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ORIGINAL ARTICLE

New insights into the systematics of North Atlantic *Gaidropsarus* (Gadiformes, Gadidae): flagging synonymies and hidden diversity

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ABSTRACT

Gaidropsarus Rafinesque, 1810 is a genus of marine fishes, commonly known as rocklings, comprising 14 living species and showing a high ecological diversity from the intertidal zone to the deep sea. The systematics of this group has been controversial due to a general lack of representative specimens and the conservative morphology exhibited. A multidisciplinary approach combining the analysis of meristic data and the DNA barcode standard was applied in a species delimitation approach. Individuals representing eight valid and three unnamed species were collected, morphologically identified and archived in several museum collections. Comparison of DNA sequences shows complex results, furthering the idea of the difficult identification of specimens based on traditional taxonomy. DNA barcoding supports synonymies, like *G. biscayensis*–*G. macrophthalmus* and *G. guttatus*–*G. mediterraneus*, agreeing with the extensive overlaps observed in the meristic variables analysed and suggesting a reduction in the number of species. Genetic distances showed pairs of closely related species like *G. granti*–*G. vulgaris* and *G. argentatus*–*G. ensis*, the latter being only distinguished by one main distinctive character. Four deep-water specimens, morphologically classified only to the genus level, constituted three independent taxa apart from the ones present in this study and with no barcode matches in the repository databases. They could represent new records for the North Atlantic or unknown species of this genus. The results obtained show that more studies will be necessary to solve the systematics of this branch of the Gadiformes.

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Introduction

The genus Gaidropsarus

The genus *Gaidropsarus* Rafinesque, 1810 shows a remarkable ecological diversity and comprises 14 living species occurring from the intertidal zone to the deep sea, from the arctic to temperate and subtropical waters. Eight of these species, *Gaidropsarus argentatus* (Reinhardt, 1837), *G. biscayensis* (Collett, 1890), *G. ensis* (Reinhardt, 1837), *G. granti* (Regan, 1903), *G. guttatus* (Collett, 1890), *G. macrophthalmus* (Günther, 1867), *G. mediterraneus* (Linnaeus, 1758) and *G. vulgaris* (Cloquet, 1824), have been described in the North Atlantic Ocean and the Mediterranean Sea and they are still currently considered valid species.

Fishes from this genus, commonly known as rocklings, are characterized by an elongated and relatively slender body, with barbels present on the chin and at each anterior nostril on the snout. The first dorsal ray is followed by a row of small fleshy filaments, the anal fin is not indented and a lateral line is uninterrupted along its entire length (Cohen et al. 1990).

The classification of the species is controversial, having been alternatively placed in the family Gaidropsaridae (Howes 1991; Iwamoto & Cohen 2016), Gadidae (Endo 2002; Teletchea et al. 2006; Roa-Varón & Ortí 2009; Nelson et al. 2016) and Lotidae (Van der Laan

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et al. 2014; Froese & Pauly 2016). In its last edition, the reference compendium 'Fishes of the World' (Nelson et al. 2016) places the genus *Gaidropsarus* in the family Gadidae, which is the classification followed in this investigation.

In spite of the taxonomic revisions of rocklings published (de Buen 1934; Svetovidov 1948, 1986a, 1986b; Iwamoto & Cohen 2016), it has been suggested that additional studies are needed. In fact, when morphology is compared to DNA data, discrepancies arise (Francisco et al. 2014). The lack of representative specimens of the known species in the collections of museums may account for the poor knowledge of the morphological variability in this genus (Balushkin 2009).

DNA barcoding

DNA barcoding has been considered an efficient aid to traditional taxonomy (Hebert & Gregory 2005; Savolainen et al. 2005), designed to facilitate fast and accurate identification of specimens from a short standardized DNA sequence (Hebert et al. 2003; Miller 2007). In its strictest sense, DNA barcoding addresses only a limited aspect of the taxonomic process, by matching DNA sequences to 'known' species, the latter being delimited with traditional (e.g. morphological) methodologies (Teletchea 2010). In this context, the role of barcodes is to provide a methodology to assign unidentified specimens to already characterized species (Hebert et al. 2003). This is a great aid to the end users of taxonomy, and it is also helping in making more rapid progress in identification of species and delimitation of species groups (Ratnasingham & Hebert 2007). However, where species are simply unknown or no attempts have been made to delimit them, the barcode approach as originally intended is inadequate in its applicability (Savolainen et al. 2005) and should be employed with caution. It is generally assumed for most vertebrate species that it is possible to use DNA markers such as the mitochondrial DNA cytochrome *c* oxidase subunit I (mtDNA-COI) to distinguish between species, and therefore the barcoding approach is based on the assumption that the variation within species of vertebrates is smaller than between species (Ratnasingham & Hebert 2007). As a consequence, DNA barcoding has the potential to aid taxonomic studies and help to clarify cases of potential synonymy (Bañón et al. 2013) and delimitation of cryptic species (Puckridge et al. 2013; Hyde et al. 2014). In order to infer species delimitations using mtDNA-COI, sequences need to take the following into consideration: retention of ancestral polymorphism, male-biased gene flow, selection on any mtDNA nucleotide, introgression following hybridization and paralogy resulting from the transfer of mtDNA gene copies to the nucleus (Moritz & Cicero 2004). Despite their benefits and pitfalls, the mtDNA-COI barcode sequences and their ever-increasing taxonomic coverage have been considered an unprecedented resource for taxonomy and systematics studies and their function as a diagnostic tool should be acknowledged (Savolainen et al. 2005).

