocleidoides* gen. n. *Journal of Parasitology* **50**, 579–584.

**Moreira, J., Scholz, T. & Luque, J.L.** (2015) First data on the parasites of *Hoplias aimara* (Characiformes): description of two new species of gill monogeneans (Dactylogyridae). *Acta Parasitologica* **60**, 254–260.

**Nadler, S.A. & De León, G.P.P.** (2011) Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology. *Parasitology* **138**, 1688–1709.

**Perkins, E.M., Donnellan, S.C., Bertozzi, T. & Whittington, I.D.** (2010) Closing the mitochondrial circle on paraphyly of the Monogenea (Platyhelminthes) infers evolution in the diet of parasitic flatworms. *International Journal for Parasitology* **40**, 1237–1245.

**Perkins, S.L., Martinsen, E.S. & Falk, B.G. (**2011) Do molecules matter more than morphology? Promises and pitfalls in parasites. *Parasitology* **138**, 1664–1674.

**Plaisance, L., Rousset, V., Morand, S. & Littlewood, T.D.J.** (2008) Colonization of Pacific islands by parasites of low dispersal ability: phylogeography of two monogenean species parasitizing butterflyfishes in the South Pacific Ocean. *Journal of Biogeography* **35**, 65–87.

**Poisot, T., Verneau, O. & Desdevises, Y.** (2011) Morphological and molecular evolution are not linked in *Lamellodiscus* (Platyhelminthes Monogenea). *PLOS One* **6**, e26252.

**Poulin, R.** (2011) Uneven distribution of cryptic diversity among higher taxa of parasitic worms. *Biology Letters* **7**, 241–244.

**Ratnasingham, S. & Hebert, P.D.N.** (2007) BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* **7**, 355–364.

**Rosim, D.F., Mendonza–Franco, E.F. & Luque, J.L.** (2011) New and previously described species of *Urocleidoides* (Monogenoidea: Dactylogyridae) infecting the gills of *Hoplias malabaricus* (Characiformes: Erythrinidae) from Brazil. *Journal of Parasitology* **97**, 406–417.

**Solé-Cava, A.M.** (2008) Código de barras de DNA: o rabo que abana o cachorro. *Ciência Hoje* **41**, 65–67.

**Takemoto, R.T., Luque, J.L., Bellay, S., Longhini, C.E. & Graça, R.J.** (2013) Monogenea. pp. 273–279 *in* Pavanelli, G.C., Takemoto, R.M. & Eiras, J.C. (*Eds*) *Parasitologia, Peixes de água doce do Brasil*. Maringá, Eduem.

**Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S.** (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

**Thatcher, V.E.** (2006) *Aquatic biodiversity in Latin America: Amazon fish parasites*. 2nd edn. Bulgaria, Pensoft.

**Thompson, J.D., Higgins, D.G. & Gibson, T.J. (**1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

**Vilas, R., Criscione, C.D. & Blouin, M.S.** (2005) A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. *Parasitology* **121**, 839–846.