FiSHMED
Fishes in Mediterranean Environments

sibic
Sociedad Ibérica de Ictiología
Sociedade Ibérica de Ictiologia

Taxonomic re-evaluation of the non-native cichlid in Portuguese drainages

João Carecho[1], Flávia Baduy[2], Pedro M. Guerreiro[2], João L. Saraiva[2], Filipe Ribeiro[3], Ana Veríssimo[4,5]*

1. Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal
2. CCMAR, Centre for Marine Sciences, Universidade do Algarve, 8005-139 Faro, Portugal
3. MARE – Marine and Environmental Sciences Centre, Faculty of Sciences, University of Lisbon, Lisbon, Portugal
4. CIBIO - Research Centre in Biodiversity and Genetic Resources, Campus Agrario de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal
5. Virginia Institute of Marine Science, College of William and Mary, Route 1208, Greate Road, Gloucester Point VA 23062, USA

* correspondence to averissimo@cibio.up.pt

SUMMARY

A non-native cichlid fish firstly reported in Portugal in 1940 was originally identified as *Cichlasoma facetum* (Jenyns 1842) based on specimens reported from "Praia de Mira" (Vouga drainage, northwestern Portugal). Currently, the species is known only from three southern Portuguese river drainages, namely Sado, Arade and Guadiana, and no other record has been made from Praia de Mira or the Vouga drainage since the original record. The genus *Cichlasoma* has since suffered major taxonomic revisions: *C. facetum* has been considered a species-complex and proposed as the new genus *Australoheros*, including many species. Given the current taxonomic re-arrangement of the *C. facetum* species group, we performed a taxonomic re-evaluation of species identity of this non-native cichlid in Portuguese drainages using morphological and molecular analyses. Morphological data collected on specimens sampled in the Sado river drainages confirmed the identification as *Australoheros facetus*. Moreover, nucleotide sequences of the cytochrome b gene obtained from specimens from Sado, Arade and Guadiana showed the existence of a single haplotype across drainages, which was 100% identical to *A. facetus* specimens collected in native Argentinean waters (i.e. Uruguay River). The current non-native distribution range of the species in Portugal results from human-mediated introductions across the southern drainages.

Keywords: Non-native fish, Cichlids, Taxonomic identification, Morphology, Cytochrome *b*

Citation: Carecho J, Baduy F, Guerreiro PM, Saraiva JL, Ribeiro F, Veríssimo A (2018) Taxonomic re-evaluation of the non-native cichlid in Portuguese drainages. Fishes in Mediterranean Environments 2018.001: 12p. https://doi.org/10.29094/FiSHMED.2018.001

DOI: 10.29094/FiSHMED.2018.001

INTRODUCTION

Non-native species, and particularly those that become invasive, are considered one of the major global threats to biodiversity, only surpassed by habitat loss and fragmentation (Mack et al. 2000; Clavero & García-Berthou, 2005; Gallardo et al., 2016). Recent estimates suggest that over 12 000 non-native species are currently present within European Union borders, of which around 10–15% are invasive (Sundseth, 2014). The economic consequences of biological invasions are remarkable and amount to about €12 billion/year over the past 20 years; yet, this amount is expected to increase considering the increasing rates of new introductions (Sundseth, 2014; Genovesi et al., 2015).

Among vertebrates, freshwater fishes are the most widely introduced species globally and comprise the best-studied group regarding invasion impacts (Rahel, 2002; Leprieur et al., 2008; Strayer, 2010). Not surprisingly, non-native fishes are considered one of the major global causes of decline in aquatic biodiversity (Cowx, 1998; Helfman, 2007). This is of particular concern within the Mediterranean biodiversity hotspot, where many regions show high levels of aquatic endemism, namely freshwater fishes (Myers et al., 2000; Smith & Darwall, 2006).

The Iberian Peninsula (Portugal and Spain) is a notable example of the current situation in Mediterranean freshwater ecosystems: non-native freshwater fish (NNFF) records are among the highest within the Mediterranean basin although the region hosts a large number of endemic freshwater fish species (Elvira & Almodóvar, 2001; Ribeiro et al., 2009). A recent revision indicated over 30 NNFF within Iberian drainages (Marr et al., 2013), and this number has since increased (e.g. Aparício, 2015; Ribeiro et al., 2015). Indeed, estimates of one new species every two years have been made for Portuguese freshwater ecosystems alone (Ribeiro et al., 2009; Ribeiro, F. unpublished data).