DNA barcoding is recognized as an important new tool that can be usefully applied to help resolve taxonomic issues in fishes based on the development of a reference library of barcode sequences from vouchered specimens (Ward et al. 2005, 2009; Zemlak et al. 2009). The analysis of validated DNA barcodes for cluster recognition provides an efficient approach for recognizing putative species (operational taxonomic units, OTU) (Kekkonen & Hebert 2014). The Barcode Index Number (BIN) system is a persistent registry for animal OTUs recognized through sequence variation in the mtDNA-COI barcode region (Ratnasingham & Hebert 2013).

On December 2016, a search of the BOLD database produced 45 specimen records of *Gaidropsarus* with barcodes comprising five species, *G. argentatus*, *G. ensis*, *G. mediterraneus*, *G. novaezealandiae* and *G. vulgaris*, of which only 22 were public. A few DNA sequences of rocklings have been obtained in relation to different attempts to infer the phylogeny of gadiform fishes employing a variety of markers (Bakke & Johansen 2002, 2005; Teletchea et al. 2006; Von der Heyden & Matthee 2008; Roa-Varón & Ortí 2009; Francisco et al. 2014) and with the molecular assignation of specimens employing the mtDNA-COI barcode (Costa et al. 2012; McCusker et al. 2013; Knebelsberger et al. 2014; Landi et al. 2014).

The aim of this investigation is to provide an insight into the systematics of the genus *Gaidropsarus* using a molecular marker and comparing the results with the morphological data available in the scientific literature. To this end, a library combining sequences obtained from voucher specimens generated in this investigation and others of already deposited BOLD public records was built up. In order to understand the barcoding results, an extensive bibliographic revision of main distinctive morphological characters was carried out. In some cases, the comparison of sequences flags incongruity in the delimitation of species of this genus, characterized by a highly conserved morphology.

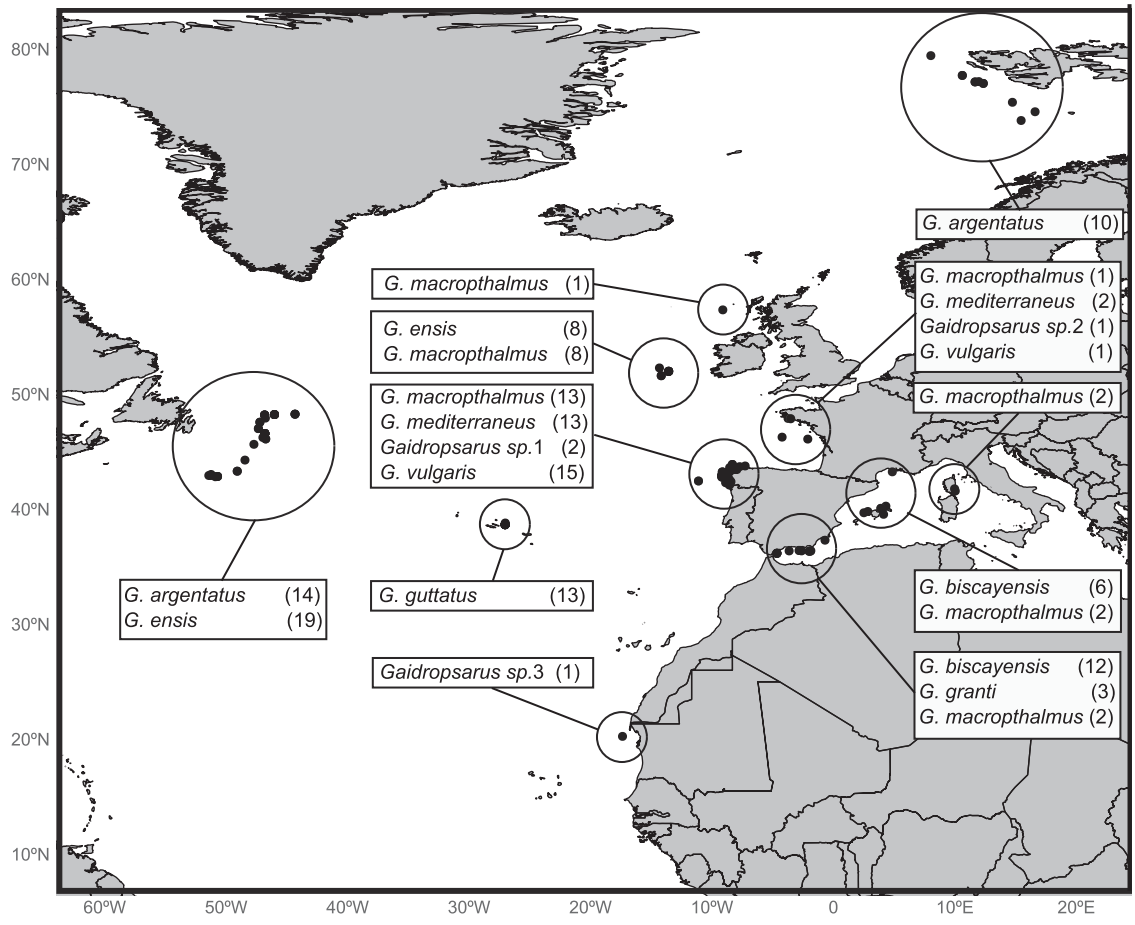

Figure 1. Sampling areas of *Gaidropsarus* in the North Atlantic Ocean and in the Mediterranean Sea, including species captured and number of specimens (shown in brackets).

Material and methods

Sample collection, morphological data and identification

Sampled at different locations in the North Atlantic and the Mediterranean were 149 specimens of rocklings (Figure 1; Table S1, supplementary material). Specimens were captured in a variety of ecological niches, from shallow coastal waters (one specimen of *G. mediterraneus* at a depth of less than 1 m in French Brittany) to deep waters (one specimen of *G. ensis* at a depth of 1458 m off Newfoundland and Labrador). Most specimens were immediately frozen and, upon transportation to the laboratory, muscle samples were removed and stored in 95% ethanol. The molecular results were compared with the main distinctive meristic characters obtained after an exhaustive bibliographical revision (Table I).

