

First record of Iberian barbel *Luciobarbus graellsii* (Steindachner, 1866) in the Tiber River (Central Italy)

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Abstract

The presence of the Iberian barbel *Luciobarbus graellsii* is recorded for the first time in the Tiber River (Central Italy). Because of its abundance at the sampling site and the presence of different size classes we assume that the species is established. Introduction of the allochthonous species is probably due to illegal release by local anglers. We suggest monitoring the expansion of the species to assess the impact on native species and the risk of hybridization and competition with the endemic *Barbus tyberinus*.

Key words: introduced species; Iberian barbel; Tiber River; DNA sequencing

Introduction

The human-mediated introduction of allochthonous species into Italian watercourses has generated alterations and negative effects on native freshwater fish fauna (Zerunian 2001; Gherardi et al. 2008). As a consequence, during recent decades many Italian autochthonous species have showed a decline, unlike many exotic species, which have expanded their ranges (Zerunian 2001). Outstanding examples of this latter trend are the European *Barbus barbus* (Linnaeus, 1758) and the Asiatic *Pseudorasbora parva* (Temminck and Schlegel, 1846), which have colonized the intermediary sections (barbel zone) of the main rivers of northern and Central Italy within a few years (Lorenzoni et al. 2006a; Kottelat and Freyhof 2007). Another exotic species, *Luciobarbus graellsii* (Steindachner, 1866), was recorded in 2001 in some rivers of Central Italy: Fiora, Ombrone and Albegna (Bianco and Ketmaier 2001). *L. graellsii* is endemic to the Iberian Peninsula; its natural range comprises the Ebro and Ter drainages on the Mediterranean slope and the Ason drainage on the Atlantic slope (Kottelat and Freyhof 2007).

The introduction and establishment of a new *L. graellsii* population in the Tiber River are now reported for the first time.

Methods

Sampling was carried out in October 2010 by electrofishing the Tiber River as part of a fish survey programme. At one sampling station, located between two of its major tributaries, the Chiascio and Nestore Rivers (42°54'36"N, 12°22'52"E), 40 barbel specimens of an unreported species were caught. For each specimen weight (W) and total length (TL) were recorded. In order to perform length-frequency analysis, the fishes were divided into 10 mm size classes. For each length interval a sample of scales (5–10) was taken from one individual and stored in ethanol (33%) for age determination, according to the microscopic scalimetric method (Bagenal and Tesch 1978). The condition of the specimens was evaluated using the relative condition factor (Le Cren 1951) expressed by the formula $K' = W(aTL^b)^{-1}$, where a and b are, respectively, the intercept on the y axis and the coefficient of the regression TL-W

Table 1. Nucleotide sequence of primers used for amplification of nuclear loci *S7* and *GH* with their references, number PCR cycles and annealing temperature used for each primer pair.

Locus	Primer name	Primer sequence (5'-3')	Source	N° cycles	T° annealing
<i>S7</i>	S7BL2F	CCCAGCTAAAGAGTTATCAAGTT	Gante et al. 2011	30	58°
	S7RPEX3R	GCCTTCAGGTCAGAGTTCAT	Chow and Hazamana 1998		
<i>GH</i>	BGH1.i3.79f	GGGGTCTGTGGAAAAGTTTGG	Gante et al. 2011	30	59.5°
	Ghe5.183R	CTACAGGGTGCAGTTGGAATC	Clement et al. 2004		

Table 2. Morphological comparison of four unidentified specimens and candidate species.

Characters	Unknown specimens	<i>L. graellsii</i>	<i>B. tyberinus</i>	<i>B. plebejus</i>	<i>B. barbuis</i>
Shape of the lower lip	Without median lobe	Without median lobe*	With median lobe*	With median lobe*	With median lobe*
Number of lateral line scales	47-49	43-51*	47-62*	58-81*	53-63*
Serration of the spine	Absent	Fine to absent*	Finely serrated***	Finely serrated***	Strongly serrated*
Peritoneal membrane	Black	Black**	Grayish**	White***	Grayish**

Reference data: *Kottelat and Freyhof (2007); **Bianco and Ketmaier (2001); ***Bianco (1995)

calculated on the whole sample. Four specimens, Lg487, Lg489, Lg531 and Lg533, were killed by overdose of an anaesthetic and transferred to the laboratory for morphological identification and genetic analyses. Morphological identification followed criteria proposed by Kottelat and Freyhof (2007), Bianco and Ketmaier (2001) and Bianco (1995). Characters analysed were: shape of the lower lip, number of scales of the lateral line, serration of the spine and colour of the peritoneal membrane.

