Biological Journal of the Linnean Society, 2002, **76**, 327–338. With 4 figures

A revision of the status of *Lepadogaster lepadogaster* (Teleostei: Gobiesocidae): sympatric subspecies or a long misunderstood blend of species?

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Molecular (partial mitochondrial 12S ribosomal DNA sequences), morphological and meristic analysis of *Lepadogaster lepadogaster lepadogaster*, *L. l. purpurea* and *L. zebrina* were performed to investigate the relationships between these taxa. On the western shore of mainland Portugal, where the two subspecies of *L. lepadogaster* occur sympatrically, they differ in microhabitat preferences and their breeding seasons are largely out of phase. This information, combined with data on distribution patterns, led to the following conclusions: *Lepadogaster l. purpurea* is considered to be a valid species, *L. purpurea* (Bonnaterre, 1788), different from *L. l. lepadogaster*, now designated *L. lepadogaster* (Bonnaterre, 1788). *L. zebrina* was found to be a synonym of *L. lepadogaster*. The two newly defined species were found to be in sympatry at Madeira and the Canary islands, the Atlantic coast of the Iberian Peninsula, and the Mediterranean at least as far as Genoa (Italy). Diagnostic characters and a list of synonyms are provided.

ADDITIONAL KEYWORDS: breeding season – *Lepadogaster purpurea* – *L. zebrina* – Mediterranean – microhabitat segregation – morphometry – north-eastern Atlantic – species revalidation – 12S rDNA.

INTRODUCTION

As defined by Briggs (1955) the genus *Lepadogaster* includes three species: *L. candollei* Risso (1810), *L. lepadogaster* (Bonnaterre, 1788) and *L. zebrina* Lowe (1839). Two subspecies of *L. lepadogaster* were recognized. According to Briggs's (1986; 1990) recent revisions, *L. l. lepadogaster* occurs in the Mediterranean from Monaco eastwards as far as Israel and in the Black Sea, and *L. l. purpurea* in the north-eastern Atlantic from Scotland to Senegal including Madeira and the Canary islands, and in the western Mediterranean eastward to Cape Roux (southern France). *L. zebrina* has a very restricted distribution limited to Madeira and the Canary Islands while *L. candollei* is the most widespread species, being present in the Atlantic coasts from England to Senegal, in Madeira and the Canary Islands and throughout the Mediterranean.

Prior to Briggs (1955), the specimens included by this author in *L. lepadogaster* had been classified in

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very diverse ways by different authors: from only one designation like *L. gouanii* (e.g. Günther, 1861; Le Danois, 1913) or *L. lepadogaster* (Fowler, 1936), to as many as four (e.g. Risso, 1826; Ninni, 1933). This confusing situation was due to the fact that many authors proposed the recognition of new species based on small differences of colour patterns and morphology of very limited numbers of specimens, often collected in restricted areas. It is interesting to note that the authors working with British populations consistently recognized the presence of a single form and all pointed to the same set of diagnostic features. On the contrary, in the Mediterranean, several authors (e.g. Risso, 1810, 1826; Canestrini, 1864; Ninni, 1933) expressed the view that at least two distinct entities should be recognized due to the sympatric occurrence of specimens with discordant traits.

On the shores of the Iberian Peninsula, apart from *L. candollei*, a single species of *Lepadogaster* was recognized, although with different designations: *L. gouanii* (Albuquerque, 1954–56) or *L. lepadogaster* (Lozano y Rey, 1960). Both authors, however, felt the need to describe two distinct patterns of body and head colouration. After the work of Briggs (1986), *L. l. purpurea* replaced the older names for the Iberian populations.

Albuquerque (1954–56) considered *L. zebrina* as a synonym of *L. gouanii*, which implied that the populations of Madeira and mainland Portugal belonged to the same species. However the revision of Briggs (1955) and all subsequent works considered *L. zebrina* a valid species for Madeira. Brito (1982) confirmed the presence of *L. zebrina* in the Canary Islands.