Specimens were identified to the species level according to Svetovidov (1986a, 1986b). Vouchers

Table I. Counts of the main distinctive characters of species of *Gaidropsarus* from the north Atlantic and Mediterranean. Species were ordered from up to down by similar species paring according to barcoding results. Abbreviations: D, dorsal; A, anal; V, pelvic; and P, pectoral.

	Vertebrae	2nd D-fin rays	A-fin rays	V-fin rays	P-fin rays	Gill rakers	Source*
G. argentatus	49–53	52–65	43–51	7–8	22–24	1+08–11	4, 5, 6, 10
G. ensis	50–54	52–64	40–48	6–7	20–27	1–2+10–11	4, 5, 6, 10
G. biscayensis	43–47	48–54	40–46	6–7	18–20	+06–07	5, 6, 13
G. macrophthalmus	45–47	53–59	45–50	6–7	17–19	+08–09	5, 6, 13
G. vulgaris	46–49	56–64	46–54	6–7	21–22	+07–09	5, 6, 13
G. granti	47	55–60	45–52	7–8	20–22	1+09	1, 5, 6, 7, 8, 9, 11, 12
G. guttatus	47–50	48–58	42–50	7	16–19	+07–09	2, 3, 5, 13
G. mediterraneus	46–50	51–63	44–52	5–6	16–19	+07–10	5, 6, 13

*Sources: 1, Regan (1903); 2, Svetovidov (1948); 3, Maul (1952); 4, Marckle (1982); 5, Svetovidov (1986a); 6, Svetovidov (1986b); 7, Zachariou-Mamalinga (1999); 8, Bañón et al. (2002); 9, Mura & Cau (2003); 10, Fahay (2007); 11, Pais et al. (2008); 12, Orsi Relini & Relini (2014); 13, Iwamoto & Cohen (2016).

were deposited in the Muséum National d'Histoire Naturelle (Concarneau and Paris, France), Museo de Historia Natural da Universidade de Santiago de Compostela (Santiago de Compostela, Spain) and Colección de Fauna Marina del Centro Oceanográfico de Málaga (CFM-IEOMA; Málaga, Spain). A project has been created in the BOLD database with the title 'Molecular identification of *Gaidropsarus* fishes' (Code GSRUS) where data, including barcoding DNA sequences of specimens, photographs and other details, are available. Sequences were also deposited in GenBank under accession numbers KY250169–KY250315, KY370533 and KY370534 (Table S1).

DNA extraction, PCR amplification and sequencing

Total DNA was purified from 25 mg of muscle tissue taken from each specimen according to the spin-column protocol of the Tissue DNA Extraction Kit (Omega-Biotek). The standard 5′ barcoding region of the COI gene (ca. 650 bp) was amplified by polymerase chain reaction (PCR) using the universal primer cocktail for fish DNA barcoding COI-3 (Ivanova et al. 2007). The following reaction conditions were applied: initial denaturation at 98°C for 30 s followed by 35 cycles of 98°C for 5 s, annealing at 52°C for 5 s and 72°C for 10 s, with a final extension at 72°C for 1 min. PCR was carried out using Phire Green Hot Start II DNA Polymerase (Thermo Scientific); mixtures contained a final volume of 25 µl and included 12.5 µl of 2× Phire Green HS II PCR Master Mix, 2 µl of primer mixture and between 50 and 100 ng of template DNA. COI amplicon bands were visualized on 1.2% agarose gels (Seakem LE Agarose) stained with ethidium bromide and reactions were purified with ExoSAP-IT (Affymetrics) following the manufacturer's instructions. DNA sequencing reactions were carried out in both directions using the M13F (−21) and M13R (−27) primers (Messing 1983). The resulting products were resolved in an ABI3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) and the consensus sequences were obtained after assembling the direct and reverse traces with SEQSCAPE v. 2.5 (Applied Biosystems).

The sequences of the 10 MNHN vouchers were obtained following protocols detailed elsewhere (Iglésias et al. 2016).

Molecular analysis and assignment of specimens

A reference dataset was built with 149 mtDNA-COI sequences derived from voucher specimens assigned to species of *Gaidropsarus*. They were aligned together with another 22 sequences retrieved from BOLD, employing the MUSCLE algorithm (Edgar 2004). The specimens used in the analysis are listed in Tables S1 and S2 and comprise 171 barcodes. The criterion for the genetic divergence estimation was the number of base differences per site between sequences, also called uncorrected *p*-distance (Nei & Kumar 2000). Its use is more accurate for the intrageneric/intraspecific level estimations and yields higher or similar identification success rates for neighbour-joining trees than K2P distance, which overestimates the genetic distances (Srivathsan & Meier 2012). The molecular analysis was conducted using the Neighbour-Joining (NJ) method (Saitou & Nei 1987) in MEGA 6.0 (Tamura et al. 2013), with confidence limits tested through a bootstrap procedure (Felsenstein 1985) with 2000 replicates. The resulting tree was edited using TreeGraph 2 (Stöver & Müller 2010). A genetic distance matrix was obtained among the species-like clusters based on the molecular analysis in order to explore the data and detect possible specimen misidentifications or hybrids, as well as synonyms or cryptic species.

The specimen assignment for every sequence was inferred from the existence of species-level assigned individuals belonging to the same cluster. In the absence of voucher specimens to compare with, specimen assignment was attempted using the identification tool present in BOLD Systems, which also allows comparison with private sequences. Sequences were grouped in representative haplotypes (Table S3) using the software DnaSP v. 5 (Librado & Rozas 2009).

