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Revalidation of the Spanish stone loach *Barbatula hispanica* (Lelek, 1987) (Teleostei, Nemacheilidae) according to morphological and mitochondrial data

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Abstract – This study revalidates *Barbatula hispanica*, previously considered a junior synonym of *B. quignardi*. This species is found in the Ebro drainage and in Cantabria (Spain) as well as in the Adour drainage (Southwestern France). It is characterized by an upper lip with a well-marked medial incision and an interorbital width 18.5–33.7% of the HL. The species delineation is corroborated by the cytochrome oxidase subunit 1 molecular marker. We provide the sequence of 12S rDNA (950 bp) as reference for environmental DNA studies, and discuss also the taxonomy of *B. quignardi* which would be restricted to the Lez River.

Keywords: Nemacheilidae / *Barbatula hispanica* / Adour / Spain / MtDNA

1 Introduction

The stone loaches *Barbatula* spp (Teleostei, Nemacheilidae) are small freshwater teleost fishes occurring in brooks of most watersheds of Eurasia (Keith *et al.*, 2020). They are rheophilic and live in high current environments, hiding under stones and vegetation (Kottelat and Freyhof, 2007; Keith *et al.*, 2020).

The taxonomy of the group has long been discussed. At the end of the 20th century, *Barbatula barbatula* (Linnaeus, 1758) was considered widespread in Europe (Kottelat, 1997). However, molecular data have delineated a multitude of evolutionary lineages in the different populations of what was previously called *B. barbatula*. Šedivá *et al.* (2008) showed the paraphyly of *B. barbatula* with the cytochrome b (cytb) marker, due to the position of *B. vardarensis* (Karaman, 1928), *B. sturanyi* (Steindachner, 1892) and *B. zetensis* (Sorić, 2000).

They delineated at least five distinct evolutionary lineages in the Danube river basin, three lineages in the North Sea basin (corresponding to the drainages of the Elbe, the Dniester, and a Vistula + Oder + Salgir group), and three Mediterranean lineages corresponding to the Ebro, the Rhône and the Ter catchments. Later, with the development of DNA barcoding projects *sensu* Hebert *et al.* (2003) using the cytochrome oxidase subunit 1 (COI) marker, other evolutionary lineages were noticed. Mendel *et al.* (2012) delineated two evolutionary lineages in Czech Republic corresponding to the Elbe and Oder drainages. Kneibelsberger *et al.* (2015) distinguished two other lineages corresponding to the Rhine and the upper Danube catchments, different from the Elbe and middle Danube drainages (Behrmann-Godel *et al.*, 2017). Finally, Norén *et al.* (2018) analysed all 1154 sequences of the FREDIE project (www.fredie.eu) and found 13 other lineages, mostly in Russia, Ukraine and western Europe. Thus, according to mitochondrial data (cytb and COI), the genetic pattern resembles the one of the European minnows *Phoxinus* spp (*e.g.* Denys *et al.*, 2020; Palandačić *et al.*, 2020), with almost

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all European catchments having at least one endemic stone loach species.

Originally, [Linnaeus \(1758\)](#) described *Cobitis barbatula* following the description made by [Artemi \(1738\)](#) and [Linnaeus \(1746\)](#). The syntype specimens came from both Germany and Sweden. Therefore, it is unclear which evolutionary lineage the syntype specimens belong to, as there are four distinct lineages in Germany and stone loaches were introduced in Sweden from Germany and eastern European countries (see [Norén *et al.*, 2018](#)). All the European stone loaches populations must therefore be considered as *Barbatula* spp.

However, several other European *Barbatula* species were described, and some are still valid, like *Barbatula quignardi* ([Băcescu-Meşter, 1967](#)). Based on his examination of the morphology of several European populations, [Băcescu-Meşter \(1967\)](#) highlighted one new subspecies in the Lez river (south of France, near Montpellier), *Noemacheilus barbatulus quignardi* [Băcescu-Meşter, 1967](#) and a Spanish form *Noemacheilus barbatulus* forma *hispanica* [Băcescu-Meşter, 1967](#). This Spanish form was described based on specimens from the Ibaizabal river at Durango (Nervión drainage) and the Tajo river. It can be characterized by a more cylindrical body, a shorter head, a larger eye diameter, rather long barbels and a rather narrow and long caudal peduncle ([Băcescu-Meşter, 1967](#)). [Lelek \(1987\)](#) made this infraspecific name available, calling it *Noemacheilus barbatulus hispanica* [Lelek, 1987](#). In 2007, [Kottelat and Freyhof \(2007\)](#) revalidated *Barbatula quignardi* ([Băcescu-Meşter, 1967](#)) based solely on morphological criteria (length of caudal peduncle 1.1 to 1.6 times in its depth (*vs.* 1.4 to 2.2 for *B. barbatula*). They extended its distribution from the Lez river to the South-West of France (Garonne, Adour and Mediterranean basins) and North-eastern Spain. And [Kottelat \(2012\)](#) designated *Noemacheilus barbatulus* forma *hispanica* [Băcescu-Meşter, 1967](#) and *Nemacheilus barbatulus hispanica* [Lelek, 1987](#) as junior synonym of *Barbatula quignardi*.

However, according to [Băcescu-Meşter \(1967\)](#), Spanish populations differ from *B. quignardi* by a more vigorous size, a shorter head, a larger eye diameter, rather long barbels, a body more cylindrical, a caudal peduncle rather narrow and long, and a colouration with brown-black variegations reaching to lower half of body. This difference had however not been checked with molecular data. Combining morphological and molecular data led to the description of *Barbatula leoparda* [Gauliard, Dettai, Persat, Keith and Denys, 2019](#) in French Catalonia, and highlighted the need for a revision of *B. quignardi* ([Gauliard *et al.*, 2019](#)).

Here we provide the analysis of two widely used mitochondrial markers, in order to verify if Spanish populations are included in *B. quignardi* or if they should be considered a distinct species. The COI is the reference marker for DNA barcoding and 12S rDNA is used for environmental DNA ([Valentini *et al.*, 2016](#); [Miya *et al.*, 2020](#)).