The Iberian NNFF include a single cichlid species, representing the oldest Neotropical ornamental fish brought alive to Europe, in 1889 (Říčan & Kullander, 2006). The first record of this cichlid in Portugal, originally identified as *Cichlasoma facetum* (Jenyns 1842), refers to specimens deposited in the Museum of Zoology of the University of Coimbra (Catalog no.s ZOO5459-5462; Figure 1). However, this record raises several questions regarding the original capture location of the specimens, as it includes four cichlid fish plus an incorrectly identified specimen of the marine fish genus *Balistes* (Catalog no. ZOO6567), reported from Mira - Praia (Figure 1), on the Atlantic coast. The presence of *Balistes* mixed in with the cichlid fishes is confusing as it invokes a marine environment as the capture location, further suggested by the mention of "Mira – Praia" (Mira Beach). However, the same record also mentions "Viveiros e Valas" (i.e. hatchery and drainage channels), alluding to the hatchery facilities at Praia de Mira ("Posto Aquícola de Praia de Mira"), a government facility from the General Forestry Directorate, that was closed down in 1980s. This hatchery facility was dedicated to acclimatize and promote the introduction of "warm" water freshwater fishes (Bochechas, J. personal communication).

In addition to the current ambiguous collection locality of the original record of *C. facetum* in Portugal, the genus *Cichlasoma* has since undergone major taxonomic revisions, and many of its species were left without a generic name until 2016 (Říčan et al., 2016). In particular, the *Cichlasoma facetum* group was thoroughly revised and the new genus *Australoheros* was proposed including several species (Říčan & Kullander, 2006, 2008; Říčan et al., 2011). Despite the recent taxonomic re-arrangement of *C. facetum*, no study has critically evaluated the species identity of the non-native cichlid species in Portuguese waters although many authors have assumed it corresponds to *Australoheros facetus* (e.g. Ribeiro et al., 2007; Baduy et al., 2016).

Given the ambiguity surrounding the first record of *C. facetum* in Portugal and the recent taxonomic re-arrangement of this particular taxon, this study aims to re-evaluate the species identification of the non-native cichlid fish present in Portuguese drainages. To this end, we conducted morphological and molecular analyses on a sample of fish specimens collected in all major Portuguese drainages where the species is currently known to occur.

Figure 1. Above, specimens associated with the original first record of *Australoheros facetus* (= *Cichlasoma facetum*) (Jenyns 1842), deposited in the Museum of Zoology of the University of Coimbra, together with a specimen of the genus *Balistes* (first from left), collected on October 13th 1940 by Helmut Helling at Mira – Praia, Viveiros (Portugal). Below, detail photographs of specimens ZOO5462 (letft) and ZOO5461 (right).

DOI: 10.29094/FiSHMED.2018.001

METHODS

Specimens were sampled through electric fishing in the Sado, Arade and Guadiana drainages (Figure 2), following protocols described in Ribeiro et al. (2007). Twenty whole fish (standard length: 37 – 80 mm) from Ribeira de Corona, Sado drainage (38.0265°N, 8.4315°W), were collected and kept frozen until morphological analysis. Morphological identification to the family-, genus- and species-levels was based on external morphological characters and taxonomic descriptions and/or keys described in Nelson et al. (2016) and in Říčan & Kullander (2006, 2008), respectively. Total and standard length were measured to the nearest millimeter, and meristics included scale counts on the upper and lower lateral lines, and on the first epaxial longitudinal series of scales (E1, *sensu* Figure 1 from López-Fernández & Taphorn 2004), as well as dorsal and anal fins pines and rays. All meristic counts were made under a dissecting scope. Fin clips were collected from a total of 41 specimens from the Sado, Arade and Guadiana drainages (Table 1), and preserved in 96% ethanol for molecular genetic analysis.