Test of the proposed assignments

A comparison between the minimum distance value to a congener sequence with the maximum divergence within species was performed for each of the 171 barcodes, with the software TaxonDNA using *p*-distance (Meier et al. 2006).

Repeated values, from the same species, were represented only once and, therefore, a final scatterplot with 48 points was obtained. The distance-based species delimitation criteria formed four quadrants, representing one or more possible explanations for the assignments proposed: (I) Concordant with current taxonomy; (II) Cryptic species; (III) Recent divergence, Hybridization or Synonymy; (IV) Probable misidentification (Hubert & Hanner 2015). Two different sequence divergence values were used as criteria for the delimitation of species to establish the quadrants; 2%, as COI divergences rarely exceed this value within a named species, and 3.9%, following the

application of the '10× rule' for the data investigated in this case (Hebert et al. 2004; Ward et al. 2009).

The different values for the two criteria established a grey zone in the scatterplot in which the interpretation can vary.

Results

Meristic traits

Bibliographical data of the main distinctive characters of the nominal *Gaidropsarus* species are summarized in Table I. An extensive overlap in the meristic variables analysed is observed, resulting in a set of conservative morphological traits. Regarding the two boreal species, *G. argentatus* and *G. ensis*, an overlap in the counts of all the characters is conspicuous. A similar result is obtained between *G. biscayensis* and *G. macrophthalmus* but to a lesser extent, with the second dorsal fin ray counts being in the range of 48–54 in the former and 53–59 in the latter. On counting the anal fin rays, the ranges of *G. biscayensis* and *G. macrophthalmus* overlap slightly (40–46 vs 45–50).

When *G. vulgaris* and *G. granti* are compared, the data collected from the literature referring to these main distinctive characters are unable to distinguish between the two species. In the case of the comparison between *G. mediterraneus* and *G. guttatus*, only the count of pelvic fin rays allows the distinction between both rocklings. In general, it can be said that the genus *Gaidropsarus* shows a highly conservative morphology.

NJ trees

The mtDNA-COI data set comprised 171 DNA sequences, represented by 52 distinct haplotypes. The alignment contained 651 nucleotide positions from which 195 were variable and 180 parsimony-informative sites. One hundred and forty-nine sequences of the reference data set constituted new additions to the global library of published COI-5P barcodes for marine fish (Table S1).

The 52 haplotypes obtained produced a NJ tree (Figure 2) with nine clades. Most of them clustered haplotypes assigned to the same species, as is the case of *G. argentatus*, *G. ensis*, *G. granti*, *G. vulgaris* and the three unknown *Gaidropsarus* spp. 1, 2 and 3. Two other clades were the result of the mixture of individuals assigned to two different species, *G. biscayensis*–*G. macrophthalmus* and *G. guttatus*–*G. mediterraneus.*

The NJ analysis of the 171 sequences (Figure S1) showed that most of the 22 mt-COI sequences obtained from the public repositories clustered according to the species assignation with few exceptions. Five *G. mediterraneus* sequences (JQ774626, KJ709762, KJ709763, KJ709764 and KP136735) are included in the *G. biscayensis*–*G. macrophthalmus* clade, one *G. vulgaris* sequence (SFM037–13) in the *G. guttatus*–*G. mediterraneus* clade and one *G. argentatus* sequence (KC015389) in the *G. ensis* clade. Therefore, seven of 22 public sequences (31.81%) were assigned to misidentified specimens (Table S2).

Genetic distances

The within-species mean distance was 0.39%, ranging from 0 to 1.38. The overall mean distance among the species of *Gaidropsarus* was 11.40% (Table II).

The between-group mean distances varied from 1.46% when comparing the clades formed by *G. granti* and *G. vulgaris* to 16.87% from *G. mediterraneus*–*G. guttatus* versus *Gaidropsarus* sp. 1. In general, they were well above 3%, with the exception of the two boreal species *G. argentatus* and *G. ensis*, which were closer (2.51%), and the comparisons of *G. vulgaris* and *G. granti* (1.46%). The genetic distances in the *G. biscayensis*–*G. macrophthalmus* complex ranged from 0 to 0.92% and from 0 to 1.1% for *G. guttatus*–*G. mediterraneus*. The within-species mean distance observed was similar to those obtained for *G. argentatus* (0.58%) and *G. vulgaris* (0.56%) (Table II). In general, the genetic distances observed showed the existence of a 'Barcoding Gap', excepting the minor distance between *G. granti* and *G. vulgaris* (1.08%) which is lower than the highest within-species value (1.38%) observed in *G. argentatus*.

The representation of the highest within-species value with the lowest between-species value for every specimen showed that the majority of the comparisons lay within the recent divergence, hybridization or synonymy quadrant III (Figure 3). As observed in the NJ tree, the individuals of species which clustered together showed the lowest between-species divergence. Even *G. granti* and *G. vulgaris*, which formed independent clades, fall into this category. The two boreal species, *G. argentatus* and *G. ensis*, are located in the overlapping zone between quadrants I and III. On the other hand, the individuals belonging to *Gaidropsarus* sp. 1 are located in quadrant I, concordant with well-delimited species. The between-species distance values of *Gaidropsarus* spp. 2 and 3 show that they are species different to the others considered in this investigation.