2 Material and methods

2.1 Abbreviations used

Cytochrome oxidase subunit 1 (COI); Fédération Départementale des Associations Agréées de Pêche et de Protection des Milieux Aquatiques, France (FDAAPPMA); head length

(HL); Muséum national d'Histoire naturelle, Paris, France (MNHN); Museo Nacional de Ciencias Naturales, Madrid, Spain (MNCN); Muzeul Național de Istorie Naturală Grigore Antipa, Bucharest, Romania (MGAB); Národní Museum, Natural History Museum, Praha, Czech Republic (JNMP); Office National de l'Eau et des Milieux Aquatiques, France (ONEMA); standard length (SL); Zoologisches Forschungsmuseum Alexander Koenig (ZFMK); Zoologische Staatssammlung Muenchen (ZSM).

2.2 Sampling

Samples were collected by electrofishing between 2009 and 2016 from 21 locations ([Fig. 1](#)). After anaesthesia, Spanish fish were fixed in formalin 5% but fin-clips were conserved in 95% EtOH. As prescribed by the French legislation (substitution of formalin, article R. 4412-66), fish from France were fixed and preserved in 95% EtOH by using progressive concentration of EtOH over a few hours in order to lower the body shrivelling induced by osmotic shock.

2.3 Morphological analysis

Counts and measurements were taken from the left side following [Kottelat and Freyhof \(2007\)](#). Measurements were taken using an electronic caliper. All measurements were made point to point, never by projection. The two last dorsal and anal fin rays were counted as one because they are connected by the same pterygiophore. External characters link to the nostril positions and the lips following [Prokofiev \(2010, 2015\)](#) were observed.

2.4 Comparative material

Barbatula leoparda [Gauliard, Dettai, Persat, Keith and Denys, 2019](#): MNHN-IC-2018-0228, holotype, 62.6 mm SL, Têt at Nefiach, 7th September 2016, Denys and ONEMA coll. – MNHN-IC-2016-0347, paratypes, 4, 32.4–61.0 mm SL, collected with holotype – MNHN-IC-2010-0997, paratype, 1, 48.2 mm SL, Tech at Elne, 14th September 2010, Denys and ONEMA coll. – MNHN-IC-2016-0351, paratype, 1, 46.5 mm SL, Tech at Céret, 9th September 2016, Denys, Hauteceur and ONEMA coll.

Barbatula quignardi ([Băcescu-Meşter, 1967](#)): MGAB77, holotype, 52.0 mm SL, Lez river, July 1962, Băcescu coll. – MGAB78, paratype (mentioned as “allotype” by [Băcescu-Meşter \(1967\)](#)), 56.5 mm SL, collected with holotype – MNHN-IC-2010-1064, 6, 31.2–41.7 mm SL, Lez at Prades-le-Lez, 24th November 2010, Denys and ONEMA coll.

2.5 Molecular analysis

Amplification, sequencing, cleaning and assembling of the complete 12S rDNA and the COI markers were performed according to [Ward *et al.* \(2009\)](#), [Dettai *et al.* \(2011\)](#) and [Gauliard *et al.* \(2021\)](#). Sequences were deposited in the Barcode of Life database (BOLD, www.boldsystems.org; [Ratnasingham and Hebert, 2007](#)) in the LOFFR project and in GenBank. Alignment was manual and straightforward, as

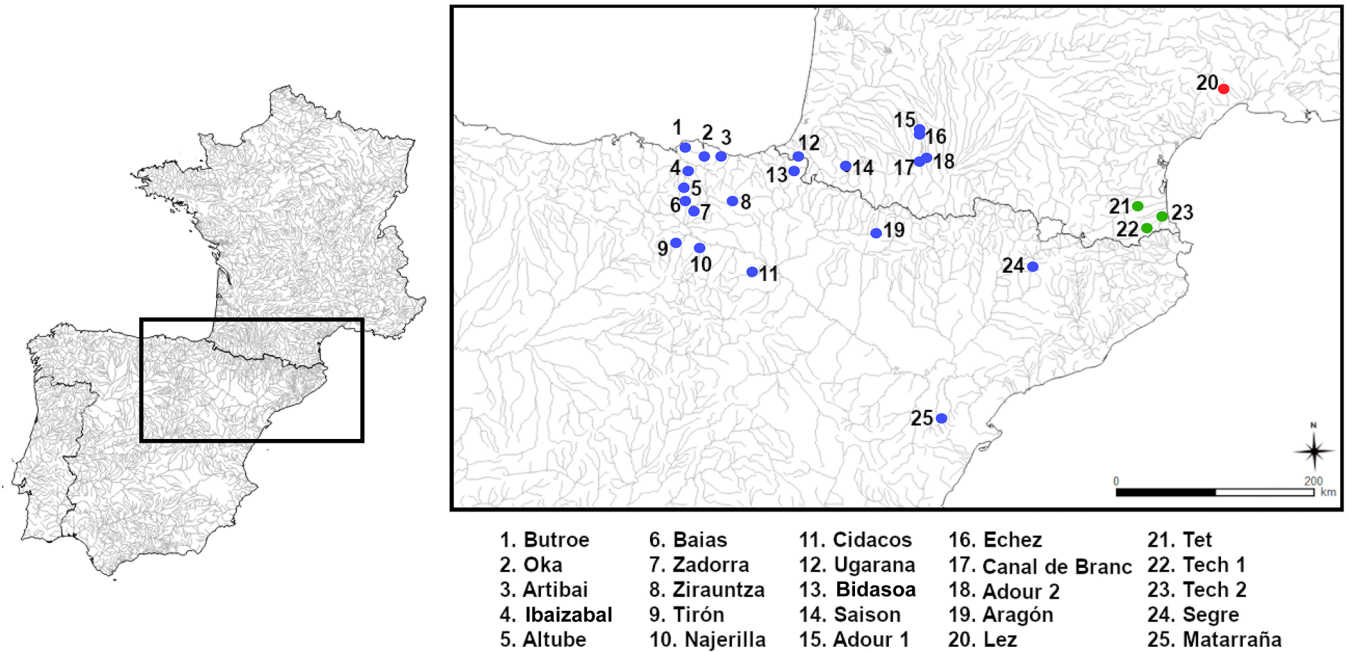


Fig. 1. Map of the 25 sampling locations of *Barbatula* spp through northeastern Spain and southwestern France. Dot colors indicate *Barbatula* species: *B. hispanica* (blue), *B. leoparda* (green) and *B. quignardi* (red).