Genomic DNA was extracted using the EasySpin® Genomic DNA Miniprep Tissue Kit (Citomed, Lisbon, Portugal) following the manufacturer's instructions. A fragment of the cytochrome *b* gene (~1200 bp) was amplified via the polymerase chain reaction (PCR) with the primers GLuDG.L-TGACTTGAARAACCAYCGTTG (Palumbi et al., 1991) and H15915-AACTGCAGTCATCTCCGGGTTACAAGAC (Irwin et al., 1991). Each sample was amplified in 10 µl reactions including 5 µl of MyTaq 2x Hot Start (BIOLINE), 0.4 µl of each primer (10 µM), 1 µl of gDNA and 3.2 µl of dH_2O. The PCR temperature profile included an initial denaturation at 94°C for 5 min, followed by 30 cycles with denaturation at 94°C for 1 min, primer annealing at 48°C for 40s, and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. Successful amplification was confirmed by 2% (w/v) agarose gel electrophoresis run at 300V in 0.5X TAE buffer. Amplicons were cleaned with 1 µl of ExoSAP-IT® PCR clean-up Kit (GE Healthcare, Piscataway, NJ, USA), and sent to Macrogen Europe (The Netherlands) for Sanger sequencing of the forward and reverse strands.

Figure 2. Adult fish captured in the Guadiana river basin with approx. 15 cm of total length.

Table 1. Information on the samples collected for molecular genetic analyses. N – number of specimens sampled (number of specimens with good quality sequences).

Location	Basin	Coordinates	N	Collection date
Ribeira de Corona	Sado	38.0265°N, 8.4315°W	20 (17)	16/Oct/2016
Ribeira de Odelouca	Arade	37.2270ºN, 8.5056ºW	7 (7)	18/Feb/2015
Ribeira de Odeleite	Guadiana	37.3650ºN, 7.4858ºW	7 (6)	18/Feb/2015
Ribeira do Vascão	Guadiana	37.5284ºN, 7.5237ºW	7 (7)	18/Feb/2015

The resulting nucleotide sequences were imported into Geneious® 10.2.2 (Biomatters Ltd., Auckland, New Zealand), checked by eye, and manually edited to remove low quality positions at the 5' and 3' ends as well as to resolve ambiguities in nucleotide assignment. Additional cytochrome *b* sequences were obtained from GenBank including representatives of the seven *Australoheros* species sampled in their native range (n=38, included in Concheiro Pérez et al., 2007; Říčan & Kullander, 2008; Říčan et al., 2011; Appendix 1), to serve for comparison against sequences from non-native Portuguese cichlid fish. All sequences were aligned in Geneous using the default algorithm, and the full alignment was trimmed to a homologous fragment and checked for gaps and absence of stop codons. Sequence comparison was performed via construction of a Neighbor Joining (NJ) tree based on Tamura-Nei's genetic distances (Tamura & Nei, 1993) between sequence pairs, and using 1000 bootstrap replicates to estimate branch support values, as implemented in Geneious.

RESULTS

The following diagnostic morphological and meristic traits for the family Cichlidae were observed, namely: one nostril on each side of snout; interrupted lateral line with 20-50 scales; moderately deep and laterally compressed body; dorsal fin with 7-25 spines and 5-30 soft rays; anal fin with 3-15 spines and 4-15 soft rays. Diagnostic morphological and meristic characteristics for the genus *Australoheros* were also observed, namely: <25 modal scale counts in E1 row; scales on chest of similar size to flank scales; three to four abdominal bars; five or more anal fin spines. Finally, the following diagnostic characteristics for *Australoheros facetus* were observed: scale counts in E1 row 23-27 (mode: 25); upper lateral line scale counts 10-18 (mode: 17); lower lateral line scale counts 7-11 (mode: 9); anal fin VI, 9-8; dorsal fin XV-XVI, 10-9; short caudal peduncle covered with 3 scale rows; mouth large and slightly upturned; mid-lateral blotch; four abdominal bars. The four abdominal bars and the anal fin spines and ray counts are the best characters supporting the studied specimens as *A. facetus* s. str. (see Říčan & Kullander, 2008).

Out of the 41 specimens sequenced for the cytochrome *b* gene fragment, 37 sequences (1087 bp) had good quality and were kept for analysis (Table 1). All sequences from Portuguese specimens showed 100% identity, resulting thus in a single unique haplotype. The final alignment including the Portuguese haplotype and the *Australoheros* sequences from GenBank corresponded to a homologous fragment of 939 bp. The resulting NJ tree showed well-supported clades corresponding to monophyletic species (Figure 3), including the clade

DOI: 10.29094/FiSHMED.2018.001

of *A. facetus* sequences with a bootstrap support value of 100%. The Portuguese haplotype was identical to a sequence from the locality La Reja, Rio de La Reconquista, (Entre Rios Province, north-eastern Argentina, GenBank Accession no. AY998667; Říčan & Kullander, 2006), belonging to the Uruguay river drainage. Three haplotypes from specimens originally identified as *A. scitulus* (from the STRI collection; STRI-4692, collected by Bermingham et al.) are also included in this clade, but were revised by Říčan et al. (2011) to be *A. facetus*.