In a recent revision of the subfamily Lepadogastrinae, Hofrichter (1995) considered *L. zebrina* a valid species, but noted that its distinction from *L. l. lepadogaster* is rather tenuous. Regarding the two subspecies of *L. lepadogaster* this author noted that their morphological characters largely overlap, and raised doubts on their subspecific status.

During preliminary observations, we noted that two distinct forms of *L. lepadogaster* were consistently found in the coast of mainland Portugal for several years, often in the same localities. Subsequently, it was also found that this overlap extends over hundreds of kilometres, from the entrance of the Gulf of Biscay, southwards at least to the mouth of the Sado River (central west coast of Portugal). The classification of the two forms using the criteria of Briggs (1955) would lead us to recognize the occurrence of the two subspecies of *L. lepadogaster* in Portugal and north-west Spain. Their extensive overlap and the finding that these two forms differ markedly in their breeding seasons (see Results), led us to consider the hypothesis that we could be in the presence of two different species instead of two subspecies.

In this paper, we re-examine the status of the forms of *Lepadogaster* previously classified as *L. l. lepadogaster* and *L. l. purpurea* and that of *L. zebrina*, which is very similar. This study combines DNA sequence data with morphological and ecological information and covers material from the Atlantic coast of the Iberian Peninsula, the Mediterranean and the Madeira island.

METHODS

As the status of the specimens studied in this work is in question, they will be designated in the Methods and Results sections as:

Group 1: Material that would be classified as *L. l. purpurea* according to Briggs (1986), including specimens from north-west Spain, west Portugal, Madeira island and the Mediterranean (Genoa, Italy);

Group 2: Material that would be classified as *L. l. lepadogaster* but collected far from the distributional area proposed by Briggs (1957, 1986) for this subspecies and well inside the distributional area of the *purpurea* subspecies. Specimens from north-west Spain, western Portugal and western Mediterranean (Malaga, southern Spain);

Group 3: Material that would be classified as the previous group but from the distribution area defined by Briggs (1957, 1986) in the Mediterranean for *L. l. lepadogaster*: southern France, Genoa (Italy) and Albania;

Group 4: Fish from Madeira island that would be classified as *L. zebrina*.

Sources of material

The specimens used for genetic and morphological analysis, their sites of origin and institutions of deposit are listed in Table 1.

Morphometric and meristic analysis

Measurements were made under a stereomicroscope with the help of a calliper to a precision of 0.05 mm. To avoid the effects of possible allometric growth the raw data were replaced by their residuals after log-log regression between each measure and standard length (SL). The measurements follow Briggs (1955) with some additions. The morphometric and meristic variables used in this study are presented in Table 2. For all collected specimens body colouration pattern and shape of the head *ocelli* were recorded.

The presence and distribution of cephalic pores were checked, but they proved to be identical among all fish groups, and thus devoided of phylogenetic information. The number and relative position of those pores

Table 1. Specimens used in each analysis (B = biometric measures; M = meristic counts; and G = genetic analysis); sites of origin: Alpertuche, Continental Portugal (38°28′N 8°59′W), Burela, Galiza, NW Spain (43°39′N 7°21′W), Varigotti, Genoa, Italy (44°11′N 8°24′E), Funchal, Madeira (32°38′N 16°54′W), Albania (exact location unknown) and Malaga, Spain (36°43 N 4°25′W); and the institutions of deposit: MO-PNA (Oceanographic Museum of Arrábida Nature Park), MMF (Museu Municipal do Funchal) and HM (Hamburg Museum). For the group/species definitions, see Methods

Analysis	No. specimens	Group/species	Origin	Deposit
B/M/G	15/15/3	1	Continental Portugal	MO-PNA
B/M/G	12/18/4	2	Continental Portugal	MO-PNA
B/M/G	8/9/2	2	NW-Spain	MO-PNA
M/G	2	1	NW-Spain	MO-PNA
B/M/G	9/9/1	3	Italy	MO-PNA
B/M/G	1	1	Italy	MO-PNA
B/M/G	12/13/3	4	Madeira	MO-PNA
B/M/G	1	1	Madeira	MO-PNA
B/M	1	3	Albania	MMF
B/M	1	2	S Spain	HM
B/M	3	*L. candollei*	Albania	MMF
G	2	*L. candollei*	Continental Portugal	MO-PNA

were in agreement with the early description of Guitel (1888) for *L. gouanii*.