neither marker includes indels. Molecular delineation by automatic barcode gap was performed using ABGD (<http://www.wabi.snv.jussieu.fr/public/abgd/abgdweb.html>) (Puillandre *et al.*, 2012) and the Barcode Index Number (BIN; Hausmann *et al.*, 2013). Phylogenetic analyses with the COI marker were performed with Bayesian inference (MrBayes 3.2, Ronquist *et al.*, 2012), with the K80+I model selected by JModelTest 2.1.1 (Darriba *et al.*, 2012) according to Bayesian criteria. Bayesian analysis was performed twice independently, launching each time two runs with 5 million generations and sampling every 100 generations. The four subsequent tree files were summarised and 10% of trees eliminated as burnin after checking for convergence. A haplotype network for the COI gene was inferred using PopART software (Leigh and Bryant, 2015) based on a Median-Joining algorithm (Bandel *et al.*, 1999). Intra- and inter-specific distances (p-distances) were calculated with MEGAX (Kumar *et al.*, 2018). Diagnostic sites for both markers were identified with the QUIDDICH package (Kühn and Hasse, 2019) for R (R Core Team, 2013).

2.6 Comparative material used for molecular study

Barbatula leoparda Gauliard, Dettai, Persat, Keith and Denys, 2019: MNHN-IC-2018-0228, holotype, Têt at Nefiach, 7th September 2016, Denys and ONEMA coll., GenBank Accession numbers COI: MK518369, 12S: MK518372 – MNHN-IC-2010-0997, paratype, FFFtag4173, Tech at Elne, 14th September 2010, Denys and ONEMA coll., GenBank Accession number COI: MZ189976.

Barbatula quignardi (Băcescu-Meşter, 1967): MNHN-IC-2010-1064, 3, FFFtag4260-4262, Lez at Prades-le-Lez, 24th November 2010, Denys and ONEMA coll., GenBank Accession numbers COI: MK518367- MK518368- MW288293, 12S: MK518370- MK518371- MW288293.

Barbatula vardarensis (Karaman, 1928): JNMP D2490, Ex33C9, Vardar at Idomeni, GenBank Accession number COI: KJ553163.

Barbatula sturanyi (Steindachner, 1892): JNMP A2943, Ex41A4, Black Drin at Dobovyani, GenBank Accession number COI: KJ553273.

Barbatula toni (Dybowski, 1869): Am-24, Shilka upstream of Shivki, 19th August 2004, Naseka coll., GenBank Accession number COI: KX039652.

Barbatula spp: no voucher, no locality but probably in France, GenBank Accession number 12S: NC_27192 (see Muriene *et al.*, 2015) – ZFMK-ICH-53038 – ZFMK-ICH-53039, Ex53C10 – Ex53C11, Eder at Vöhl, 28th May 2012, Duempelmann coll., GenBank Accession numbers COI: KM286481- KM286482-ZFMK-ICH-58090 – ZFMK-ICH-58352 – ZFMK-ICH-58363, SCEX02C10 – SCEX04B5 – SCEX04B11, Sieg at Hennef, 27th June 2012, Steinmann and Ennenbach coll., GenBank Accession numbers COI: KM286473 - KM286474 - KM286480 – ZSM-Pis-033871, BayFi 04623, Lech at Augsburg, 8th October 2005, GenBank Accession number COI: KM373656-ZSM-Pis-032901, BayFi 02584, Isen at Oberndorfen, 15th April 2005, GenBank Accession number COI: KM373639-ZSM-Pis-032918, BayFi 02531, Paar at Ottmaring, 13th April 2005, GenBank Accession number COI: KM373640.

3 Results

3.1 Molecular delineation

ABGD and BIN analyses as well as the phylogenetic tree for the COI marker (609 bp) inferred from 55 sequences of *Barbatula* discriminate 3 clades within the ingroup (Fig. 2). The first clade is a single haplotype (three sequences) of

