Neotropical Ichthyology, 15(1): e160141, 2017
DOI: 10.1590/1982-0224-20160141

Journal homepage: www.scielo.br/ni
Published online: 03 April 2017 (ISSN 1982-0224)
Printed: 31 March 2017 (ISSN 1679-6225)

Using ancient DNA to unravel taxonomic puzzles: the identity of *Deuterodon pedri* (Ostariophysi: Characidae)

Priscilla C. Silva, Maria C. Malabarba and Luiz R. Malabarba

Accurate identification is essential for any study exploring biodiversity. Unfortunately, museum type specimens preserved for more than a hundred years are often not informative enough for precise identification of the species represented by the name-bearing type. The use of ancient DNA can help solve taxonomic problems when name-bearing types no longer have diagnostic morphological features that allow for an accurate identification of the species involved. That is the case for *Deuterodon pedri*, an endemic species from a small drainage in the rio Doce basin in Minas Gerais, Brazil, for which the type material is in poor condition. Specimens of *D. pedri* were collected in 1865 by the Thayer Expedition to Brazil and fixed in spirits, enabling them to yield viable DNA. As the morphology alone of the type material does not allow for an accurate identification, we used both morphological and ancient DNA (aDNA) methods to decisively establish the identity of *D. pedri*. This identification allowed us to recognize the species among recently collected specimens and then, based on them, redescribe the species. A genetype for the lectotype of *D. pedri* is presented.

Keywords: Lectogenetype, Mini-Barcode, Primers, Rio Doce, Thayer Expedition.

Uma identificação acurada é fundamental para qualquer estudo que explora a biodiversidade. Infelizmente, espécimes de museu descritos há mais de cem anos, algumas vezes não são informativos o suficiente para uma identificação precisa da espécie representada pelo tipo. O uso de DNA antigo pode ajudar a resolver problemas taxonômicos, quando espécimes tipos não apresentam mais as características morfológicas diagnósticas que permitem a identificação precisa das espécies. Esse é o caso de *Deuterodon pedri*, uma espécie endêmica de uma pequena drenagem na bacia do rio Doce, em Minas Gerais, Brasil cujo material tipo encontra-se em condições precárias. Espécimes de *D. pedri* foram coletados em 1865 pela Expedição Thayer ao Brasil e fixados em "cachaça", o que permite apresentar DNA viável. Como apenas o exame morfológico do material tipo não permitiria a identificação precisa, nós usamos ambos os dados de análises morfológicas e DNA antigo (aDNA) para estabelecer decisivamente a identidade de *D. pedri*. Esta identificação permitiu reconhecer a espécie entre exemplares coletados recentemente e, com base neles, redescrever a espécie. É apresentado um genetipo para o lectótipo de *D. pedri.*

Palavras-chave: Expedição Thayer, Lectogenetipo, Mini-Barcode, Primers, Rio Doce.

Introduction

Taxonomy is fundamental to the biological sciences. More than merely labeling biodiversity, taxonomy is essential for any study exploring biodiversity. Thus, the accurate identification of life forms is crucial not only to understanding biodiversity but also for any taxonomy-based study, whether phylogenetic, evolutionary, inventorial, ecological or conservation-focused (Buerki, Baker, 2016; Vecchione *et al*., 2000). As the traditional repository for biological specimens, and recent years also for tissue samples, museum collections are a valuable resource for mapping and naming biodiversity. However, independent of the collecting and storage methods, museum materials tend to degrade over time. Century-old name-bearing types are often involved in nomenclatural doubts and ambiguities because they no longer exhibit the diagnostic features that allow an accurate identification (Cappellini *et al*., 2013). The use of ancient DNA (aDNA) can help to solve these taxonomical problems.

Ancient DNA techniques were first used in 1984 to recover DNA from a 150-year-old museum specimen of an extinct subspecies of the Plains Zebra *Equus quagga* (Higuchi *et al.*, 1984). In that study, aDNA was used to solve identification problems and was sufficient for determining the phylogenetic relationships of the species, which allowed the development of a project for breeding and re-introducing the Quaggas (http://www.quaggaproject.com/quagga-dna-results.htm).

Departamento de Zoologia and Programa de Pós-Graduação em Biologia Animal, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, RS, Brazil. (PCS) pricarola@gmail.com (corresponding author), (MCM) claudia.malabarba@ufrgs.br, (LRM) malabarb@ufrgs.br

Deuterodon pedri Eigenmann, 1908 was collected in 1865 by Ward during the Thayer Expedition to Brazil (see www.mcz.harvard.edu/Departments/Ichthyology/expeditions_thayer_hassler.html; Higuchi, 1996). As usual then, the specimens were fixed in an available spirit ("cachaça" in this case), which could yield viable DNA sequences (De Bruyn *et al*., 2011). The adoption of formaldehyde fixation made DNA difficult to amplify from more recent collections because formaldehyde fixation degrades DNA and cross-links DNA, DNA to protein, and protein to protein (Schander, Halanych, 2003). Eigenmann (1908) briefly described the species based on seven specimens, which were in very poor conditions (Fig. 1). Later, Eigenmann (1927:348) provided a more complete description of the species, indicating the same seven damaged specimens as cotypes (=syntypes) and again emphasized their poor conditions. In a more recent redefinition of the genus *Deuterodon* Eigenmann, 1907, Lucena, Lucena (2002) placed the species *D. pedri* as *incertae sedis* in Characidae. After inspection of two of the syntypes, Lucena, Lucena (2013) designated a lectotype (MCZ 21081) and consequently four paralectotypes (MCZ 170510) for *D. pedri,* observing that only five specimens (not seven) were registered as syntypes at the collection database of the Museum of Comparative Zoology. Lucena, Lucena (2013) also commented that the only paralectotype they examined clearly belonged to a species different from the lectotype.

In 2015, as part of her doctoral dissertation, one of the authors (PCS) had the opportunity to examine the types in an attempt to identify *D. pedri* and recognize it among recently collected material from the type locality. However, more than a hundred years after the description, the original specimens had deteriorated so severely that they no longer provide morphological information sufficient for a definitive identification of the species based on morphological traits alone. Thus, we sought to resolve the identity of *D. pedri* by combining the scant morphological information available with DNA data from the type specimens.

Ancient specimens usually contain highly degraded nucleic acid molecules (Linderholm, 2016), which directly complicate the amplification process. In addition, probably because of their poor conditions, in 1978 the syntypes of *D. pedri* (Fig. 1) were re-fixed in 10% formalin for 28 days, which further degraded the DNA and made the amplification and sequencing even more difficult. We applied a DNA protocol that proved successful for accessing the ancient information stored in the genomes of these museum materials. The sequences obtained from selected *Cytochrome oxidase c subunit 1* (COI) regions of the type specimens were compared with those of fresh specimens from the type locality and nearby localities to find a match and definitively identify the species. The aDNA sequences from the lectotype matched those from some of the fresh specimens, enabling the correct recognition and redescription of *D. pedri*. The *D. pedri* lectogenetype here presented corresponds to the DNA sequence obtained from the lectotype. The term was proposed by Chakrabarty (2010), to indicate DNA sequences generated from type specimens.

Fig. 1. *Deuterodon pedri*, MCZ 21081, lectotype, 78.56 mm SL and original labels.