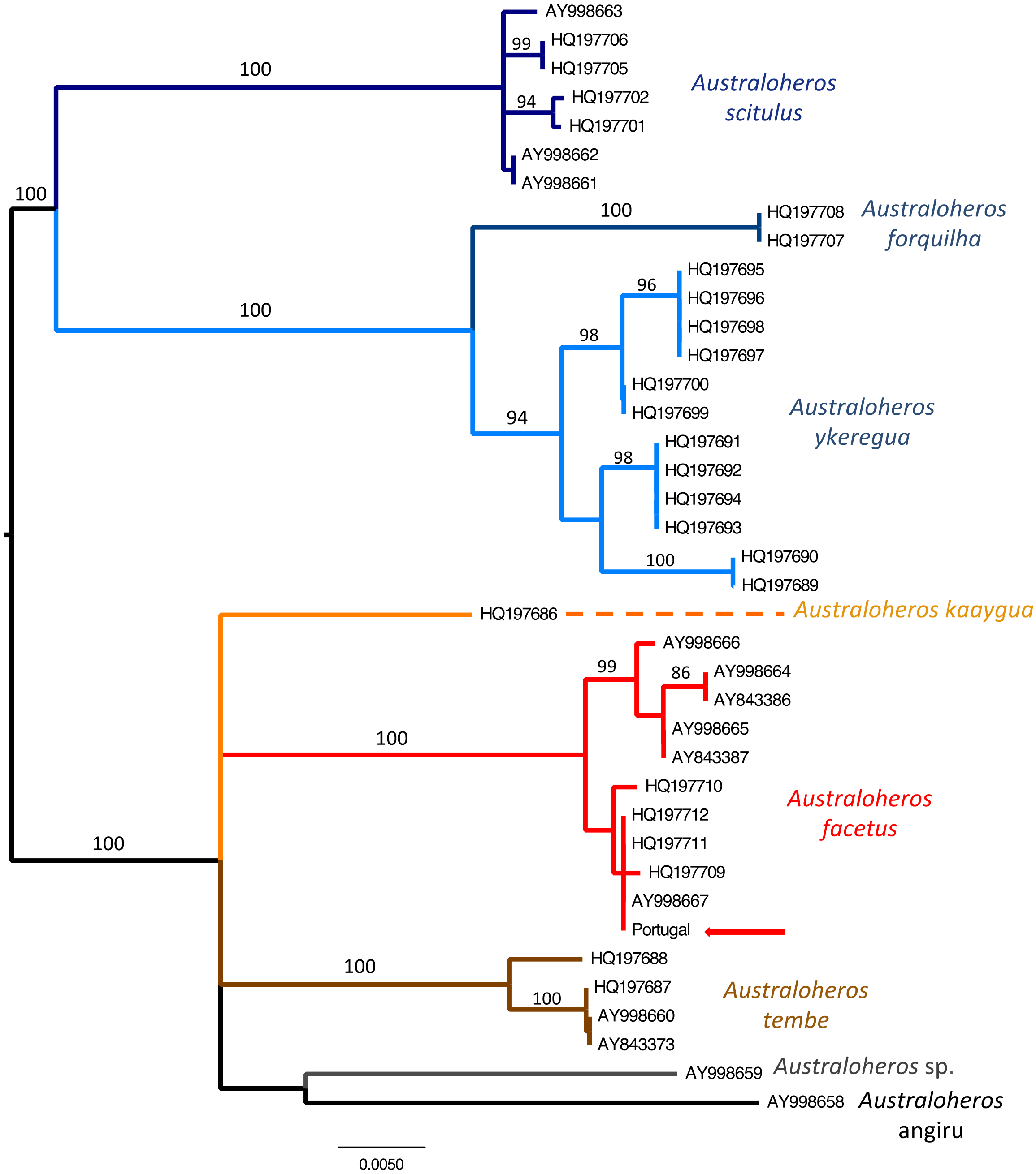

Figure 3. Neighbor Joining tree based on Tamura-Nei genetic distances of cytochrome *b* sequences of *Australoheros* species included in present study, using midpoint rooting. Numbers at nodes represent bootstrap support values >85. Species clades are colour-coded, and the cyt*b* haplotype retrieved from the Portuguese specimens is marked with a red arrow.