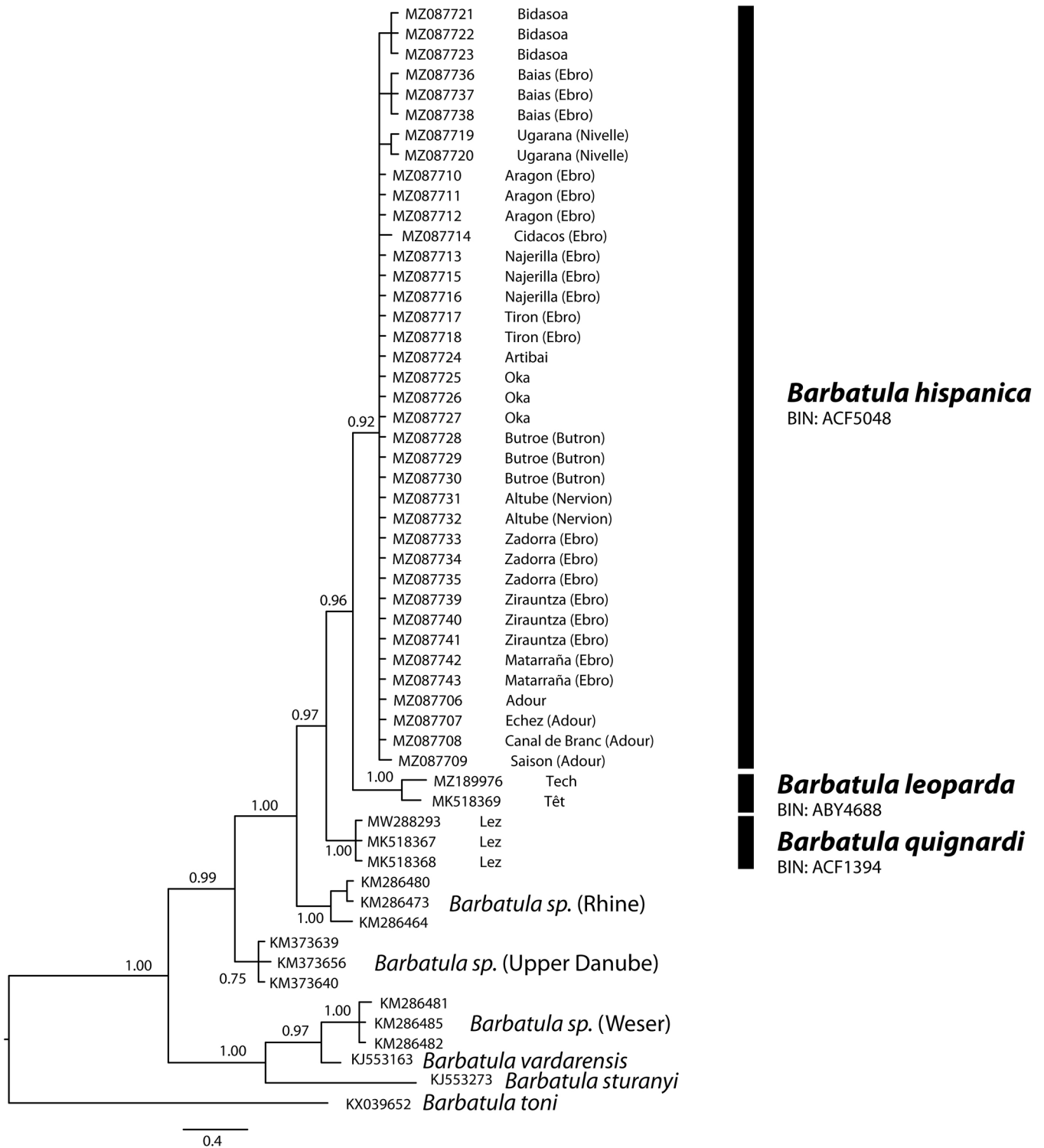


Fig. 2. Phylogenetic tree by Bayesian inference with the COI marker (609 bp) on 55 sequences of *Barbatula* spp. Black vertical bars represent ABGD and BOLD BIN delineations. Posterior probability values are indicated above the nodes.

B. quignardi from its type locality (Lez River). Its sister group is composed by one clade with the two specimens (2 haplotypes) of *B. leoparda*, and a second clade with specimens from the Adour drainage (Southwestern France), Ebro drainage and Basque coastal catchments (Artibai, Butrón,

Nive, Nivelle, Nervión and Oka; Northeastern Spain). This last clade is separated from *B. quignardi* and *B. leoparda* by an average divergence of respectively 1.6 and 2.1%. Within this clade, the seven haplotypes have very little geographical structuration and an average divergence of 0.1%.

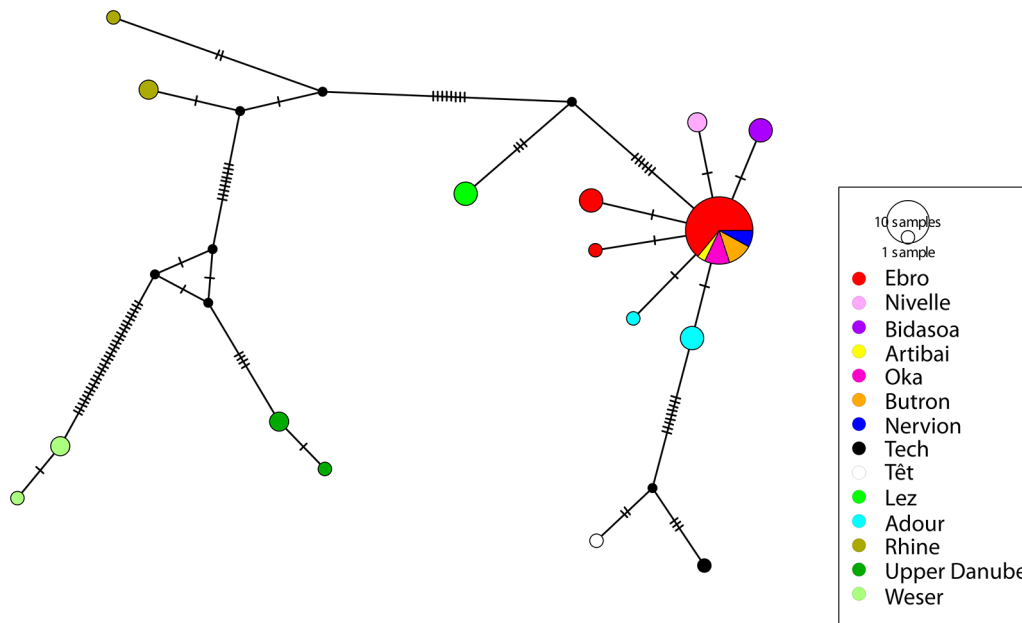


Fig. 3. Haplotype network with the COI marker (609 bp) on 55 sequences of *Barbatula* spp. Circles indicate haplotypes. Each colour represents the catchment of origin of the sample. Notches indicate the number of mutations.



Fig. 4. Specimens of *Barbatula* spp in lateral view. A: *Barbatula hispanica* alive specimen from the stream Cadagua (Nervión drainage) (scale bar = 1 cm; photo credits: I. Doadrio); B: *Barbatula hispanica* MGAB400.151, 51.9 mm SL, Ibaizabal at Durango (Nervión drainage), 24th October 1949, Calderon coll.; C: *Barbatula hispanica* MNCN_ICHTYO 278716-278735, 70.9 mm SL, Ugarana at Dantxarinea (Nivelles drainage), 11th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll.

The COI haplotype network is congruent with the clades recovered in the phylogenetic analysis (Fig. 3). It highlights that samples from the Adour, Nive, Nivelles and Baias catchments each have their own haplotypes.

3.2 Taxonomy

Barbatula hispanica (Lelek, 1987).

EN: Spanish stone loach, FR: Loche franche espagnole (Figs 4, 5a, 6a, 7a, 8a).

3.2.1 Synonyms

Noemacheilus barbatulus forma hispanica Băcescu-Meşter, 1967.

Nemacheilus barbatulus hispanica Lelek, 1987.