Material and Methods

Tissue collection, DNA extraction and sequence generation for museum specimens. A 2 mg fragment of epaxial muscle tissue was removed from the right side of the body by incision from the lectotype (MCZ 21081) and one of the paralectotypes (MCZ 170510, 58.6 mm SL) of *D. pedri.* The tissue samples were processed in the molecular biology facilities of the Smithsonian National Museum of Natural History. DNA was extracted using the QIAamp DNA micro kit (Qiagen) following the manufacturer's protocol. A negative control containing no sample was prepared and analyzed following the same procedure used for the ancient samples. The extractions were conducted in a dedicated laboratory area free from DNA and PCR products (amplicons); meanwhile PCRs were carried out in an isolated section of a different laboratory to avoid contamination from other DNA extracts (Gilbert *et al*., 2005). Given the highly fragmented nature of the aDNA (Linderholm, 2016), the primers were designed in this study to flank small fragments of 100 - 150 bp of the COI gene (see Tab. 1). We designed 5 sets of primers to cover the entire COI gene, but only the two first sets were successfully amplified. PCRs were performed in a volume of

10 µL of a Promega Hotstart Master Mix under commercial recommendations. The PCR products were purified by the Exosap enzymatic method (25% exonuclease, 25% Shrimp Alkaline Phosphatase and 50% deionized water), and sequencing was performed at the Laboratory of Analytical Biology at National Museum of Natural History, Smithsonian, Washington DC. Each fragment of sequence was independently aligned using Clustal W in MEGA 6.0 software (Tamura *et al*., 2013). The p-distance between the ancient sequence and modern ones was estimated using the default conditions (Kimura 2-parameter model; d: Transitions + Transversions; uniform rates; Pairwise deletion; three codon positions selected) of the MEGA 6.0 software (Tamura *et al*., 2013). To illustrate the relationship among the sequences, a Neighbor Joining tree (using the same default conditions for calculating of p-distance) was constructed in MEGA. Additionally, polymorphic sites were identified using DnaSP software (Librado, Rosas, 2009) and a haplotype network was drawn using Network 5.0 software (Fluxus technology Ltd.).

Tab. 1. COI DNA primers designed for this study.

	Primer Sequence Left	Primer Sequence Right
COI - 1	5' GTATTYGTTGCCTGAGCYGG 3'	5' TATRACRAARGCATGTGCGG 3'
COI - 2	5' WTCCCTTTTAGGTGAYGACC 3'	5' KGGRGGAAGAAGYCARAAGC 3'

Tissue collection, DNA extraction and sequencing for modern specimens. For the comparison with museum samples, we sequenced 699 base pairs of the COI gene for 47 individuals from 30 species (**S1-** Available only as online supplementary files accessed with the online version of the article at http://www.scielo.br/ni). Tissues previously fixed in 96% ethanol from the fish collection of the Departamento de Zoologia, Universidade Federal do Rio Grande do Sul (UFRGS), Museu de Zoologia João Moojen da Universidade Federal de Viçosa (MZUFV), Royal Ontario Museum (ROM), and Universidade Federal da Bahia (UFBA). DNA was extracted from the gill filaments, muscles, or liver tissue of the samples using the "Phire Animal Tissue Direct PCR Kit" developed by Thermo Scientific® under commercial recommendations. COI was amplified with the primer cocktails FishF1t1 and FishR1t1 (Ivanova *et al*., 2007).

The PCR reactions were conducted in a reaction volume of 20 µL [10.3 µL of H2O, 2 µL of 10× reaction buffer (Platinum®Taq), 0.6 µL of MgCl2 (50 mM), 2 µL of dNTPs (2 mM), 2 µL of each primer (2 µM), 0.1 µL (5 U) of Platinum® Taq (Invitrogen), and 100 ng of template DNA]. The PCR conditions were as follows: an initial DNA denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 52°C for 40 s, and 72°C for 1 min and a final extension at 72°C for 10 min.

The PCR products were purified using the Exosap enzymatic method (25% exonuclease, 25% Shrimp Alkaline Phosphatase and 50% deionized water), and the sequencing was performed by Macrogen Inc, Seoul, South Korea, and by Ludwig Biotec at Porto Alegre, Brazil. The sequences were aligned using Clustal W in MEGA 6.0 software (Tamura *et al*., 2013), and the alignments were visually inspected for any obvious base miscall (base incorporated at the sequence different from the color pic showed at chromatograms). All work involving modern DNA was performed at the molecular biology laboratory of Departamento de Zoologia (UFRGS, Porto Alegre, RS, Brazil), with separately ordered primers (Ishida *et al.*, 2011).

Morphological techniques. Measurements and counts followed Fink, Weitzman (1974), with the exception of the number of scale rows below the lateral line, which were counted from the scale row ventral to the lateral line to the scale row nearest to the origin of the first pelvic-fin ray.

The measurements were taken point to point with an electronic caliper on the left side of specimens. Measurements are expressed as the percentage of standard length (SL) except for subunits of the head, which are recorded as percents of head length (HL). The counts of vertebrae, supraneurals, and procurrent caudal-fin rays were taken from cleared and stained specimens (c&s). The vertebral counts included the four vertebrae of the Weberian apparatus, and the terminal centrum counted as a single element.

Institutional abbreviations. MCN, Museu de Ciências Naturais da Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre; MCP, Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre; MCZ, Museum of Comparative Zoology of Harvard University, Cambridge; MNRJ, Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro; MZUSP, Museu de Zoologia, Universidade de São Paulo, São Paulo; UFRGS, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Results

Molecular analyses of type sequences. In the lectotype (MCZ 21081), only two sets of the five designed primers directly in-line succeeded in recovering the first third of the COI sequence with 136 (COI-1: starting at bp1) and 179 (COI-2: starting at bp115) base pairs each. Since the COI-1 sequence showed a p-distance 0.1 or 10% from recently collected material from the Santo Antônio River basin, which is the type locality of *D. pedri*, and a distance greater than 0.1 from all other characid species included in the alignment, it was excluded from further analysis (see

additional comments on the Discussion). Concerning the paralectotype (MCZ 170510, 58.6 mm SL), although the DNA extraction was successful, the amplification (PCR) failed. The COI-2 lectotype sequence was compared separately with the characid sequences of 48 species of *Astyanax* Baird & Girard, *Deuterodon*, *Hyphessobrycon* Durbin, *Jupiaba* Zanata, *Myxiops* Zanata & Akama, and *Probolodus* Eigenmann from the coastal and the rio Doce drainages. All data referring to this comparative material, including the GenBank accession numbers, (are listed in **S1** - Available only as online supplementary files accessed with the online version of the article at http://www.scielo.br/ni). The lectotype COI-2 sequence (accession number KY345055) showed the lowest p-distance (p=0.01=1%; **S2** - Available only as online supplementary files accessed with the online version of the article at http://www.scielo.br/ni) to a characid fish population recently collected from the rio Santo Antônio in the rio Doce basin (MCP 47661 and UFRGS 17543), indicating that these specimens actually correspond to *D. pedri* (Fig. 2).