Assignment of unknown specimens

Four individuals were tentatively identified as *Gaidropsarus* sp. after morphological examination. The NJ tree

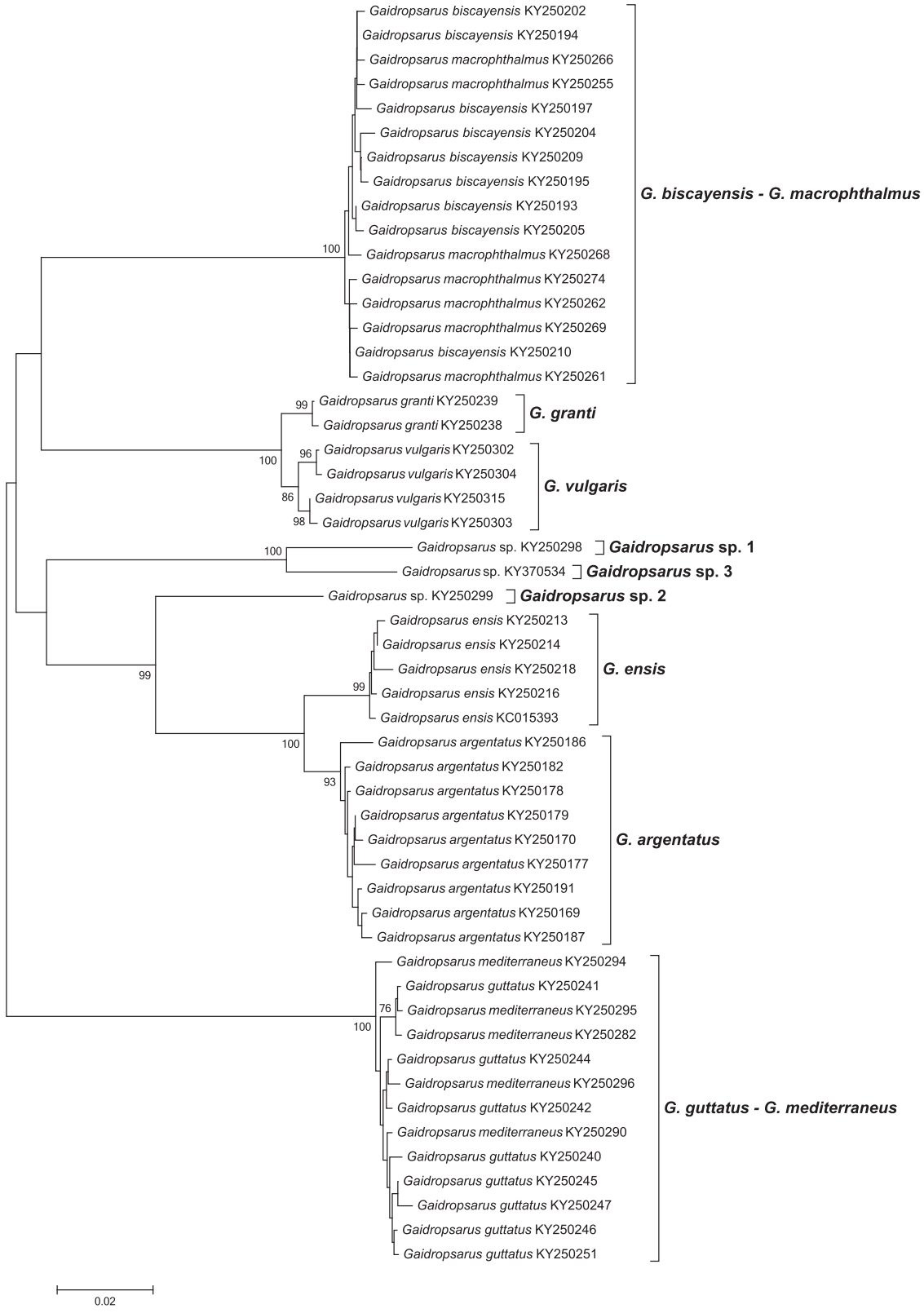

Figure 2. Neighbour-joining tree of COI haplotypes of *Gaidropsarus* fishes based on *p*-distances. Numbers at the main nodes are bootstrap percentages after 2000 replicates. Only values higher than 70% are shown. Subtrees include species assignations.

analysis placed them in three independent clades, *Gaidropsarus* spp. 1, 2 and 3, respectively, distinct from those assigned to known species. The specimen identification requests performed through the BOLD identification tool yielded different results. The sequence KY250298 representing *Gaidropsarus* sp. 1

Table II. Mean nucleotide distances (% of *p*-distance) within and between species of *Gaidropsarus* (range values shown in brackets).

		Between species							
Species[a] (n)	Within sp.	Gar	Gbi-Gma	Gen	Ggr	Ggu-Gme	Gvu	Gsp1	Gsp2
Gar (29)	0.58 (0–1.38)								
Gbi-Gma (47)	0.51 (0–0.92)	14.51 (13.98–14.90)							
Gen (34)	0.37 (0–0.61)	2.51 (2.00–3.07)	14.89 (14.44–15.05)						
Ggr (3)	0.15 (0–0.15)	13.08 (12.75–13.36)	12.37 (11.98–12.60)	13.96 (13.67–14.29)					
Ggu-Gme (33)	0.58 (0–1.1)	14.76 (14.59–15.05)	15.69 (15.05–16.28)	15.03 (14.59–15.67)	15.24 (14.75–15–67)				
Gvu (21)	0.56 (0–0.92)	13.17 (12.90–13.52)	12.43 (11.98–12–90)	14.04 (13.67–14.44)	1.46 (1.08–1.84)	15.01 (14.44–15–21)			
Gsp1 (2)	0	13.69 (13.36–14.13)	15.75 (15.36–16.13)	14.16 (14.13–14.44)	13.29 (13.21–13.36)	16.87 (16.59–17.36)	14.13 (13.98–14.29)		
Gsp2 (1)	–	7.95 (7.68–8.29)	13.58 (13.21–13.82)	8.08 (7.83–8.29)	12.06 (11.98–12.14)	14.65 (14.44–14.90)	12.90 (12.75–13.06)	12.90	
Gsp3 (1)	–	13.98 (13.67–14.29)	14.83 (14.44–15.05)	14.62 (14.44–14.90)	13.59 (13.52–13.67)	16.32 (15.82–16.59)	14.06 (13.98–14.13)	4.92	13.67