DISCUSSION

The morphological and molecular genetic results confirm the presence of the chameleon cichlid *A. facetus* (Jenyns 1842) in the three Portuguese drainages sampled - Sado, Arade and Guadiana. Accordingly, with the new systematics re-arrangements for the *Australoheros* genus, the species is native to Argentina and Uruguay, and has been reported as introduced in Chile in addition to Spain and Portugal (Říčan & Kullander, 2006; Ribeiro et al., 2007; Říčan et al., 2011; Vila & Habit, 2015). Its Iberian range is currently confined to southern Portuguese drainages, namely Sado, Arade, and Guadiana (including the Spanish section), occurring in the lower Guadalquivir and, possibly, Segura (Ribeiro et al., 2007; Fernandéz-Delgado et al., 2014). The species was also recently detected on the freshwater upstream reaches of Lagoa de Albufeira, Portugal (38.527010ºN, 9.140071ºW) based on a photo voucher (Cabral, H., personal communication). This new record refers to a small coastal drainage leading to a coastal lagoon (Lagoa de Albufeira), not connected to any of the drainages where the species was previously reported, and suggests a progressive, human-driven northwards extension of the introduced range. In fact, *A. facetus* is commonly found in artificial urban lakes in Lisbon (northern to Lagoa de Albufeira), which indicates that it is used as ornamental species by citizens and aquarists.

No evidence has been found of the recent presence of *A. facetus* in its original recorded location in Praia de Mira. A comprehensive species survey and inventory of the coastal lagoons of Mira conducted in 2014 has not detected any cichlid fish (Varandas et al., 2014). The known populations of *A. facetus* in southern Portuguese drainages are about 300 km from Praia de Mira (Vouga drainage) and in independent river drainages (i.e. Sado, Arade and Guadiana). In fact, the first record of a wild population of *A. facetus* in Portugal was in the Guadiana drainage (Moura, Ardila river) in 1965 (Almaça, 1965). This implies human-mediated introductions of this cichlid species into southern Portuguese drainages, most likely from the Mira fish hatchery (Vouga drainage, central Portugal). Additionally, the occurrence in the Mira drainage (southwestern Portugal) was probably an erroneous citation by previous authors (Collares-Pereira, 1985), since *A. facetus* was absent in past extensive surveys done across this basin (Magalhães et al., 2002). The reduced genetic diversity at the cytochrome *b* gene in the 37 Portuguese specimens (i.e. exhibiting a single haplotype) compared to the higher levels of haplotype diversity found in a smaller sample size from fish in their native range (e.g. 10 specimens from 6 sites exhibited 10 distinct haplotypes; Říčan et al., 2011) suggest the establishment of this non-native species from few individuals in all Portuguese river drainages. The species introduction in the southern part of the Iberian Peninsula is assumed to be accidental and mediated by aquarists (Doadrio, 2001), among which there is a keen interest for this species (e.g. "Associação Portuguesa de Ciclídeos"; http://www.apciclideos.org/).

There is currently very limited information about the biology of *A. facetus* in Iberian waters. It is known that it has strong parental care (Baduy et al., 2017), reaches a maximum total length of 180 mm, and exhibits an extremely wide physiological tolerance (Ribeiro, 2008). These findings are in line with studies on the wide environmental tolerance range reported for the species, that can survive, under laboratory conditions, to water temperatures varying from ~5ºC to ~37ºC, and adults can cope well with salinities from freshwater to 12ppt, while juveniles can tolerate up to 18ppt (Baduy et al., 2016; Baduy, 2017). The species feeds predominantly on insects, small molluscs and crustaceans, and plant materials, but has an opportunistic feeding behaviour (Ribeiro, 2008). It exhibits a high degree of territoriality, especially during the breeding season (April-August; Ribeiro, 2008) when it is most aggressive and territorial (Doadrio, 2001; Kodde et al., 2016; Baduy et al., 2017). The above species' traits have the potential to facilitate the establishment and spread of *A. facetus* within Iberian drainages (Figure 4; Supplementary Material), and to significantly affect the host ecosystems via resource competition or predation (Kodde et al., 2016; Baduy, 2017).

In sum, this study unambiguously clarifies the taxonomic identity of the non-native cichlid fish present in Portuguese drainages as *A. facetus*, which is an essential first step in any future biological studies.