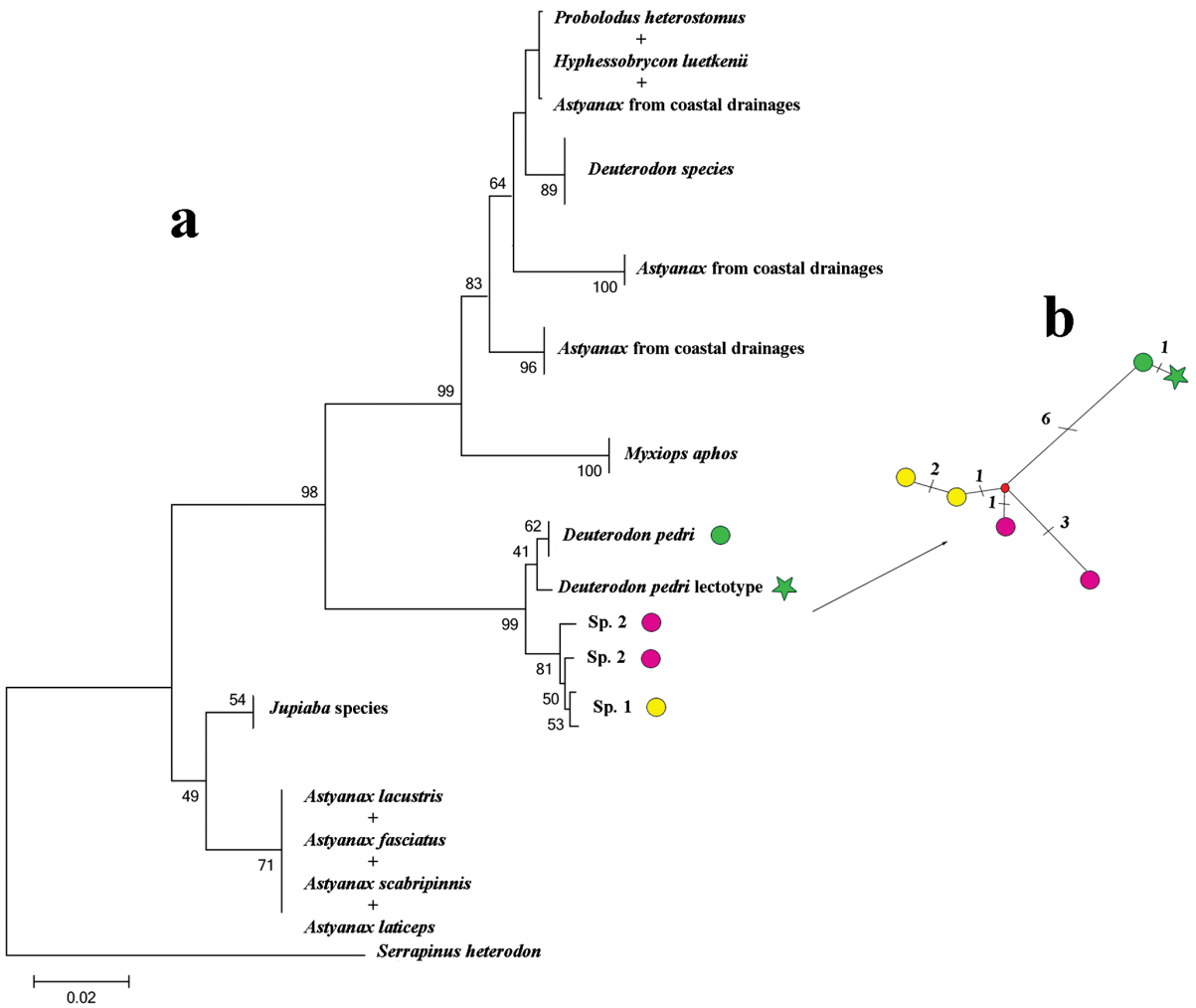

Fig. 2. Neighbor Joining tree and Haplotype network showing high similarity between specimens collected at rio Santo Antônio basin and sequence of the lectotype of *Deuterodon pedri*. **a.** Neighbor joining tree with bootstrap values. **b.** Haplotype network of *D. pedri* clade. Numbers in each branch of the net refer to number of mutational steps between haplotypes.

Morphological analyses of type specimens and of specimens identified by Eigenmann as *Deuterodon pedri*. The examination of the type series of *D. pedri* detected two different species among the syntypes. The lectotype (MCZ 21081, 78.6 mm SL) and two of the paralectotypes (MCZ 170510, 72.1 and 76.3 mm SL) have dentary teeth decreasing gradually in size posteriorly, bearing seven cusps each (Figs. 3a,c). This same tooth arrangement is observed in the characid samples from the rio Santo Antônio and rio Piracicaba populations, both of which are western tributaries of the rio Doce. That similarity is coincident with the analysis of the aDNA amplified from the lectotype, and also matches the DNA from the Santo Antônio river population. As with the lectotype, these two paralectotypes and the fresh specimens from these populations do not differ in other counts and measurements. Thus they are considered conspecific and the newly-collected samples from the rio Doce basin are used below in the redescription of *D. pedri.*

Two of the paralectotypes (MCZ 170510, 58.6 and 71.4 mm SL), however, have dentary teeth decreasing abruptly in size after the fifth (Figs. 3b,d), with five cusps on the three anterior dentary teeth instead of seven. These do not belong to *D. pedri*. Previously, Lucena, Lucena (2013) noted that at least one paralectotype was distinct from the lectotype, but it was left as an unidentified species. Comparison with recent material from Santana de Ferros (Ferros, state of Minas Gerais) and with the type series of *Astyanax intermedius* Eigenmann, 1908, allowed the identification of these two paralectotypes of *D. pedri*, as *A. intermedius*.

Furthermore in the original description of *Deuterodon pedri*, Eigenmann listed five lots (MCZ 20956-20960; Fig. 4) collected by Dom Pedro II, the Brazilian Emperor, at Santa Cruz, Rio de Janeiro State. Although Eigenmann (1908, 1927) did not include this additional material as part of the type series, he mentioned the possibility that they belong to *D. pedri*. Due to the poor conditions of the types, Eigenmann (1908:99, 1927:348) claimed that an "absolute morphologically identification is impossible." Examination of two of these five lots (MCZ 20956 and 20958) allowed us to reject their assignment to *D. pedri*. These specimens present bony hooks distributed on the anal-, dorsal-, pectoral-, pelvic- and caudal-fin rays, instead of bony hooks only on the anal-fin rays, as observed in *D. pedri*. In addition, these specimens have fewer lateral line scales (35-37 *vs*. 39-41 in *D. pedri*) and a humeral spot with the dorsal portion expanded like a large dot and the ventral portion narrow and curved anteriorly (like a comma) rather than bar-shaped as in *D. pedri*. In view of that, we propose that these lots collected by Dom Pedro II be assigned to *Astyanax hastatus* Myers, 1928, which presents these same features.