[a]Gar, *Gaidropsarus argentatus*; Gbi-Gma, *Gaidropsarus biscayensis–Gaidropsarus macrophthalmus*; Gen, *Gaidropsarus ensis*; Ggr, *Gaidropsarus granti*; Ggu-Gme, *Gaidropsarus guttatus–Gaidropsarus mediterraneus*; Gvu, *Gaidropsarus vulgaris*; Gsp1, *Gaidropsarus* sp. 1; Gsp2, *Gaidropsarus* sp. 2; Gsp3, *Gaidropsarus* sp. 3.

exhibited the highest similarity value (95.89%) with a sequence belonging to *G. novaezealandiae* (Hector, 1874), captured in the southern Atlantic Ocean (no public access in BOLD). The sequence KY250299 named as *Gaidropsarus* sp. 2 showed the highest similarity value (92.40%) with several individuals of *G. argentatus*. The comparison of sequence KY370534 belonging to *Gaidropsarus* sp. 3 resulted in a similarity of 95.24% with the same sequence as *Gaidropsarus* sp. 1. Curiously, these two species showed the highest similarity value with a South Atlantic sequence.

Discussion

General morphological traits

When the main morphological characters traditionally used as distinctive traits among *Gaidropsarus* are

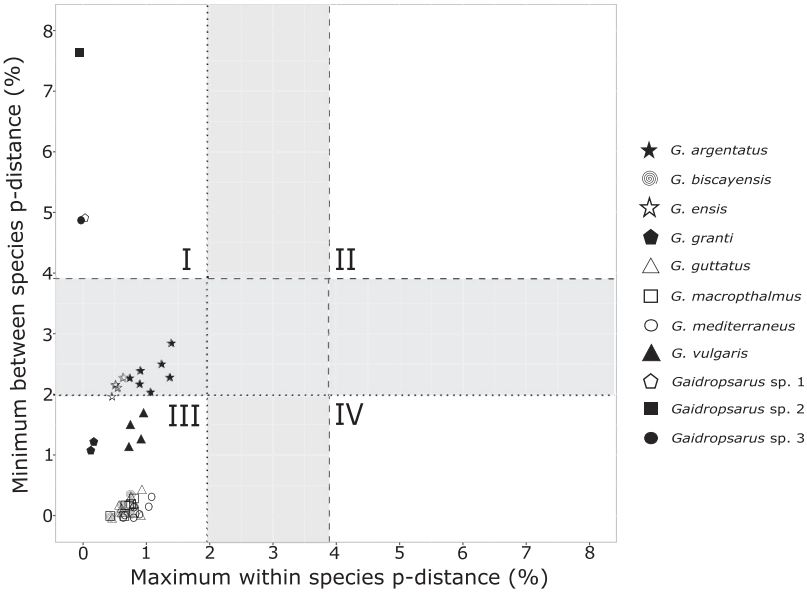

Figure 3. Scatterplot of maximum within-species distances compared to minimum between-species distances. Two different cut-off values were chosen to discriminate among species, the 2% criterion and the 10× rule. This creates a grey area where the delimitation is unclear and depends on the criterion selected. The graph is also divided into four quadrants representing different categories: (I) Concordant with current taxonomy, when the value of maximum within-species distance is below the cut-off and the minimum between-species distance is above the cut-off; (II) Cryptic species, when both distances are above the cut-off value selected; (III) Recent divergence, hybridization or synonymy, when both distances are under the cut-off value; (IV) Probable misidentification, when the maximum within-species distance is above the cut-off and the minimum between-species distance is under the cut-off.

compared, including biometric and meristic traits but also colouration pattern and length of first dorsal ray, the conspicuous overlaps observed in the measurements show that rocklings exhibit a conservative morphology, which hampers their identification based on traditional taxonomy. Original descriptions based on only a few specimens and meristic and biometric data have been successively repeated since the pioneering work (Svetovidov 1948) to the most recent (Iwamoto & Cohen 2016), without a critical revision. In the past, descriptions of new species were generally somewhat inconsistent, based on few specimens and morphological traits. Furthermore, the knowledge of the taxonomic status of fish species is unequal and clearly imbalanced in favour of coastal and/or commercial species, compared with the less-known deep-water and/or non-commercial ones.

Gaidropsarus guttatus–Gaidropsarus mediterraneus

G. guttatus was described as a new species by the comparison of several morphological characters, mainly the body height, body width, size of scales, teeth and eyes and number of ventral fin rays, although its similarity with *G. mediterraneus* was already reported when it was described for the first time (Collett, 1890). Further investigations declared that the *G. guttatus* form was close, if not identical, to *G. mediterraneus* (Svetovidov 1948). Recently, it has been stated that both species can be distinguished by the number of anal fin rays and their colour patterns (Iwamoto & Cohen 2016). However, the bibliographical revision of the meristic counts increases the number of anal fin rays, invalidating it as a diagnostic character. The colour pattern can also be discarded as a taxonomical character due to its high variability among rocklings (Cohen & Russo 1979). *G. guttatus* and *G. mediterraneus* have a similar habitat consisting of intertidal pools and shallow waters, where the ecosystem is highly variable and a cryptic colouration supposedly an adaptive advantage. The fact that the former species exhibits a darker colour pattern could probably correspond to an adaptation to the tones of the volcanic sea bed in the Macaronesian islands.