To analyse the relationships between the four groups, a discriminant analysis that included their closest relative *L. candollei* was performed. Cluster analysis on morphometric and meristic data was performed using each individual as an OTU based on their euclidian distances and unweighted pair-group average method (UPGMA). Phylogenetic analysis of morphological data was performed with the program 'CONTML' of the software package PHYLIP (Felsenstein, 1989), using a maximum likelihood method with *L. candollei* as an outgroup.

GENETIC ANALYSIS

Total genomic DNA was extracted from muscle tissue using a proteinase K/SDS based extraction buffer, purified by phenol/chloroform and ethanol precipitation (Maniatis *et al*., 1982).

Polymerase chain reaction (PCR) was used to amplify a segment of 433 base pairs from the third domain of the 12S rDNA. Primers were designed from highly conserved areas of the 12S rDNA sequences of six different fish families (accession numbers AF023183, AF023188, AF038484, NC001606, M91245, NC001717, NC001960, AB000667, X99772, Z21921). Primer sequences are 12SFor 5′-AACTGGGATTAGA TACCCCA-3′ and 12SRev 5′-GGGAGAGTGACGG GCGGTGTG-3′ and correspond to regions of 100% homology between aligned sequences. The positions from 5′ to 3′ of both primers correspond to positions 421–441 and 923–903, respectively, of the human 12S rDNA (see Horai *et al*., 1995).

Amplifications were obtained in a total volume of 20 µL with 1.5 µM MgCl2, 200 µM each dNTP, 0.5 µM each primer, 0.5 U of *Taq* polymerase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA), ≈ 20 ng of genomic DNA and 2 µL of buffer supplied by the manufacturer. PCR was performed in a Biometra thermocycler (Biometra, Trio-Thermblock, Göttingen, Germany) and the amplifications consisted in 4 min at 94°C, 30 cycles of 1 min at 94°C, 1 minute at 55°C, 1 min at 72°C and a final extension period of 10 min at 72°C. Each PCR product was purified from the gel and cloned into the pGEM-T easy vector following the recommendations of the manufacturer (Promega, Madison, WI, USA). After an alkaline-lysis extraction of the DNA, manual sequencing was performed following the dideoxynucleotide chain termination method (Sanger *et al*., 1977).

Sequence of alignments were made using CLUSTAL W (Thompson *et al*., 1994) with default settings (gap opening = 10; gap extension = 0.05). Adjustments to refine the alignments were made according to the secondary structure model of piranhas (Ortí *et al*., 1996). Segments were defined as stems and loops according to their base pairing and were folded to secondary structure by eye.

In order to assess the phylogenetic relationships between taxa, the data set was analysed with three methods of phylogenetic inference: maximum parsimony (Fitch, 1971), maximum likelihood (Kimura, 1980; Felsenstein, 1981) and neighbour joining (Saitou & Nei, 1987). Several weighting combinations were performed for transition (Ts)/transversion (Tv) (Ts/Tv = 1, Ts/Tv = 1/2 and Ts/Tv = 1/4) and stem (St)/loop (Lp) (St/Lp = 1, St/Lp = 1/2 and St/Lp = 1/4) to account

Table 2. Morphometric and meristic variables used in this study. Marked (*) variables were not included in discriminant analysis. Except for TL and SL the remaining variables had zero variance in some fish groups