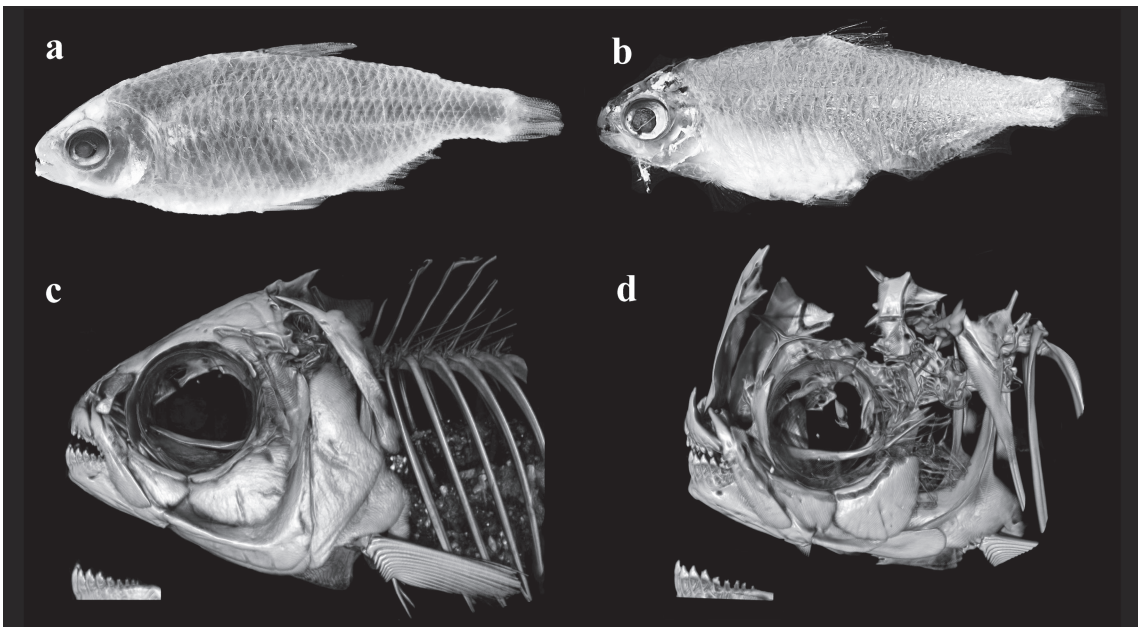

Fig. 3 Two paralectotypes of *Deuterodon pedri*, MCZ 17510. **a-c.** Photograph and X-ray computed tomography of the paralectotype with 72.1 mm SL, showing the dentary teeth decreasing gradually in size, corresponding to *D. pedri*. **b-d.** Photograph and X-ray computed tomography of the paralectotype with 58.6 mm SL, showing the dentary teeth decreasing abruptly, corresponding to *Astyanax intermedius*.

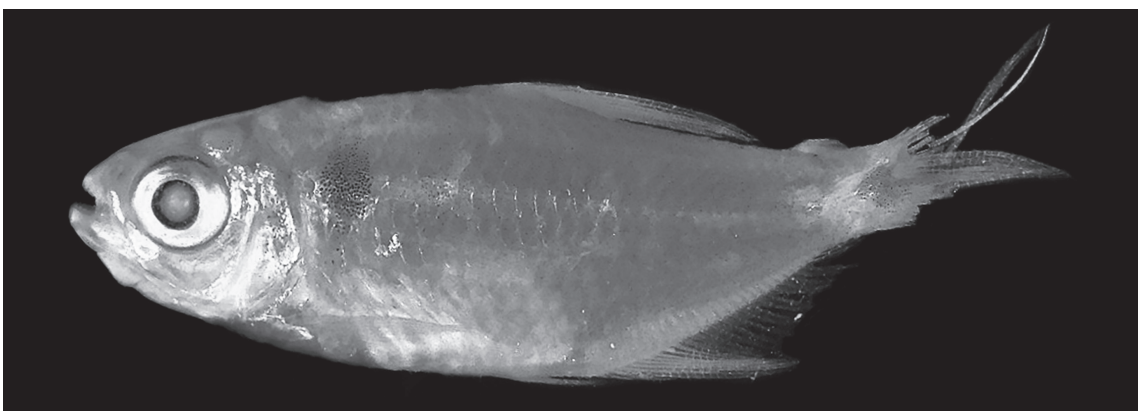

Fig. 4. Specimen of *Astyanax hastatus* from Santa Cruz, Rio de Janeiro (MCZ 20958, 33.0 mm SL), tentatively identified as *Deuterodon pedri* by Eigenmann (1908).

***Deuterodon pedri* Eigenmann, 1908**

Figs. 1, 3a-b, 5-7, Tab. 2

Deuterodon pedri Eigenmann, 1908:98 (brief description). -Eigenmann, 1927:348 (description; type locality: Santa Anna de Ferros, Minas Gerais, Brazil). -Lucena, Lucena, 2002: 119 (placed as *incertae sedis* in Characidae). -Lucena, Lucena, 2013: 598 (MCZ 21081 designated as lectotype). -Coutinho-Sanches, Dergam, 2015: 9 (Cytogenetic data).

Diagnosis. *Deuterodon pedri* can be distinguished from all congeners by the following combination of characters: a characteristic pigmentation on the two or three longitudinal and dorsolateral series of scales below the dorsal fin, each scale showing an arched and well-delineated strip at the distal margin of the free border; this strip is either dark brown when chromatophores are expanded (Fig. 5a) or translucent when chromatophores are contracted (Fig. 5b) – in either case it is clearly distinguishable from the pigmentation of the whole scale; dentary teeth decreasing gradually in size; number of anal-fin rays 18-21 in females (n=26) and 22-24 in males (n=3); longitudinal lateral silver band starting 5 or 6 scales posterior to upper margin of the opercle; humeral spot bar-shaped, vertically elongated above and below the lateral line; bony hooks only on anal-fin rays of mature males.

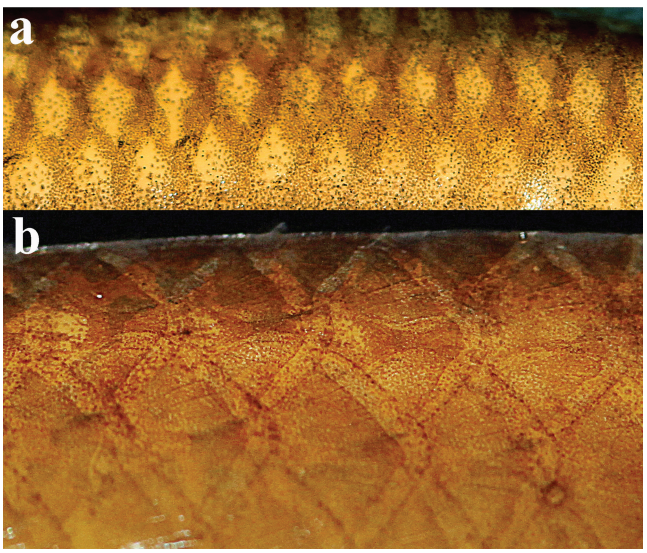

Fig. 5. Detailed images of the dorsolateral series of scales in *Deuterodon pedri*, showing their pigmentation pattern, each scale showing an arched and well delineated strip at the distal margin of the free border that is either dark brown when chromatophores are expanded (**a.** MNRJ 38463, 51.18 mm SL) or translucent when chromatophores are contracted (**b.** MCN 19698, 85.70 mm SL).

Deuterodon pedri can be further differentiated from morphologically similar species of the genus *Astyanax* that occur in Atlantic coastal river basins by the higher number of perforated scales in the lateral line (39-41 *vs*. 35-38 in most of species), except *A.* aff. *fasciatus* (Cuvier, 1819), *A. parahybae* Eigenmann, 1908 and *A. taeniatus* (Jenyns, 1842). From *A.* aff. *fasciatus*, *A. parahybae* and *A. scabripinnis* (Jenyns, 1842) it can be distinguished by the dentary teeth decreasing gradually in size posteriorly (*vs*. dentary teeth decreasing abruptly after the fourth tooth). From *A. taeniatus*, *D. pedri* can be distinguished by the absence of a gap between the symphyseal teeth of dentary and by a rectangular and vertically elongate humeral spot (*vs*. the presence of a gap between the symphyseal dentary teeth and humeral spot shaped like a comma).