Despite *G. guttatus* being considered an endemic species of the Azores (Avila et al. 2014), the majority of the Azorean marine biota seems to comprise species that have arrived predominantly from the Eastern Atlantic, where *G. mediterraneus* is distributed (Morton & Britton 2000). According to this, the hypothesis of a colonization by *G. mediterraneus* of the Azores islands cannot be discarded.

The genetic distances between these two nominal species fall within the typical intraspecific values measured in marine fishes (Ward et al. 2009). A different hypothesis could explain these results, such as recent divergence, hybridization, synonymy or misidentification of specimens, the latter being the first to be considered when COI sequence comparisons show incongruent results. In this case, this is unlikely because these two species could easily be distinguished, either by the colouration and/or distribution (Svetovidov 1986a, 1986b). Previously, using phylogenetic methods, the lack of evolutionary divergence was revealed with mitochondrial fragments (12S, 16S and Cytb) as well as with a nuclear marker (rhodopsin) (Francisco et al. 2014). Therefore, a hybridization event between these species can be rejected.

DNA barcoding results argue in favour of a synonymy and, although morphological data and distribution areas do not disagree with the former idea, they highlight the existence of a possible population structure or speciation process. The analysis of more rapidly evolving DNA markers, such as microsatellites, would be needed in order to test the latter hypothesis.

Gaidropsarus biscayensis–Gaidropsarus macrophthalmus

In its description as a new species, it was stated that *G. biscayensis* could be distinguished from the similar *G. macrophthalmus* by a smaller head and different colouration and dentition (Collett 1905). According to a recent revision (Iwamoto & Cohen 2016), which does not change the values reported by the reference one (Svetovidov 1986a), both species can be distinguished by the number of second dorsal fin rays and by the number of anal fin rays. The bibliographic revision of the meristic counts slightly overlaps the second dorsal and the anal fin ranges, discarding these characters as distinctive.

The description of both species also takes into account their separated distribution ranges, with *G. biscayensis* having a southern distribution from the Iberian Peninsula south to Morocco (24°N) and Madeira, and also from the western Mediterranean, Adriatic and Aegean Seas, whereas *G. macrophthalmus* ranges from the Faeroe Islands towards the south along the west coast of the British Isles to the Bay of Biscay and even to the south of the Azores Islands (Svetovidov 1986a; Cohen et al. 1990; Iwamoto & Cohen 2016). However, *G. macrophthalmus* has been also reported south of this area, in Galician waters (Bañón et al. 2010) and in Portugal (Carneiro et al. 2014). The identification of several *G. macrophthalmus* specimens

in the western Mediterranean discards the distribution area as a criterion to differentiate these species. Molecular results suggest the existence of a unique species with an Atlantic–Mediterranean distribution, as occurs in the cases of *G. mediterraneus*, *G. vulgaris* and *G. granti*. Contrary to what was widely believed, the Gibraltar sill is not an impenetrable barrier for fishes and a certain number of species supposedly endemic to the Mediterranean Sea have also been captured in the Atlantic Ocean or made synonyms of Atlantic species (Danovaro et al. 2010). For example, barcoding data together with morphological analysis shows a synonymy between the Atlantic *Lepidion eques* (Günther, 1887) and the Mediterranean *Lepidion lepidion* (Risso, 1810) morids, resulting in the latter being the only valid species with an Atlantic and Mediterranean distribution (Bañón et al. 2013; Barros-García et al. 2016).

Moreover, some morphological differences found among Atlantic and Mediterranean specimens of the same species can be attributed to their size, shown to be larger in the Atlantic Ocean (Massutí et al. 2004), and to geographical variations related to different environmental conditions, mainly temperature (Barlow 1961; Bañón et al. 2013).

All the individuals assigned to *G. biscayensis* and *G. macrophthalmus* grouped together in the same cluster. Different explanations could account for this result, including specimen misidentification, synonymy or hybridization events. The latter could be favoured by the existence of an overlap in the distribution areas between these two species, as occurs in the western Mediterranean. The large number of individuals sampled in their distribution areas makes hybridization processes or misidentification unlikely. The same result was observed when the phylogenetic relationships among these species were inferred using both mitochondrial and nuclear markers (Francisco et al. 2014).

Because information in the literature is restricted to but a few individuals, the morphological diversity could have being underestimated. Therefore, a further sampling is required in order to describe real distinctive traits, which would possibly show an overlap in the morphological data, agreeing with the barcoding and suggesting that *G. biscayensis* is a junior synonym of *G. macrophthalmus*.

Low genetic divergence between Gaidropsarus granti *and* Gaidropsarus vulgaris

G. granti was first described by Regan (1903) based on a specimen caught in the Azores Islands. It is a little-known species, with few specimens described in the literature. Although it was believed that this rockling was only distributed in the Azores and the Canary Islands (Svetovidov 1986b), recent records have also found this species in different areas of the Atlantic and the Mediterranean (García 2015). Morphological analysis pointed out *G. granti* as a species close to *G. mediterraneus* and *G. guttatus* (Svetovidov 1986a), although molecular data show that it is closer to *G. vulgaris*, from which it differs mainly in its colouration pattern, habitat and distribution (Svetovidov 1986b). *G. vulgaris* is a common species found on the continental shelf up to a depth of 120 m and is characterized by the presence of numerous dark spots (Svetovidov 1986b). Meanwhile, *G. granti* is a rare species mainly distributed on seamounts and islands between depths of 120 and 830 m and with a characteristic white stripe on the body (García 2015).