AUDIO-VISUAL SUPPLEMENTARY MATERIAL

Australoherus facetus at Ribeira da Foupana, Portugal. FiSHMED Youtube channel

www.youtube.com/watch?v=gejQfKJImTE

Australoheros facetus among aquatic plants during late summer (October 7th 2014), occupying shallow areas with slow currents in the Foupana river (Guadiana drainage), together with native cyprinids (*Squalius alburnoides*). Forty seconds footage obtained with a Go-Pro Hero 4 Silver camera, operator Marta Vargas.

AUTHORS CONTRIBUTIONS

FR, FB, PMG, JLS contributed to sample collection, JC performed the laboratory work and data analyses. FR and AV planned, supervised, and coordinated the work. JC wrote the paper with major contributions from FB, PMG, AV and FR.

AKNOWLEDGEMENTS

The authors wish to thank Cristina Rufino for her availability and precious help in accessing the collection at the Museu de Zoologia da Universidade de Coimbra. This study was developed under the project FRISK (PTDC/AAG-MAA/0350/2014) funded by Portuguese national funds through FCT/MCTES (PIDDAC) and co-funded by the European Regional Development Fund (FEDER) through COMPETE— Programa Operacional Factores de Competitividade (POFC). Strategic plan of MARE – Marine and Environmental Sciences Centre (UID/MAR/04292/2013) supported the sampling. Collection of specimens in southern streams was possible with additional funding from FCT grant CCMAR/Multi/04326/2013. AV and JLS were funded by Fundação para a Ciência e Tecnologia (SFRH/BPD/77487/2011 and SFRH/BPD/67008/2009 respectively). FB was recipient of a doctoral fellowship from the Brazilian CNPq Program Science without Borders (245971/2012-2).

CITED REFERENCEES

Almaça, C. (1965) Contribution à la connaissance des poissons des eaux intérieures du portugal. *Revista da Faculdade de Ciências de Lisboa* **13**, 225–262.

Aparício, E. (2015) First record of a self-sustaining population of Alpine Charr *Salvelinus umbla* (Linnaeus, 1758) (Actinopterygii, Salmonidae) in Spain. Graellsia **71**, e034.

Baduy, F., Soares, J., Silva, M., Canário, A. V. M., Saraiva, J. & Guerreiro, P. M. (2016) Critical thermal maximum and minimum in *Australoheros facetus*, a neotropical invader in the Iberian Peninsula. *FiSHMED Fishes in Mediterranean Environments* 2016.012, 3 pp.

Baduy, F., Guerreiro, P.M., Canário, A.V.M. & Saraiva, J.L. (2017) Social organization and endocrine profiles of *Australoheros facetus*, an exotic freshwater fish in southern Portugal. *Acta Ethologica* **20**, 263-277.

Baduy, F. (2017) *Tolerância ambiental e comportamento social e reprodutivo de Australoheros facetus (Cichlidae): que mecanismos fisiológicos e moleculares permitem a adaptação desta espécie invasora em Portugal?* Tese de Doutoramento. Universidade do Algarve, Portugal, 244 pp.

Clavero, M. & García-Berthou, E. (2005) Invasive species are a leading cause of animal extinctions. *Trends in Ecology & Evolution* **20**, 110.

Collares-Pereira, M. J. (1985) *Ciprinídeos do Alentejo*. In Primeiro Congresso sobre o Alentejo, pp. 537–545.

Concheiro Pérez, G. A., Říčan, O., Ortí, G., Bermingham, E., Doadrio, I. & Zardoya, R. (2007) Phylogeny and biogeography of 91 species of heroine cichlids (Teleostei: Cichlidae) based on sequences of the cytochrome *b* Gene. *Molecular Phylogenetics and Evolution* **43**, 91–110.

Cowx, I. G. (1998) *Stocking and Introduction of Fish*. Oxford: Fishing New Books, Blackwell Science.

Doadrio, I. (2001) *Atlas y libro rojo de los peces continentales de España*, 2nd ed. Dirección General de Conservación de la Naturaleza.

Elvira, B. & Almodovar, A. (2001) Freshwater fish introductions in Spain: facts and figures at the beginning of the 21st century. *Journal of Fish Biology* **59**, 323–331.

Fernández-Delgado, C., Rincón, P. A., Gálvez-Bravo, L., De Miguel, R. J., Oliva-Paterna, F. J., Pino, E., Ramiro, A., Moreno-Valcárcel, R. & Peña, J. P. (2014) *Distribución y estado de conservación de los peces dulceacuícolas del río Guadalquivir. Principales áreas fluviales para su conservación*. Ministerio de Agricultura, Alimentación y Medio Ambiente. Confederación Hidrográfica del Guadalquivir.