Description. Morphometric data are summarized in Tab. 2. Body compressed and elongated; deepest at dorsal-fin origin. Snout profile slightly rounded from margin of upper lip to vertical through anterior nostrils. Dorsal profile of head straight between vertical through posterior nostril and tip of supraoccipital spine. Body profile convex from tip of supraoccipital spine to dorsal-fin base; ventrally slanted from this point to caudal peduncle. Ventral profile of body convex from margin of lower lip to pelvic-fin origin, and straight from that point to anal-fin origin. Body profile along anal-fin base dorsally slanted. Caudal peduncle elongated and nearly straight to slightly concave along both dorsal and ventral margins.

Head small. Mouth terminal or slightly sub-terminal. Maxilla extending posteriorly to vertical through anterior margin of orbit, slightly oblique. Anterodorsal border of maxilla slightly concave, posterodorsal border slightly convex, and ventral border convex.

Premaxilla with two tooth rows; outer row with three (6) or four (5) teeth bearing four, five or six cusps with central cusp longer. Five teeth (11) on inner row, gradually decreasing in size from first to fifth teeth. Symphyseal premaxillary teeth of inner series distinctively narrower than other teeth and asymmetrical, with two or three short cusps shorter on medial side near symphysis, followed by a high cusp and another three or four short cusps on lateral side of tooth. Teeth with five to nine cusps, with central cusp longer and as broad as other cusps. Maxilla with three (10) or four (1) teeth with five to seven cusps (usually 5 or 6), central cusp longest. Seven or six anteriormost dentary teeth larger than other teeth, with five to eight cusps, followed by three or four teeth gradually decreasing with three to five cusps. Central cusp in all teeth as long and broad as other cusps. Symphyseal teeth of dentary narrower than others with seven or eight cusps.

Dorsal-fin rays ii,9(27). Distal margin of dorsal fin straight or slightly convex. Dorsal-fin origin approximately at middle of SL. Anal-fin rays ii-iv, 18(5), 19(15), 20(4), 21(2) in females and 22(1), 23(1), 24(1) in males. Anal-fin distal border concave, with rays decreasing in size, with anterior-most rays much longer than others. Anal-fin origin located approximately on vertical through base

of posterior third portion of dorsal-fin. Pectoral-fin rays i, 11(4), 12(15), 13(9). Pectoral-fin tip falls one or two scales short of vertical through pelvic-fin insertion or reaching pelvic-fin origin in some specimens. Pelvic-fin rays i(9), ii(18), 7(27). Dorsal-fin origin located at vertical line through first third part of pelvic-fin. Tip of adpressed pelvic fin falls one or two scales short of anal-fin origin. Caudal-fin forked with 18(1), 19(22), 20(2) principal rays. Dorsal procurrent rays 11(1) or 12(1). Ventral procurrent rays 10(2).

Lateral line slightly curved anteriorly, completely pored, with 38(2), 39(10), 40(13) or 41(4) (mean= 39.7, n = 29) perforated scales. Horizontal scale rows between dorsal-fin origin and lateral line 5(26) or 6(1). Horizontal scale rows between lateral line and pelvic-fin origin 4(27). Pre-dorsal scales 9(1), 10(5), 11(15), 12(6), arranged in regular or irregular series. Thirteen (12) or fourteen (13) scale rows around caudal peduncle. Scale sheath along anal-fin base formed by six to ten scales in a single series and covering base of anteriormost rays.

Precaudal vertebrae 16(2); caudal vertebrae 21(2); total vertebrae 37(2). Supraneurals 5(2). First gill-raker upper limb of 6(9), 7(4), or 8(2) + lower branch 11(8), 12(6) or 13(1). Anal pterygiophores 20(2). Dorsal pterygiophores 10(2).

Coloration in alcohol. Dorsal and dorsolateral portions of head light brown. Infraorbitals, preopercle and opercular bones silver, without chromatophores or rarely a few. Lips yellow to light brown, snout with concentration of few chromatophores. Dorsal and dorsolateral portion of body dark brown. Scales above lateral band showing an arched and well-delineated strip bordering the posterior margin. This strip is either dark brown when chromatophores are expanded (Fig. 5a) or translucent when chromatophores are contracted (Fig. 5b); in any case it is clearly distinguishable from the pigmentation of the whole scale. A conspicuous dark or silver midlateral band extending from two scales after humeral spot to the middle caudal-fin rays crossing a slightly rectangular caudal spot. Humeral spot vertical and bar-shaped, extended for two or three scales above and one or two scales below lateral line. Pectoral-, pelvic-, and anal-fins hyaline. Dorsal fin usually hyaline; in some cases with disperse chromatophores on distal portion of rays. Caudal-fin border slightly black (Fig. 6).

Sexual dimorphism. One pair of bony hooks per segment are present on each lepidotrichium along the anal-fin rays of mature males. They are delicate and narrow, distributed from the 4th unbranched ray to the 12th to 19th branched rays. The number of segments bearing bony hooks decreases gradually from the anterior to the posterior rays on all observed males. Males have a higher number of rays (22-24) than females (18-21). Gill glands were not observed on first gill arch in either males or females.

Geographical distribution. Until recently, *Deuterodon pedri* was considered endemic to the rio Santo Antônio basin (its type locality). The latest collecting trips have found the species in the rio Guanhães, which is a tributary of the rio Santo Antônio, and at the confluence of the rio Brumadinho and the rio Caraça, which are tributaries of the rio Piracicaba. All of these are sub-drainages of the rio Doce (Fig. 7).

Tab. 2. Morphometric data for *Deuterodon pedri*, Lectotype (Lec), Paralectotypes (Par) and non-type specimens. SD = standard deviation.

Character	Lec	Par (n=2)				Males (n= 2)				Females (n= 23)			
		Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD
Standard length (mm)	78.5	72.06	76.27	74.17	-	86.9	87.18	87.04	-	43.5	89.23	72.40	-
Percents of standard length													
Head length	24.3	23.7	24.8	24.2	0.78	21.8	23.4	22.6	1.11	22.3	27.4	24.4	1.50
Predorsal distance	48.0	49.5	50.7	50.1	0.87	48.1	50.00	49.00	1.34	46.6	52.7	50.3	1.40
Prepelvic distance	45.7	42.9	47.9	45.4	3.50	43.4	44.4	43.9	0.73	43.4	49.9	46.9	1.32
Prepectoral distance	24.5	23.00	23.6	23.3	0.48	23.3	25.3	24.3	1.44	21.6	25.9	23.6	1.27
Preanal distance	65.1	64.4	65.3	64.8	0.67	62.7	63.5	63.1	0.53	62.9	71.5	66.6	1.94
Depth at dorsal-fin origin	32.3	30.6	30.7	30.7	0.12	31.1	31.9	31.5	0.61	27.6	37.6	32.0	2.32
Caudal peduncle depth	10.4	10.7	11.0	10.9	0.20	10.0	10.2	10.1	0.14	9.6	12.0	10.9	0.64
Caudal peduncle length	15.9	11.8	14.5	13.2	1.95	12.6	14.4	13.5	1.29	13.1	16.4	14.8	0.96
Anal-fin base	23.9	-	24.3	-	-	28.9	30.5	29.7	1.12	20.3	26.2	24.3	1.16
Dorsal fin length	-	21.8	23.8	22.8	1.43	24.2	24.6	24.4	0.31	19.0	26.3	23.8	1.59
Pelvic fin length	14.3	15.5	15.6	15.5	0.05	16.4	16.9	16.6	0.33	14.5	16.7	15.5	0.66
Pectoral fin length	15.7	17.5	20.61	19.0	2.17	21.0	21.9	21.5	0.60	15.8	23.3	21.0	1.45
Percents of head length													
Snout length	24.0	22.9	23.8	23.4	0.62	24.1	24.9	24.5	0.58	20.7	27.8	23.6	1.75
Upper jaw length	39.3	38.9	39.0	38.9	0.62	34.4	41.1	37.8	4.75	30.4	38.3	34.7	1.76
Orbital diameter	32.3	33.9	34.9	34.4	0.67	37.0	41.3	39.2	3.02	38.6	44.0	40.9	1.45
Interorbital width	32.3	33.8	33.9	33.9	0.09	33.6	37.0	35.3	2.44	27.6	35.8	32.2	2.34