Molecular techniques were applied to test further the morphological identification. We used sequences of the Cytochrome *b* (*Cytb*) mitochondrial gene, sequences of the *S7* ribosomal protein (*S7*) and Growth Hormone (*GH*) nuclear genes useful to identify morphologically similar species of barbels (Doadrio et al. 2002; Gante 2009; Buonerba 2010). Moreover nuclear genes sequences, due to their biparental inheritance, are also useful in detecting hybridisation events (Gante 2009; Buonerba 2010). Total genomic DNA was extracted from scales using QIAGEN® DNeasy Blood and Tissue extraction kit. *Cytb* was amplified by polymerase chain reaction (PCR) using primers Glu-F and Thr-R (Zardoya and Doadrio 1998) and then sequenced, in both directions, with the same primer pair. Species belonging to genera *Luciobarbus* and *Barbus* are tetraploids, therefore nuclear loci have to be selectively amplified and sequenced using paralog-specific primers (Gante et al. 2011). The same primer pairs utilized in PCR reactions were used later for sequencing nuclear loci in both

directions (Table I). Heterozygous specimens for insertion or deletion (indels) were manually phased, analysing the complementary information carried by the forward and reverse sequences (Flot et al. 2006). Generated *Cytb* gene sequences were manually aligned with previously published sequences of Iberian *Luciobarbus* and *Barbus* species (Bianco and Ketmaier 2001; Machordom and Doadrio 2001; Doadrio et al. 2002; Kotlík et al. 2004; Mesquita et al. 2007). Sequences of nuclear genes were manually aligned with available sequences of *L. graellsii*, *B. tyberinus*, *B. plebejus* and *B. barbuis* (Gante et al. 2011; Buonerba 2010). For both mitochondrial and nuclear sequences genetic divergence was calculated as p-distance and then a neighbour-joining analysis, tested with 1,000 bootstrap replicates, was performed.

Results and discussion

Morphological characters of the unidentified specimens were first compared to *Barbus* species known to be present in Tiber River: namely *Barbus tyberinus* Bonaparte 1839, *Barbus plebejus* Bonaparte 1839, *Barbus barbuis* (Lorenzoni et al. 2006) and subsequently compared to *Luciobarbus* species, leading to their identification as *Luciobarbus graellsii* (Figure 1). The four specimens have thick lower lip without a median lobe, a typical trait of genus *Luciobarbus* (Kottelat and Freyhof 2007). The peritoneal membrane is black, unlike the white peritoneum of *B. plebejus* (Bianco 1995) and the greyish one of *B. barbuis* and *B. tyberinus* (Bianco and Ketmaier 2001).

Figure 1. *Luciobarbus graellsii* specimen collected in October 2010 in the Tiber River. Photograph by L. Pompei.

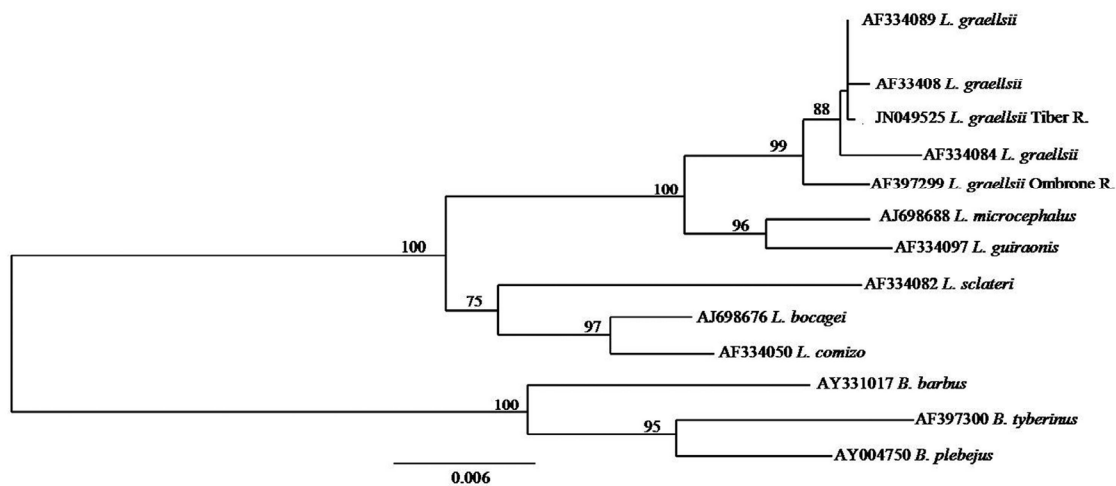


Figure 2. Neighbour-joining cluster analysis of 1140 bp Cytb haplotypes. Bootstrap values are given above the branches. Each haplotype label includes GenBank Accession Number and species name.

Scales along the lateral line are larger and less numerous than those of *B. plebejus*, *B. tyberinus* and *B. barbus* (Bianco 1995; Bianco and Ketmaier 2001). Moreover body coloration, greyish green dorsally, fading to whitish yellow on the flanks to white on the belly, is very different from the typical body coloration of the other *Barbus* species already known to be present in the Tiber River basin (Bianco 1995; Bianco and Ketmaier 2001). Another diagnostic character is the serration along the posterior margin of first dorsal ray, absent in these specimens, but strongly serrated in *B. barbus* and finely serrated in *B. plebejus* and *B. tyberinus* (Bianco 1995). Absence of serration along the spine also distinguishes *L. graellsii* specimens from the very similar Iberian

barbel *Luciobarbus bocagei* (Steindachner, 1864), with which it could be easily confused (Kottelat and Freyhof 2007) (Table 2).