3.2.2 Material examined

MGAB400.141, 7, 44.5–58.9 mm SL, Ibaizabal at Durango, 24th October 1949, Calderon coll. – MGAB400.151, 9, 31.4–59.9 mm SL, Ibaizabal at Durango, 24th October 1949, Calderon coll. – MNCN_ICHTYO 278716-278735, 3, 57.2–70.9 mm SL, Ugarana at Dantxarinea, 11th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. – MNCN_ICHTYO 279064-179125, 3, 45.8–66.2 mm SL, Bidasoa at Arrayoz, 12th October 2009, MNCN_ICHTYO Doadrio, Garzón, Pedraza and Ornelas coll. – MNCN_ICHTYO 279188-279189, 1, 70.5 mm SL, Artibai at Amalloa, 12th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. – MNCN_ICHTYO 279227-279231, 3, 39.7–60.5 mm SL, Oka at Mugica, 12th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. – MNCN_ICHTYO 279280-279297, 3, 39.4–59.5 mm SL, Butron at Fruiz, 13th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. – MNHN-IC-2010-0483, 3, 47.8–52.3 mm SL, Echez at Larreule, 23th June 2010, Persat, Denys and Delacoste coll. – MNHN-IC-2010-0488, 1, 54.4 mm SL, Adour at Tarbes, 23th June 2010, Persat, Denys and Delacoste coll. – MNHN-IC-2010-0507, 9, 46.7–83.2 mm SL, canal de Branc at Juillan, 23th June 2010, Persat, Denys and Delacoste coll. – MNHN-IC-2010-1034, 9, 47.6–65.7 mm SL, Saison at

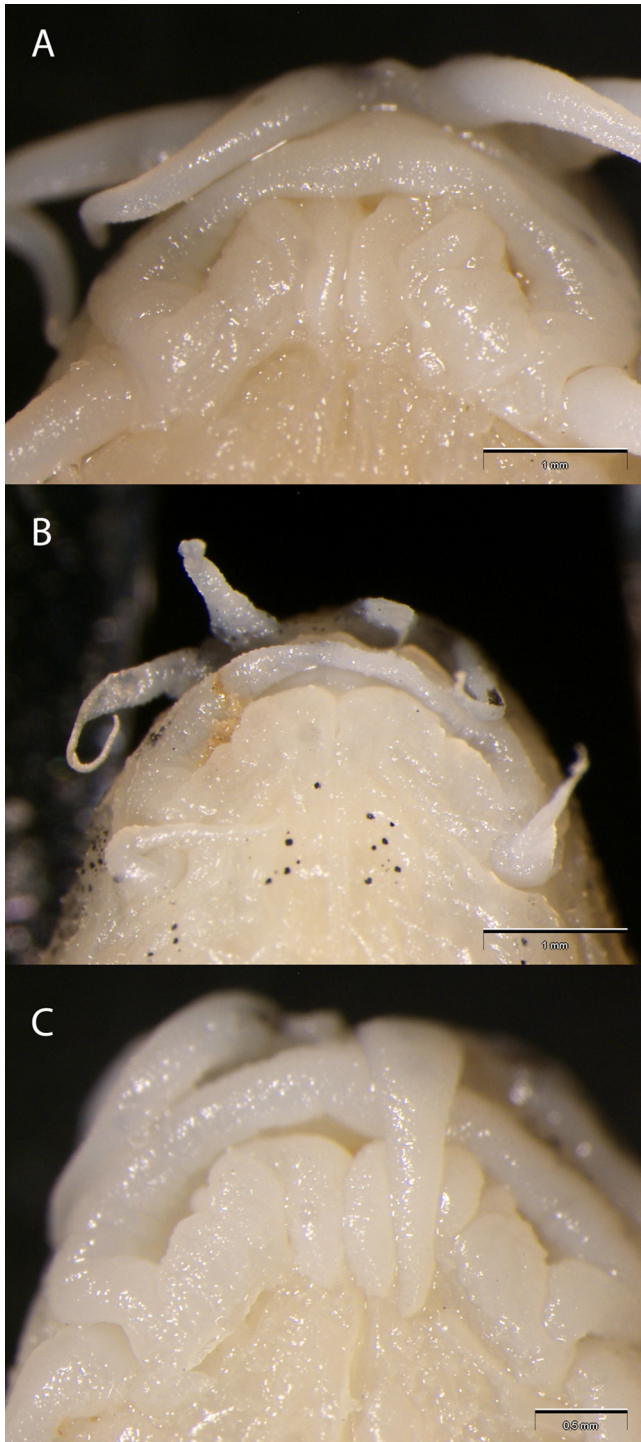


Fig. 5. Mouth of *Barbatula* spp: A: *Barbatula hispanica* MNHN-IC-2016-0067, Adour at Estirac, 29th August 2013, Denys and ONEMA coll.; B: *Barbatula leoparda* MNHN-IC-2016-0347, Têt at Nefiach, 7th September 2016, Denys and ONEMA coll.; C: *Barbatula quignardi* MNHN-IC-2010-1064, Lez at Prades-le-Lez, 24th November 2010, Denys and ONEMA coll.

Menditte, 8th October 2010, Denys and ONEMA coll. – MNHN-IC-2016-0067, 2, 41.2–42.6 mm SL, Adour at Estirac, 29th August 2013, Denys and ONEMA coll.