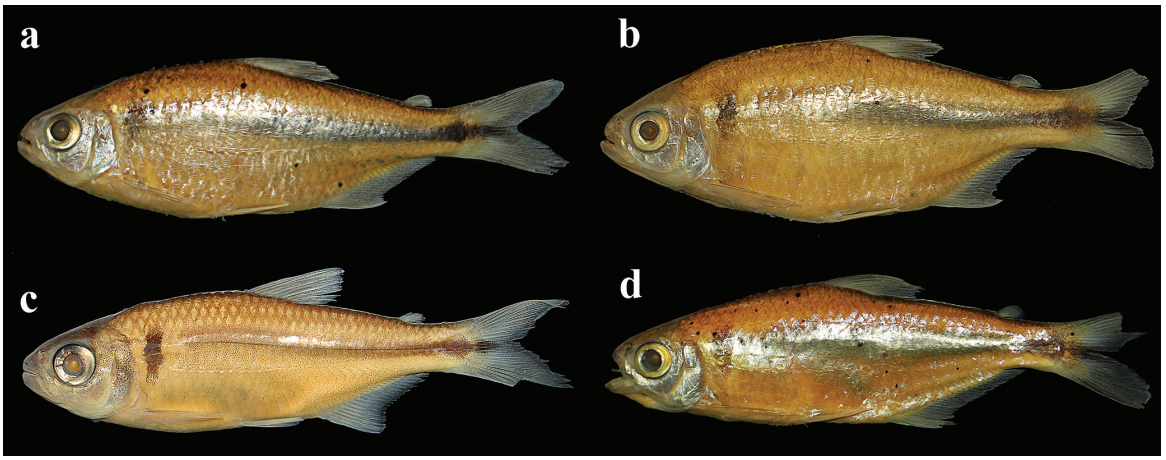

Fig. 6. Recently collected specimens of *Deuterodon pedri* showing variability according to sex and body size: a, female, MCN 19697, 79.82 mm SL; b, female, MCN 19698, 85.70 mm SL; c, juvenile, MNRJ 38463, 51.18 mm SL; d, male, UFRGS 17543, 86.47 mm SL.

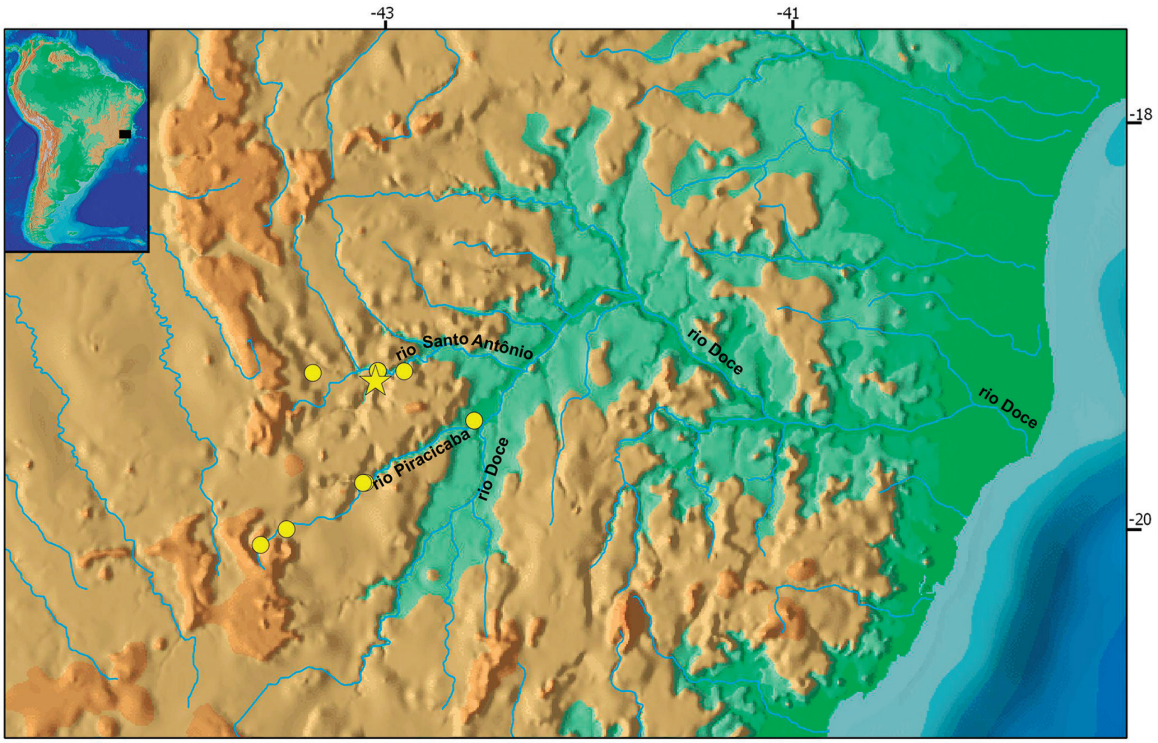

Fig. 7. Rio Doce drainage with the distribution of *Deuterodon pedri*, southeastern Brazil. The star indicates the type locality.

Ecological notes. *Deuterodon pedri* is found in localities with rapid-to-median speed dark waters with substrates of rocks and sand. At the type locality, this species is syntopic with other endemic species from the rio Santo Antônio basin (*e.g.*, *Henochilus wheatlandii* Garman).

Conservation status. According to IUCN criteria, we recommend that *D. pedri* be classified as an Endangered species (EN). The species is known from an Extent of Occurrence (EOO) of approximately 4,900 km^2 (B1). There is a significant continued decline in habitat quality [criterion B1b(iii)] from continuous extensive iron ore quarrying and from the rupture of two dams in 2015 that released a large amount of iron ore waste, which contaminated most of the rio Doce, including the area between the rio Santo Antônio and rio Piracicaba, where the species is found. This accident has been reported to have eliminated all endemic flora and fauna in the affected waterways (Lambertz, Dergam, 2015). Additionally, the habitat of the species has been severely fragmented by hydroelectric power dams that modify river hydrodynamics, by quarrying activities that renders river stretches uninhabitable and due to the presence of 35 exotic fish species in that drainage (Barros *et al*., 2012).