For each specimens (N=4) a nucleotide sequence of 1140 bp long, corresponding to a partial region of the Cytb gene, was analyzed. No variable sites were detected and the unique recovered haplotype was deposited in GenBank: accession number JN049525. Sequencing of nuclear genes yielded 1171 aligned sites (584 bp GH; 587 bp S7). Specimens analysed showed 3 and 2 alleles for S7 and GH respectively. Identical alleles were recorded by Gante (2009) for Iberian *L. graellsii* specimens. Genetic divergence of Cytb sequences revealed 0.0%–0.4% difference among detected haplotype and *L. graellsii* sequences. In

particular the haplotype from the Tiber River JN049525 was very similar to *L. graellsii* AF334089 differing just for one transition (A/G) at position 402 bp. Higher divergences were recorded in the comparisons with remaining *Luciobarbus* (1.4%–3.2%) and *Barbus* species (6.3%–7.2%). Sequence divergence for nuclear loci varied from 0.0% among *L. graellsii* alleles and 2.0%–2.8% among *L. graellsii* and *Barbus* species. Nuclear genetic distances were in agreement with those previously reported by Gante (2009) and Buonerba (2010). Neighbour-joining analysis clustered, as expected, the specimens from Tiber River with *L. graellsii* sequences analysed, and distinctly separated from the other Iberian barbels and *Barbus* species for all genetic markers (Figure 2, *GH* and *S7* trees not shown). Nuclear sequences showed no evidence of hybridisation, indeed all detected alleles were confidently attributed to the Iberian barbel.

Length-frequency analysis of 40 specimens revealed a well structured population with specimens of 115–364 mm TL (Figure 3) and 20–500 g in weight. Age determination confirmed the presence of both juveniles and adults; age classes most represented in the sample were subadults (2+: 50.28%) and adults (3+: 20.51%). The condition of the specimens can be considered good, since the condition factor presented an average value (\pm S.E) of 1.000 ± 0.008 .

Genetic analyses, supported by the high number of molecular markers used, were in agreement with the morphological identification of the specimens as *L. graellsii*. The most probable explanation for the occurrence of *L. graellsii* could be an illegal introduction carried out by unauthorized local anglers. Unauthorized fish introduction is one of the main causes that led the spread of alien fish species and represents a serious problem facing fisheries management since also the isolated introduction of few specimens can cause severe impacts to entire aquatic ecosystems (Rahel 2004). However, translocation of specimens from the Ombrone River, where *L. graellsii* was previously recorded, seems to be very improbable due to the high divergence of their *Cytb* haplotypes (9 substitution) and the wide geographic distance.

The high relative abundance in the sampling site (27.5% of the entire biomass sampled) combined with the length frequency distribution and age composition, which reveal the presence of juveniles and adults, and the good condition of the specimens, suggest a viable establishment of the species. It is therefore likely that in the

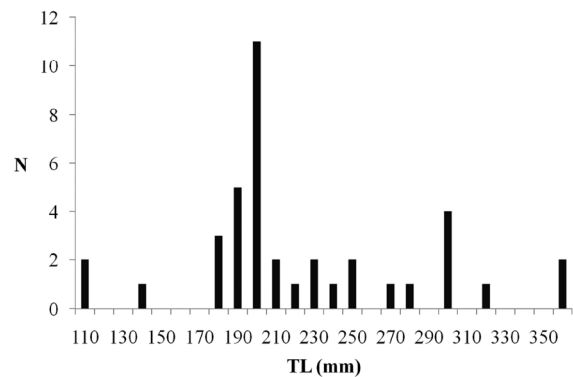


Figure 3. Length-frequency distribution of 40 *Luciobarbus graellsii* collected in October 2010 in the Tiber River. N: number of specimens; TL: total length.

future *L. graellsii*, as observed for other exotic species introduced into the Tiber (Lorenzoni et al. 2006a), could spread throughout the main course of the river; the presence of numerous bridges however, could prevent the spread of the species upstream into secondary waterways. As documented for other alien species (Zerunian 2001), *L. graellsii* could be a source of risk for the fish fauna of the Tiber River, but in particular for *B. tyberinus*, an endemic species of Central Italy. *L. graellsii*, as *B. tyberinus*, is a gregarious species inhabiting middle reaches of rivers with moderate current and usually it feeds on invertebrates, plants and algae (Kottelat and Freyhof 2007). From May to August, reproductive specimens migrate upstream to areas with faster current and gravel or stone bottom for spawning (Kottelat and Freyhof 2007). These biological characteristics make *L. graellsii* a potential competitor for resources of *B. tyberinus*, already threatened by the presence of the allochthonous *B. barbus* (Lorenzoni et al. 2006b; Giannetto et al. 2012). Preliminary results showed no evidence of hybridisation between *L. graellsii* and *B. tyberinus*. However, this possibility should be seriously taken into consideration since genetic pollution already affects several *B. tyberinus* populations of Tiber River due to hybridisation with *B. barbus* (Buonerba 2010). Data reported in this study concerning hybridisation and population status should be considered as preliminary. A more in-depth monitoring of this new species in the Tiber River should be conducted to better understand its ecological impact on the fish community and should be included in future conservation and management plans.

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