In DNA barcoding, species delimitation depends on the cut-off value employed, able to distinguish within-species diversity from between-species divergence (Ward et al. 2009). Different criteria have been proposed, of which the '10× rule' implies that two sequences with a divergence higher than 10 times the average within-species value could be considered as belonging to different species (Hebert et al. 2004). Lately, after surveying more than 1000 species of marine fish, it was stated that two barcodes with a 2% COI divergence value show a conspecific probability of only 3% (Ward et al. 2009). The combination of both criteria defines a 2%–3.9% range in which species delimitation would be uncertain.

The results obtained in the distances scatterplot between *G. granti and G. vulgaris* show that all the values are under 2%, falling in the zone where hybridization, synonymy or recent divergence are possible. Despite this fact, these specimens form independent clades concordant with current taxonomy in the NJ analysis, making hybridization phenomena unlikely. Nevertheless, more individuals of *G. granti* and the use of nuclear markers would be necessary in order to discard this hypothesis.

Taking into account all the available data, it would appear that *G. granti* and *G. vulgaris* are two valid but closely related species.

Low genetic divergence between G. argentatus *and* G. ensis

These two boreal species are separated by low genetic distances, with the smallest value being 2%, a fact also observed in an Atlantic marine fishes study in Canada (McCusker et al. 2013). In a brief phylogenetic review, a group with *G. ensis* and *G. argentatus* appeared as sister to *G. vulgaris*, *G. biscayensis*, *G. macrophthalmus*

and *G. granti* (Francisco et al. 2014). Depending upon the criterion put into practise to delimitate species, the results may vary. Considering the 2% criterion, both species are concordant with current taxonomy, but taking into account the 10× rule, which yields a cut-off value of 3.9%, the relationship between them could be explained as the result of recent divergence, synonymy or hybridization. The latter can be discarded considering the sampling carried out and the existence of two well-defined clusters, each one representing one of the species. The length of the first dorsal fin ray and the presence/absence of a median supratemporal pore clearly distinguish both species morphologically (Cohen & Russo 1979; Svetovidov 1986a, 1986b), making synonymy unlikely. Therefore, *G. argentatus* and *G. ensis* should be considered two closely related valid species.

Unidentified specimens

Four specimens were captured in the eastern North Atlantic, between depths of 500 and 1230 m. Despite the fact that this study deals with all the recognized species from the North Atlantic and Mediterranean, their barcodes branched into three independent clusters and are, therefore, referred to as *Gaidropsarus* spp. 1, 2 and 3. Two possibilities arise from these results: new records of previously recognized southern species in the North Atlantic or the discovery of new deep-water species. In any case, these findings reflect the general lack of knowledge of the deep-sea environments even in such well-characterized areas as the North Atlantic Ocean and the Mediterranean Sea (UNEP 2006).

Misidentified public records

The comparison of newly generated barcodes with published data may help to detect misidentifications, taxonomic uncertainties or real cases of haplotype sharing among species (Knebelsberger & Thiel 2014). In this study, the comparison between a self-created barcoding database curated by expert taxonomists with all publically available sequences deposited in the repositories has flagged the presence of several misidentifications of *Gaidropsarus* voucher records in the latter. Most of the misidentifications found in the repository databases are related to the construction of DNA barcode reference libraries where only one sequence was employed and not compared with other *Gaidropsarus* barcodes (Costa et al. 2012; Knebelsberger et al. 2014; Landi et al. 2014).

Indeed, accumulating FISH-BOL data suggest that initial specimen misidentification appears to be considerably more worrying than complications caused by hybridization (Ward et al. 2009). This fact can have serious implications for end users of reference libraries and once a name has been added to a database, it may be difficult for a third party to convince data managers that it should be changed (Collins & Cruickshank 2013).

Final remarks

The results of this investigation suggest that morphology-based identification and taxonomy can be challenging in *Gaidropsarus*, even within regions as well characterized as the North Atlantic Ocean and the Mediterranean Sea, and have highlighted the need for further detailed taxonomic examinations of this genus.

In some species, the apparent contradictions between molecular and morphological data could be explained by the low number of individuals examined, with countable traits difficult to distinguish. This lack of sampling could lead to underestimations of the morphological variability, showing false distinctive values in their meristic. Therefore, an updated identification key of rocklings, based on increased sampling sizes and broader geographical areas, is required to reflect their real morphological variability.

DNA barcoding can be used to distinguish species of *Gaidropsarus* with a high degree of accuracy with some exceptions related to its challenging systematics and complex evolutionary history. The high contribution in number of specimens and diversity of species, and the detection of misidentifications in the public repositories, could make the task for future investigations of this genus easier.

The results suggest a more complex evolutionary history than expected, with low genetic distances observed between pairs of species that are morphologically distinguishable, which could be explained by recent or on-going speciation processes. What is more, the fact that COI-sequences obtained from unknown deep-water specimens are more similar to a South Atlantic record, despite their collection site, could suggest a connection between northern and southern hemisphere species. The impossibility of species-level assignation of four specimens captured in deep-water environments highlights the general lack of knowledge of these ecosystems. Furthermore, these results show that the existence of different and little known types of deep habitats could hold an undetermined number of new species of *Gaidropsarus*.

An integrative taxonomy approach, considering not only morphology and barcoding but also phylogeography, population genetics, ecology, development and behaviour, could be necessary to delineate correct species boundaries in this genus.

Data accessibility

Photographs, DNA sequence data and other details of the reference data set of specimens employed in this investigation are available in the Barcode of Life Database in the project entitled 'Molecular identification of *Gaidropsarus* fishes', code GSRUS. Barcodes have also been deposited in GenBank under accession numbers KY250169–KY250315, KY370533 and KY370534.

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