Discussion

The use of aDNA recovered from the lectotype associated with the examination of new and representative samples of characid fish species from the type locality and nearby proved to be a powerful framework to solve the taxonomical puzzle of *Deuterodon pedri*. According to Marinho *et al*. (2015), the most challenging genera of the Characidae (*Astyanax*, *Bryconamericus* Eigenmann, *Hemigrammus* Gill, *Hyphessobrycon* and *Moenkhausia* Eigenmann) present serious taxonomic problems mainly due to the existence of poorly known widespread species complexes, old and short descriptions, and poorly preserved type specimens, most of which have unknown type localities and uncertain geographical distributions. The case study of *D. pedri* typifies the problems exposed by Marinho *et al*. (2015). The species was described based on poorly preserved material (Eigenmann, 1908); the type series included different species (Lucena, Lucena, 2013 and our results), and its assumed distribution was overly broad because of the incorrect assignation of specimens of a third species (our results). Not surprisingly, the species has not been cited in the literature for a long time, and it has remained an enigma for ichthyologists. Recently, the description of a new species of Diptera (Marshall, Evenhuis, 2015) that designated a photo as the holotype has triggered the discussion of whether preserved specimens are needed for species descriptions (Amorim *et al.*, 2016; Pape, 2016; Krell, 2016; Ceríaco *et al.*, 2016). *Deuterodon pedri* provides a sound argument for the importance and utility of preserved types in this discussion.

The result obtained from the first fragment (COI-1), with a p-distance equal or larger than 0.1 when compared with all characid species included at the alignment, may be explained by nucleotide misincorporation in aDNA (Sawyer *et al.*, 2012). Studies with this kind of sample have revealed an increased occurrence of depurination of the DNA followed by hydrolysis of the phosphate-sugar backbone (Briggs *et al.*, 2007). An alternative explanation for COI-1 result is that the primers amplified a numt instead COI-1. The amplification of numts seems to increase with the usage of primers to amplify small fragments and is common in studies with aDNA (Tex *et al.*, 2010).

The second fragment (COI-2), although short (179 base pairs), is located in a variable region of the gene and was sufficient to determine the identity of the lectotype of *D. pedri*. Long DNA barcode sequences are no longer considered essential, since even a few base pairs may be sufficiently informative in solving taxonomical questions (see Hajibabaei *et al.*, 2006). The use of such a mini-barcode has been proposed as an alternative when it is not possible to obtain the entire fragment because of the degraded nature of the samples (Boyer *et al.*, 2012). Our data further demonstrates that mini-barcodes can be as effective as the entire COI gene in determining species identities.

Deuterodon pedri is a valid species but should be maintained as *incertae sedis* in Characidae as previously proposed by Lucena, Lucena (2002) since it does not share the synapomorphies that define the genus: (1) the anterior region of the toothed portion of the maxilla deeper than the posterior region of the toothed portion; (2) the ventral margin of toothed portion of maxilla arching toward the ventral margin of the premaxilla, determining an alignment between maxillary and premaxillary teeth; and (3) posterior region of the maxilla without teeth smaller than anterior toothed region. An ongoing and more comprehensive study is being developed to reconstruct *D. pedri's* relationships and determine its most appropriate generic assignment.

Increasing advances are making molecular techniques more accessible, facilitating the use of aDNA (Linderholm, 2016) as a complement to solve taxonomical problems. In cases such as that exemplified herein with *D. pedri* (see discussion in Marinho *et al.*, 2015), the aDNA of museum types associated with DNA and morphological studies of new samples will be the key to help solving problems related to the species identity and relationships. Notwithstanding the degraded nature of aDNA prevents the amplification of full COI fragments, we herein demonstrate that the usage of mini-barcode is a powerful tool in the resolution of taxonomical problems. So, we strongly recommend the use of small fragments of aDNA for taxonomic resolution in cases with high complexity.

Material examined. *Deuterodon pedri*: Lectotype: MCZ 21081, 78.6 mm SL, Brazil, Minas Gerais State, Santa Anna de Ferros, rio Doce basin at rio Santo Antônio, approx. 19º17'S 43º02'W, T. Ward, 1865. Lectogenetype: GenBank accession number KY345055. Paralectotypes: MCZ 170510, 2 of 4, 58.64-76.27 mm SL, collected with the lectotype.

Non type-specimens. All from Brazil, Minas Gerais State: MCP 47661, 7, 76.71-86.90 mm SL, Ferros, rio Santo Antônio basin, 19º13'55"S 43º01'17"W. UFRGS 17543, 6, 88.07-74.97 mm SL, and UFRGS 17544, 1 c&s, 89.23 mm SL, same locality as MCP 47661. MCN 19700, 1, 69.99 mm SL, Ferros, rio Esmeralda, 19º14'01"S 42º53'30"W. MCN 19697, 4, 76.4-79.4 mm SL (counts only), Ferros, rio Santo Antônio, 19º13'55"S 43º01'17"W. MCN 19698, 2, 85.82-73.61 mm SL, Ferros, rio Santo Antônio, 19º13'55"S 43º01'17"W. MNRJ 38463, 10 of 90, 30.49-54.51 mm SL (4, 43.52-54.51 mm SL), Catas Altas, confluence of the rio Brumadinho and rio Caraça, 20º00'34"S 43º28'15"W. MNRJ 38444, 6, 29.73-61.55 mm SL (3, 44.75-61.55 mm SL), Catas Altas, rio Conceição, 20º05'11"S 43º35'54"W. MZUSP 75389, 15, 56.51-72.08 mm SL, Morro do Pilar, rio Preto, on the road between Morro do Pilar and Santo Antônio, 19º14'29"S 43º20'29"W. MZUSP 73133, 5, 52.30-37.53 mm SL (counts only), Dores de Guanhães, rio Guanhães, road km 351, 19°03'22"S 42°55'53"W. MZUSP 110653, 1, 66.59 mm SL (counts only), João Monlevade, rio Santa Bárbara, 19º46'50"S 43º05'W. MZUSP 110671, 4, 32.69-64.65 mm SL (counts only), João Monlevade, rio Piracicaba, 19º46'55"S

43º05'38"W. MZUSP 75340, 30 of 134 specimens 43.70-60.79 mm SL (counts only), Dores de Guanhães, rio Guanhães, road to quarry, Maria das Dores farm, approximately 8 km N of Dores de Guanhães, 19°00'33.23"S 42°56'20"W. MZUSP 104709, 6, 53.20-62.67 mm SL (counts only), Conceição do Mato Dentro, córrego São João, 19º02'29"S 49º20'34"W.

Acknowledgments

We are indebted to Karsten Hartel (MCZ) for the permission to study and to sample tissues from type specimens, and to Andrew Williston (MCZ) for providing CTscans; both of them supported the visit by PCS to MCZ. The present study was largely accomplished during a 10-month visit of the first author to the NMNH (Smithsonian Institution, Washington DC) in 2015-2016, financially supported by a CNPq scholarship (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil). In this sense, we are indebted to the NMNH staff, in particular that of the Fish Division, Lynne Parenti and David Johnson, for guidance. The authors would like to thank Richard Vari (*in memoriam*) who encouraged, facilitated and supported the execution of this study at the Smithsonian. The molecular work was undertaken at LAB (SI), and we thank Lee Weight and Jeff Hunt for their support and Jeff Clayton and Chris Murphy for help with fish collection at NMNH and MSC, respectively. We are also grateful to Jorge Dergam (UFV) for collecting an important amount of fresh material and kindly making it available for this study; to Oswaldo Oyakawa for taking photos and tissue samples from the lectotype; and to Angela Zanata for providing tissues from *Myxiops*. We thank the editor Brian Sidlauskas and three anonymous reviewers for comments that greatly improved this manuscript. Financial support was provided by BRBOLD Project for the sequencing. CNPq, CAPES and FAURGS provided grants and scholarships to the authors (PCS, MCM, LRM).