Gallardo, B., Clavero, M., Sanchez, M. I., & Vila, M. (2016) Global ecological impacts of invasive species in aquatic ecosystems. *Global Change Biology* **22**, 151–163.

Genovesi, P., Carnevali, L. & Scalera, R. (2015) *The Impact of invasive alien species on native threatened species in Europe* - Technical Report for the European Commission. Rome.

Helfman, G. S. (2007) *Fish conservation: a guide to understanding and restoring global aquatic biodiversity and fishery resources*. Washington, D.C.: Island Press.

Irwin, D. M., Kocher, T. D. & Wilson, A. C. (1991) Evolution of the cytochrome *b* gene of mammals. *Journal of Molecular Evolution* **32**, 128–144.

Kodde, A., Gkenas, C., Cheoo, G., Ribeiro, F. & Magalhães, M. F. (2016) Uninvited dinner guests: the effect of invasive fish and temperature on the foraging efficiency of southern Iberian chub. *FiSHMED Fishes in Mediterranean Environments* 2016.011, 3 pp.

Leprieur, F., Beauchard, O., Blanchet, S., Oberdorff, T. & Brosse, S. (2008) Fish invasions in the world's river systems: when natural processes are blurred by human activities. *PLoS Biology* **6**, e28.

López-Fernández, H. & Taphorn, D. C. (2004) *Geophagus abalios*, *G. dicrozoster* and *G. winemilleri* (Perciformes: Cichlidae), three new species from Venezuela. *Zootaxa* **439**, 1-27.

Mack, R. N., Simberloff, D., Lonsdale, W. M., Evans, H., Clout, M., & Bazzaz, F. A. (2000) Biotic invasions: causes, epidemiology, global consequences, and control. *Ecological Applications* **10**, 689–710.

Magalhães, M. F., Batalha, D. C. & Collares-Pereira, M.J. (2002) Gradients in stream fish assemblages across a Mediterranean landscape: contributions of environmental factors and spatial structure. *Freshwater Biology* **47**, 1015–1031.

Marr, S. M., Olden, J. D., Leprieur, F., Arismendi, I., Ćaleta, M., Morgan, D. L., Nocita, A., Šanda, R., Serhan Tarkan, A. & García-Berthou, E. (2013) A global assessment of freshwater fish introductions in Mediterranean-climate regions. *Hydrobiologia* **719**, 317–329.

Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B. & Kent, J. (2000) Biodiversity hotspots for conservation priorities. *Nature* **403**, 853–858.

Nelson, J. S., Grande, T. C. & Wilson, M. V. H. (2016) *Fishes of the World*, 5th ed. Hoboken, New Jersey: John Wiley & Sons, Inc.

Palumbi, S. R., Martin, A., Romano, S., Stice, L. & Grabowski, G. (1991) *The Simple Fool's Guide to PCR*. Honolulu.

Rahel, F. J. (2002) Homogenization of freshwater faunas. *Annual Review of Ecology and Systematics* **33**, 291–315.

Ribeiro, F. (2008) Patterns and Processes of Fish Invasions in Iberian Rivers: The Lower Guadiana Drainage as a Case Study. Doctoral dissertation, University of Lisbon, 216 pp.

Ribeiro, F., Orjuela, R. L., Magalhães, M. F. & Collares-Pereira, M. J. (2007) Variability in feeding ecology of a south American cichlid: a reason for successful invasion in Mediterranean-type rivers? *Ecology of Freshwater Fish* **16**, 559–569.

Ribeiro, F., Collares-Pereira, M. J. & Moyla, P. B. (2009) Non-native fish in the fresh waters of Portugal, Azores and Madeira Islands: a growing threat to aquatic biodiversity. *Fisheries Management and Ecology* **16**, 255–264.

Říčan, O. & Kullander, S. O. (2006) Character- and tree-based delimitation of species in the '*Cichlasoma*' *facetum* group (Teleostei, Cichlidae) with the description of a new genus. *Journal of Zoological Systematics and Evolutionary Research* **44**, 136–152.

Říčan, O. & Kullander, S. O. (2008) The *Australoheros* (Teleostei: Cichlidae) species of the Uruguay and Paraná River drainages. *Zootaxa* **1724**, 1–51.