3.2.3 Material used for the molecular study

No voucher, MNCN-ADN 118999-119000, Altube at Ziorraga, 18th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. GenBank accession numbers COI: MZ087731-MZ087732, GenBank accession numbers 12S: MZ088041-MZ088042 – MNCN_ ICHTYO 278716-278735, MNCN-ADN 119004-119005, Ugarana at Dantxarinea, 11th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. GenBank accession numbers COI: MZ087719-MZ087720 – MNCN_ ICHTYO 279064-179125, MNCN-ADN 119001 to 119003, Bidasoa at Arrayoz, 12th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. GenBank accession numbers COI: MZ087721 to MZ087723 – MNCN_ ICHTYO 279188-279189, MNCN-ADN 119006, Artibai at Amalloa, 12th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. GenBank accession number COI: MZ087724 – MNCN_ ICHTYO 279227-279231, MNCN-ADN 119007 to 119009, Oka at Mugica, 12th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. GenBank accession numbers COI: MZ087725 to MZ087727 – MNCN_ ICHTYO 279280-279297, MNCN-ADN 119010 to 119012, Butroe at Fruiz, 13th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll. GenBank accession numbers COI: MZ087728 to MZ087730 – MNCN_ ICHTYO 281596-281602, MNCN-ADN 119013 to 119015, Aragon at Jaca, 3rd October 2009, Doadrio, Garzai, Solis and Polo coll. GenBank accession numbers COI: MZ087710 to MZ087712 – MNCN_ ICHTYO 281832-281840, MNCN-ADN 119016, Cidacos at Arnedo, 4th October 2009, Doadrio, Garzón, Solis and Polo coll. GenBank accession number COI: MZ087714 – MNCN_ ICHTYO 275902-275919, MNCN-ADN 119017 to 119019, Najerilla at Uruñuela, 5th October 2009, Doadrio, Garzón, Solis and Polo coll. GenBank accession numbers COI: MZ087713 - MZ087715 - MZ087716 – MNCN_ ICHTYO 276098-276119, MNCN-ADN 119020-119021, Tirón at Herramelluri, 5th October 2009, Doadrio, Garzón, Solis and Polo coll. GenBank accession numbers COI: MZ087717-MZ087718 – MNCN_ ICHTYO 283034-283037, MNCN-ADN 119022 to 119024, Zadorra at Nanclares de la Oca, 19th October 2009, González, Prieto, Pedraza and Ornelas coll. GenBank accession numbers COI: MZ087733 to MZ087735 – MNCN_ ICHTYO 296724-296757, MNCN-ADN 119025-119026, Matarraña at Valderrobes, 23th October 2009, Doadrio, Garzón and González coll. GenBank accession numbers COI: MZ087742-MZ087743 – no voucher, MNCN-ADN 119028 to 119030, Baias at Zuazo de Kuartango, 19th October 2009, Doadrio, Garzón and González coll. GenBank accession numbers COI: MZ087736 to MZ087738 – no voucher, MNCN-ADN 119031 to 119033, Zirauntza at Albeniz, 23th October 2009, Doadrio, Garzón and González coll. GenBank accession numbers COI: MZ087739 to MZ087741 – MNHN-IC-2010-0483, FFFtag4100, Echez at Larreule, 23th June 2010, Persat, Denys and Delacoste coll. GenBank accession number COI: MZ087707, GenBank accession number 12S: MZ088041 – MNHN-IC-2010-0488, FFFtag4093, Adour at Tarbes, 23th June 2010, Persat, Denys and Delacoste coll. GenBank accession number COI: MZ087706 – MNHN-IC-2010-0507, FFFtag4119, canal de Branc at Jullian, 23th June 2010, Persat, Denys and Delacoste coll. GenBank accession number COI: MZ087708 – MNHN-IC-2010-1034, FFFtag4174, Saison at Menditte, 8th October 2010, Denys and

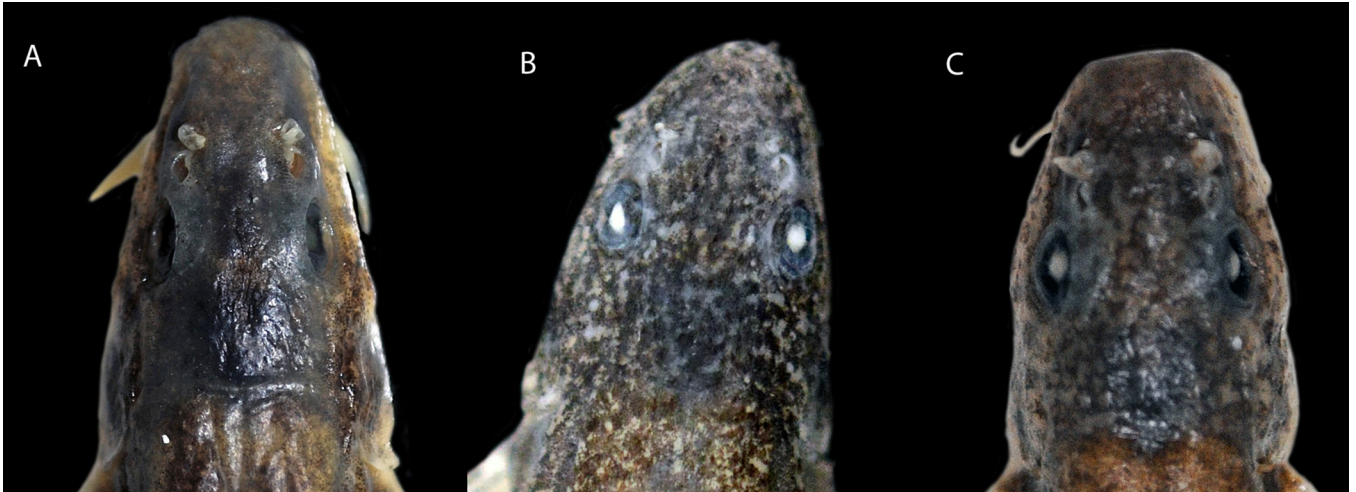


Fig. 6. Heads of *Barbatula* spp in dorsal view: A: *Barbatula hispanica* MNCN_ICHYTO 278716-278735, 3, 57.2–70.9 mm SL, 57.2 mm SL, Ugarana at Dantxarinea, 11th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll.; B: *Barbatula leoparda* holotype MNHN-IC-2018-0228, 62.6 mm SL, Têt at Nefiach, 7th September 2016, Denys and ONEMA coll.; C: *Barbatula quignardi* holotype MGAB77, 52 mm SL, Lez River, July 1962, Băcescu coll.



Fig. 7. Heads of *Barbatula* spp in lateral view: A: *Barbatula hispanica* MNCN_ICHYTO 279280-279297, 59.5 mm SL, Butroe at Fruiz, 13th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll.; B: *Barbatula leoparda* holotype MNHN-IC-2018-0228, 62.6 mm SL, Têt at Nefiach, 7th September 2016, Denys and ONEMA coll.



Fig. 8. Specimens of *Barbatula* spp in ventral view: A: *Barbatula hispanica* MNCN_ICHYTO 279064-179125, 45.8 mm SL, Bidasoa at Arrayoz, 12th October 2009, Doadrio, Garzón, Pedraza and Ornelas coll.; B: *Barbatula leoparda* holotype MNHN-IC-2018-0228, 62.6 mm SL, Têt at Nefiach, 7th September 2016, Denys and ONEMA coll.

ONEMA coll. GenBank accession number COI: MZ087709, GenBank accession number 12S: MZ088042.

3.2.4 Diagnosis

Barbatula hispanica is distinguished from *B. leoparda* by an upper lip with a well-marked medial incision (vs. with a medial incision not exceeding the half of its width; Fig. 5), a fleshy upper lip (vs. slim; Fig. 5), well-marked mental lobes on the lower lip (vs. not well marked; Fig. 5), an interorbital width 18.5–33.7% HL (vs. 35.5–41.8% HL; Tab. 1; Fig. 6), a post-

Table 1. Morphometry of *Barbatula hispanica*, *B. leoparda* and *Barbatula quignardi*. Values in parentheses: mean and standard deviation. Values of holotypes are included in range.