References

Amorim DS, Santos CMD, Krell FT, Dubois A, Nihei SS, Oliveira OMP *et al*. Timeless standards for species delimitation. Zootaxa. 2016; 4137(1):121-28.

Barros LC, Santos U, Zanuncio JC, Dergam JA. *Plagioscion squamosissimus* (Sciaenidae) and *Parachromis managuensis* (Cichlidae): a threat to native fishes of the Doce River in Minas Gerais, Brazil. PlosOne [serial on the Internet]. 2012; 7(6):e39138. Available from: http://dx.doi:10.1371/journal.pone.0039138

Boyer S, Brown SDJ, Collins RA, Cruickshank RH, Lefort MC, Malumbres-Olarte J, Wratten SD. Sliding window analyses for optimal selection of mini- barcodes, and application to 454 pyrosequencing for specimen identification from degraded DNA. PlosOne [serial on the Internet]. 2012; 7(5):e38215. Available from: http://dx.doi:10.1371/journal.pone.0038215

Briggs A, Stenzel U, Johnson PL, Green R, Kelso J, Prüfer K *et al*. Patterns of damage in genomic DNA sequences from a Neanderthal. Proc Natl Acad Sci. 2007; 104(37):14616-21.

De Bruyn M, Parenti LR, Carvalho GR. Successful extraction of DNA from archived alcohol-fixed white-eye fish specimens using an ancient DNA protocol. J Fish Biol. 2011; 78(7):2074-79.

Buerki S, Baker WJ. Collections based research in the genomic era. Biol J Linn Soc. 2016;117(1):5-10.

Cappellini E, Gentry A, Palkopoulou E, Ishida Y, Cram D, Roos AM, *et al*. Resolution of the type material of the Asian elephant, *Elephas maximus* Linnaeus, 1758 (Proboscidea, Elephantidae). Zool J Linn Soc. 2013; 170(1):222-32.

Ceríaco LMP, Gutiérrez EE, Dubois A, Abdala CS, Alqarni AS, Adler K, *et al*. Photography-based taxonomy is inadequate, unnecessary, and potentially harmful for biological sciences. Zootaxa. 2016; 4196(3):435-45.

Chakrabarty P. Genetypes: a concept to help integrate molecular phylogenetics and taxonomy. Zootaxa. 2010; 2632(1):67-68.

Coutinho-Sanches N, Dergam J. Cytogenetic and molecular data suggest *Deuterodon pedri* Eigenmann, 1907 (Teleostei: Characidae) is a member of an ancient coastal group. Zebrafish. 2015; 12(15):357-65.

Eigenmann CH. Preliminary descriptions of new genera and species of tetragonopterid characins. (Zoölogical Results of the Thayer Brazilian expedition.). Bull Mus Comp Zool. 1908; 52(6):91-106.

Eigenmann CH. The American Characidae. Cambridge: Printed for the Museum; 1927. (Memoirs of the Museum of Comparative Zoology at Harvard College; vol 43, part 4).

Fink W, Weitzman S. The so-called cheirodontin fishes of Central America with descriptions of two new species (Pisces: Characidae). Washington (DC): Smithsonian Institution Press; 1974. (Smithsonian contributions to Zoology; No. 172).

Gilbert MT, Bandelt HJ, Hofreiter M, Barnes I. Assessing ancient DNA studies. Trends Ecol Evol. 2005; 20(10):541-44.

Hajibabaei M, Smith MA, Janzen DH, Rodriguez JJ, Whitfield JB, Hebert PDN. A minimalist barcode can identify a specimen whose DNA is degraded. Mol Ecol Notes. 2006; 6(4):959-64.

Higuchi H. An updated list of ichthyological collecting stations of the Thayer Expedition to Brazil (1865–1866). 1996. Available from: www.mcz.harvard.edu/Departments/Ichthyology/docs/Higuchi_1996_Thayer_Formated_prelim.pdf (30/Sep/2016).

Higuchi R, Bowman B, Freiberger M, Ryder O, Wilson A. DNA sequences from the quagga, an extinct member of the horse family. Nature. 1984; 312(5991):282-84.

Ishida Y, Demeke Y, van Coeverden de Groot PJ, Georgiadis NJ, Leggett KE, Fox VE, Roca AL. Distinguishing forest and savanna African elephants using short nuclear DNA sequences. J Hered . 2011; 102(5):610-16.

Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN. Universal primer cocktails for fish DNA barcoding. Mol Ecol Notes. 2007; 7(4):544-48.

Krell FT. Taxonomy: preserve specimens for reproducibility. Nature. 2016; 539(7628):168.

Lambertz M, Dergam JA. Mining disaster: huge species impact.

Nature. 2015; 528(7580):39.

Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009; 25(11):1451-52.

Linderholm A. Ancient DNA: the next generation - chapter and verse. Biol J Linn Soc. 2016; 117(1):150-60.

Lucena CAS, Lucena ZMS. Redefinição do gênero *Deuterodon* Eigenmann (Ostariophysi: Characiformes: Characidae). Comum Mus Ciênc Tecnol, Sér Zool. 2002; 15(1):113-35.

Lucena C, Lucena Z. Designation of a lectotype for *Deuterodon pedri* Eigenmann, 1908 (Characiformes: Characidae). Zootaxa. 2013; 3635(5):598.

Marshall SA, Evenhuis NL. New species without dead bodies: a case for photo-based descriptions, illustrated by a striking new species of *Marleyimyia* Hesse (Diptera, Bombyliidae) from South Africa. Zookeys [serial on the Internet]. 2015; 525:117-27. Available from: http://dx.doi.org/10.3897/zookeys.525.6143

Marinho MMF, Camelier P, Birindelli JLO. Redescription of *Astyanax guianensis* Eigenmann 1909 (Characiformes: Characidae), a poorly known and widespread fish from the Amazon, Orinoco and Guiana Shield drainages. Zootaxa. 2015; 3931(4):568-78.

Pape T. Taxonomy: species can be named from photos. Nature. 2016; 537(7620):307. Available from: http://dx.doi.org/10.1038/537307b

Sawyer S, Krause J, Guschanski K, Savolainen V, Pääbo S. Temporal patterns of nucleotide misincorporations and DNA fragmentation in ancient DNA. PlosOne [serial on the Internet]. 2012; 7(3):e34131.Available from: http://dx.doi.org/10.1371/journal.pone.0034131

Schander C, Halanych KM. PCR and formalinized animal tissue - a short review and protocols. Org Divers Evol. 2003; 3(3):195-205.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013; 30(12):2725-29.

Tex R-Jd, Maldonado JE, Thorington R, Leonard JA. Nuclear copies of mitochondrial genes: another problem for ancient DNA. Genetica [serial on the Internet]. 2010; 138(9-10):979-84 Available from: http://dx.doi.org/10.1007/s10709-010-9481-9

Vecchione M, Mickevich MF, Fauchald K, Collette BB, Williams AB, Munroe TA, Young RE. Importance of assessing taxonomic adequacy in determining fishing effects on marine biodiversity. ICES J Mar Sci. 2000; 57(3):677-81.

Submitted October 6, 2016
Accepted March 8, 2017 by Brian Sidlauskas