Říčan, O., Piálek, L., Almirón, A. & Casciotta, J. (2011) Two new species of *Australoheros* (Teleostei: Cichlidae), with notes on diversity of the genus and biogeography of the Río de La Plata basin. *Zootaxa* **2982**, 1–26.

Smith, K. G. & Darwall, W. R. T. (2006) *The Status and distribution of freshwater fish endemic to the Mediterranean Basin*. IUCN. Gland and Cambridge.

Strayer, D. L. (2010) Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. *Freshwater Biology* **55**, 152–174.

Sundseth, K. (2014) *Invasive Alien Species: A European Response*. Brussels.

Tamura, K. & Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512–526.

Varandas, S., Assunção, T., Almeida, P., Cortes, R., Crespí, A., Ferreira, P., Froufe, E., Hinzmann, M., Jesus, J., Lopes-Lima, M., et al. (2014) *PAELORIS: Guia de Flora e Fauna aquáticas das lagoas costeiras do sítio Rede Natura 2000: Dunas de Mira, Gândara e Gafanhas* (Código PTCON0055). Bragança.

Vila I, Habit E (2015) Current situation of the fish fauna in the Mediterranean region of Andean river systems in Chile. *Fishes in Mediterranean Environments* 2015.002: 19p.

Appendix. List of *Australoheros* cytochrome *b* sequences included in this study with information on the drainage and the country where the specimens were sampled, and the corresponding GenBank Acession number (GenBank#). *These specimens are listed with the original species names but species identifications were reviewed by Říčan et al. (2011).

Species	Drainage(s)	Country	GenBank#
Australoheros sp.	Arade, Sado, Guadiana	Portugal	This study
Australoheros angiru	Iguazu	Brazil	AY998658
Australoheros facetus	La Plata	Argentina	AY998664
Australoheros facetus	La Plata	Argentina	AY843386
Australoheros facetus	Paraná	Paraguay	HQ197709
Australoheros facetus	Paraná	Paraguay	HQ197710
Australoheros facetus	Paraná	Argentina	HQ197711
Australoheros facetus	Paraná	Argentina	HQ197712
Australoheros facetus	Uruguay	Argentina	AY843387
*Australoheros scitulus**	Uruguay	Argentina	AY998665
Australoheros facetus	Uruguay	Argentina	AY998667
Australoheros facetus	La Plata	Uruguay	AY998666
Australoheros forquilha	Uruguay	Brazil	HQ197707
Australoheros forquilha	Uruguay	Brazil	HQ197708
Australoheros kaaygua	Iguazu	Argentina	HQ197686
Australoheros scitulus	Uruguay	Argentina	HQ197705
Australoheros scitulus	Uruguay	Argentina	HQ197706
Australoheros scitulus	Uruguay	Argentina	HQ197701
Australoheros scitulus	Uruguay	Argentina	HQ197702
Australoheros scitulus	La Plata	Uruguay	AY998662
Australoheros scitulus	La Plata	Uruguay	AY998661
Australoheros scitulus	Uruguay	Argentina	AY998663

Species	Drainage(s)	Country	GenBank#
Australoheros tembe	Paraná (Uruguay stream)	Argentina	HQ197687
Australoheros tembe	Paraná (Uruguay stream)	Argentina	HQ197688
Australoheros tembe	Paraná (Uruguay stream)	Argentina	AY998660
Australoheros tembe	Paraná (Uruguay stream)	Argentina	AY843373
Australoheros ykeregua	Uruguay	Argentina	HQ197689
Australoheros ykeregua	Uruguay	Argentina	HQ197690
Australoheros ykeregua	Uruguay	Argentina	HQ197691
Australoheros ykeregua	Uruguay	Argentina	HQ197692
Australoheros ykeregua	Uruguay	Argentina	HQ197693
Australoheros ykeregua	Uruguay	Argentina	HQ197694
Australoheros ykeregua	Uruguay	Argentina	HQ197695
Australoheros ykeregua	Uruguay	Argentina	HQ197696
Australoheros ykeregua	Uruguay	Argentina	HQ197697
Australoheros ykeregua	Uruguay	Argentina	HQ197698
Australoheros ykeregua	Uruguay	Argentina	HQ197699
Australoheros ykeregua	Uruguay	Argentina	HQ197700
*Australoheros sp. Uruguai**	Uruguay	Uruguay	AY998659