N	<i>Barbatula hispanica</i>	<i>Barbatula leoparda</i>		<i>Barbatula quignardi</i>	
	53	Holotype	7	Holotype	8
Standard length SL (mm)	31.4-83.2 (53.0; 10.4)	62.6	33.2-62.6 (47.3; 11.4)	52.1	31.2-56.5 (39.8; 9.6)
In percent of SL					
Head length HL	20.6-27.3 (23.7; 1.5)	22.1	22.1-24.0 (23.0; 0.6)	23.3	23.3-26.7 (25.6; 1.3)
Predorsal length	48.9-57.0 (53.3; 1.7)	52.3	50.4-55.0 (52.6; 1.7)	52.0	52.1-56.8 (54.0; 1.5)
Postdorsal length	32.2-39.4 (36.7; 1.6)	34.1	31.7-38.6 (35.7; 2.5)	35.3	32.7-36.9 (35.0; 1.2)
Prepectoral length	20.5-29.1 (25.1; 1.7)	22.8	22.7-26.4 (24.1; 1.4)	23.7	23.8-29.1 (27.1; 1.8)
Prepelvic length	51.7-59.5 (54.6; 1.6)	56.1	51.1-56.2 (54.6; 1.9)	54.3	53.4-57.0 (55.4; 1.3)
Preanal length	71.8-82.2 (77.6; 1.5)	76.8	74.0-80.3 (78.0; 2.4)	78.7	74.6-82.0 (78.4; 2.2)
Caudal peduncle length CPL	13.4-19.4 (17.3; 1.1)	17.0	13.8-17.9 (16.7; 1.4)	16.1	14.5-16.9 (15.6; 0.8)
Caudal peduncle depth CPD	9.1-13.3 (11.5; 1.0)	12.9	11.7-13.0 (12.4; 0.5)	11.9	8.9-12.4 (10.4; 1.4)
Body depth	12.2-20.5 (14.7; 1.6)	14.2	13.4-18.5 (15.0; 1.7)	16.3	13.4-19.5 (16.1; 1.9)
Dorsal-fin length	17.9-23.9 (21.2; 1.3)	17.1	17.1-22.7 (20.6; 1.8)	23.5	17.5-23.5 (21.5; 2.0)
Anal-fin length	13.8-18.7 (16.6; 1.2)	14.2	14.2-17.2 (15.9; 1.2)	17.7	13.9-18.5 (16.9; 1.7)
Ratio CPL/CPD	1.2-2.0 (1.5; 0.2)	1.3	1.1-1.5 (1.4; 0.1)	1.4	1.2-1.7 (1.5; 0.2)
In percent of HL					
Snout length	31.2-48.0 (41.7; 3.2)	45.8	39.5-50.3 (45.3; 4.1)	41.3	41.3-47.7 (44.6; 2.3)
Eye diameter	15.0-22.0 (18.6; 1.6)	18.0	15.6-19.5 (17.9; 1.6)	20.4	14.9-21.5 (19.5; 2.3)
Postorbital length	36.9-48.2 (42.5; 2.4)	46.9	45.7-51.6 (48.0; 2.5)	45.8	40.0-45.8 (43.9; 2.1)
Interorbital width	18.5-33.7 (26.9; 3.1)	41.8	35.5-41.8 (38.3; 2.8)	31.7	29.3-36.2 (33.1; 2.6)

Table 2. Diagnostic sites for *Barbatula hispanica*, *Barbatula leoparda*, *Barbatula quignardi* and *Barbatula sp* from [Murienne et al. \(2015\)](#) on the 12S marker.

	GenBank Accession	37	134	178	363	384	385	477	616	639	642	875	889	890
<i>Barbatula hispanica</i>	MZ088039 to MZ088042	A	G	G	A	A	C	A	G	A	A	T	C	A
<i>Barbatula leoparda</i>	MK518372	.	.	.	G	.	.	G	A	.	G	.	.	.
<i>Barbatula quignardi</i>	MK518370, MK518371, MW288293	G	.	A	.	G	T	.	.	G	.	C/T	.	G
<i>Barbatula sp</i>	NC_27192	.	A	T	.

orbital length representing 36.9–48.2% of HL (*vs.* 45.7–51.6% HL; [Tab. 1](#)), the third pair of barbels at the corner of the lips reaching the posterior eye border (*vs.* not reaching to the posterior eye border; [Fig. 7](#)), and a belly and jugular area without blotches (*vs.* presence of blotches; [Fig. 8](#)).

Barbatula hispanica is distinguished from *B. quignardi* by an upper lip with a well-marked medial incision (*vs.* with a medial incision not exceeding the half of its width; [Fig. 5](#)) and an interorbital width 18.5–33.7% HL (*vs.* 29.3–36.2% HL; [Tab. 1](#); [Fig. 6](#)).

3.2.5 Distribution and habitat

Barbatula hispanica occurs in the Ebro and in the eastern Cantabria coastal catchments in Spain, as well as in the Adour drainage (Southwestern France) ([Fig. 1](#)). Like other *Barbatula* species, it lives in fresh, clear waters with current and grounds of stones, pebbles, gravels, sand or marl ([Neveu, 1981](#); [Mastrorillo et al., 1996](#); [Doadrio et al., 2011](#)).

3.2.6 Molecular characterization

On the COI marker, *B. hispanica* can be differentiated from *B. leoparda* and *B. quignardi* by three diagnostic sites: A (*vs.* G) in position 205, T (*vs.* C) in position 319 and G (*vs.* A) in position 376.

For the 12S rDNA sequences there is no molecular synapomorphy of *B. hispanica* ([Tab. 2](#)). However, it can be distinguished from *B. leoparda* by four diagnostic sites: A (*vs.* G) in positions 363, 477 and 642, and G (*vs.* A) in position 616. It is also distinguished from *B. quignardi* by six diagnostic sites: A (*vs.* G) in positions 37, 384, 639 and 890, G (*vs.* A) in position 178, and C (*vs.* T) in position 385.

3.2.7 Nomenclatural note

[Băcescu-Meşter \(1967\)](#) described *Noemacheilus barbatulus* forma *hispanica* from eight specimens from the river Ibaizabal at Durango (Nervión drainage) and three specimens from the Tajo River. However, no catalog number is mentioned

in the article. There is only a figure illustrating the taxon with a male (length 8.6 cm TL) caught in the Nervión drainage in April 1961 (Băcescu-Meşter, 1967: Fig. 6A-B). There are two lots of *Barbatula* from the Nervión drainage (river Ibaizabal) at Durango in the MGAB collections (MGAB400.141 and MGAB400.151) and both were caught in October 1949 but we have no certainty on whether they belong to the syntype series (Iftime, pers. com.). Moreover, according to Doadrio *et al.* (2011), no loach inhabits the Tajo River. So we do not know for sure the origin of the three syntypes from this locality, and they may even correspond to another evolutionary lineage or species. By consequence, according to the art. 74.7 of the International Code of Zoological Nomenclature, we designate as lectotype of *Noemacheilus barbatulus* forma *hispanica* Băcescu-Meşter, 1967 the specimen illustrated by Băcescu-Meşter (1967: Fig. 6A-B). And then, we designate this same specimen as lectotype of *Nemacheilus barbatulus hispanica* Lelek, 1987.

4 Discussion

Both molecular and morphological data agree on the delineation of *Barbatula hispanica*. It therefore needs to be revalidated. This species occurs in Spain in the Ebro drainage and in the eastern Cantabria coastal catchments as well as in the Adour drainage (Southwestern France). This distribution is identical to the one of two other species with which it co-occurs, *Gobio lozanoi* Doadrio and Madeira, 2004 and *Phoxinus bigerri* Kottelat, 2007 (Doadrio and Madeira, 2004; Kottelat and Persat, 2005; Kottelat, 2007; Doadrio *et al.*, 2011; Corral-Lou *et al.*, 2019; Denys *et al.*, 2020). These three species would share the same biogeographical history in the Iberian Peninsula, with the Pleistocene glaciation cycles and the apparition of Mediterranean climate (see Corral-Lou *et al.*, 2019), as well as a possible connection between southern France and northern Spain during the Pleistocene when water levels decreased due to glaciation cycles (Patarnello *et al.*, 2007). The low intraspecific genetic distance (0.1%) in addition to the low geographical structuration observed for the COI marker may suggest a recent colonization of some catchments, like the Ebro drainage, due to the glaciers melting during the Late Pleistocene in the Cantabrian belt (Serrano *et al.*, 2013, 2017; García-Ruiz *et al.*, 2016). Local introductions like in northern Europe (Lundberg and Svanberg, 2010; Norén *et al.*, 2018) or like in the Neretva catchment in Croatia (Tutman *et al.*, 2017) cannot be excluded.

Barbatula hispanica can be distinguished from both other species with the COI marker (Fig. 2). The genetic distance from *B. leoparda* (>2%) is usual for teleosts (e.g. Ward *et al.*, 2009; Dettai *et al.*, 2011; Geiger *et al.*, 2014). The genetic distance with *B. quignardi* is lower (1.6%) but in line with the interspecific distances observed for 11.3% of Mediterranean freshwater fishes (Geiger *et al.*, 2014). Both ABGD and BOLD detected barcode gaps between these species, highlighting distinct evolutionary lineages.

Barbatula hispanica is also distinguished from both other species by morphological characters. Reevaluating the diagnosis of Băcescu-Meşter (1967), we disagree with the use of the head, the caudal peduncle, the barbels lengths and the eye diameter to distinguish *B. hispanica* from *B. quignardi*.

The integration of more specimens in our study made overlaps apparent between these characters.

Barbatula quignardi seems to be restricted to the Lez River, contrary to what Kottelat and Freyhof (2007) thought. It differs from *B. hispanica* by an upper lip with a medial incision not exceeding the half of its width (vs. with a well-marked medial incision; Fig. 5) and an interorbital width –29.3–36.2% HL (vs. 18.5–33.7% HL; Tab. 1; Fig. 6). It also differs from *B. leoparda* by an upper lip with a well-marked medial incision (vs. with a medial incision not exceeding the half of its width; Fig. 5), a fleshy upper lip (vs. slim; Fig. 5), well-marked mental lobes on the lower lip (vs. not well marked; Fig. 5), an interorbital width 29.3–36.2% HL (vs. 35.5–41.8% HL; Tab. 1; Fig. 6), a post-orbital length representing 40.0–45.8% of HL (vs. 45.7–51.6% HL; Tab. 1), the third pair of barbels at the corner of the lips reaching the posterior eye border (vs. not reaching to the posterior eye border) and a belly and jugular area without blotches (vs. presence of blotches). Geiger *et al.* (2014) grouped samples from the Rhône river with this species according to mitochondrial data. However, Băcescu-Meşter (1967) and Gauliard *et al.* (2019) highlighted morphological differences between the populations from the Lez River and the Rhône catchment. We prefer to consider each population separated until more data is available. This situation is similar to the one of the Lez sculpin, *Cottus petiti* Băcescu and Băcescu-Meşter, 1964, endemic to the Lez River. It is close to *Cottus gobio* Linnaeus, 1758 occurring in the Rhône catchment, different according to morphological data (Freyhof *et al.*, 2005) but not distinguishable with molecular data (Eppe *et al.*, 1999; Šlechtová *et al.*, 2004; Geiger *et al.*, 2014).

This study furthers the evolving taxonomic knowledge on *Barbatula* in Europe, with several species described or revalidated (Sorić, 2000; Geiger *et al.*, 2014; Gauliard *et al.*, 2019) and multiple cryptic lineages highlighted (Šedivá *et al.*, 2008; Knebelsberger *et al.*, 2015; Behrmann-Godel *et al.*, 2017; Norén *et al.*, 2018; Behrens-Chapuis *et al.*, 2021). *Barbatula hispanica* is the fourth stone loach species known in France, and the second species known for Spain after *B. cf. barbatula*. However, the population in the Ter catchment (Northeastern Spain) belongs to a distinct evolutionary lineage from the Ebro populations and those of other European catchments (Šedivá *et al.*, 2008), and they are also morphologically different (Aparicio *et al.*, 2016). Moreover, a population introduced in the Duero drainage is also morphologically different (Doadrio *et al.*, 2011). These populations were originally affiliated to *B. barbatula* (Perea *et al.*, 2011), but without molecular data. They could actually correspond to any *Barbatula* sp.

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