*Total length	TL
*Standard length	SL
Body depth	Bd
Head length	Hl
Head width	Hw
Sucking disc length	SDl
Sucking disc width	SDw
Secondary sucking disc length	sSDl
Secondary sucking disc width	sSDw
Distance between tip of snout and the anterior margin of the sucking disc	S-D
Distance between the posterior margin of the sucking disc and the anus	SD-A
Distance between the anus and the origin of the anal fin	A-A
Dorsal fin length	Dl
Anal fin length	Al
Predorsal distance	pD
Preanal distance	pA
Caudal peduncle length	CPl
Caudal peduncle depth	CPd
Eye diameter (mean of left and right eye)	Ey d
Inter-orbital distance	Iod
Pre-orbital distance (mean of left and right preorbital distance)	Pod
Length of posterior nostril's tentacle	pNt
Length of anterior nostril's tentacle	aNt
N° of dorsal rays	Dr
N° of papillae rows in anterior disc region	papA
N° of papillae rows in posterior disc region	papB
*N° of papillae rows in central disc region	papC
*Body colour pattern	BP
*Head *ocelli*	HO

for the lack of independence among substitutions in stems. Neighbour joining results were obtained using Kimura 2-parameter distance. In order to identify the primitive characters, *L. candollei* was used as an outgroup. Analysis were performed with PAUP 4.0 beta 2 version (Swofford, 1997) and PHYLIP (Felsenstein, 1989).

Sequences were deposited in GenBank database and the accession numbers are: AY036587, AF388176 (*L. candollei*); group 1: AY036599, AY036600, AY036601, AY036602, AY036603, AY036604, AY036605 (*L. purpurea*); group2: AY036589, AY036590, AY036591, AY036592, AY0 36593, AY036594; group 3: AY036598 (*L. lepadogaster*); group 4: AY036595, AY036596, AY036597 (*L. zebrina*).

Breeding seasons and microhabitats

Ecology and reproduction of groups 1 and 2 were studied in the west coast of Portugal at Alpertuche, Arrábida (38°28′N–8°59′W), with additional observations at Parede (38°41′N–9°22′W). To determine the breeding season of each group, standard transects parallel to the shoreline were inspected monthly at low tide (from December 1998 to December 2000). Transects were 44 m long and 4 m wide. Up to 120 boulders and stones were inspected per transect and the presence of egg masses and fish, as well as their identity and size were recorded. After inspection, fish and stones were carefully placed as they where before. Similar observations were performed at Parede during the years 1993–94.

Some dives in the adjacent subtidal were performed and stones were lifted to check if the data obtained for the breeding season in the intertidal were also valid in subtidal conditions. The number of fish and the presence of egg masses were recorded.

To characterize the microhabitats of the two groups, data from transects inspected between January and August 2000 were used. The beach was divided in two zones (A and B) crossed by the transects and differing in the predominant type of stones. The stones were measured and the texture of their underside surface was qualitatively classified on a simple three-point scale: (1) smooth, (2) intermediate and (3) rough. The diversity of the biological cover was estimated based on the number of categories of benthic organisms that were attached. To avoid the effect of very rare organisms**,** only taxa that covered at least 10% of the underside of the stone were considered. The types of organisms found were: algae, sponges, cnidarians, bryozoans, annelids, barnacles, amphipods, decapods, gastropods, echinoderms, tunicates and fish. Fish abundance of each group was compared using Wilcoxon matched pairs test, and stone characteristics of the two zones were compared using ANOVA.

Phylogenetic analysis of morphological data was performed with the program 'CONTML' of the software package PHYLIP (Felsenstein, 1989). All other statistical treatments were performed with the software package STATISTICA 5.0 (© StatSoft, Inc).

RESULTS

The same pattern of relationship between groups was observed in all analysis (cluster analysis on morphological data, Figure 1; discriminant analysis for morphological data—Table 3; analysis of genetic data, Fig. 2; and genetic distance between groups, Table 4). After the separation of *L. candollei* specimens, two distinct groups emerged: one includes all specimens of group 1, fish that would be classified as *L. l. purpurea*

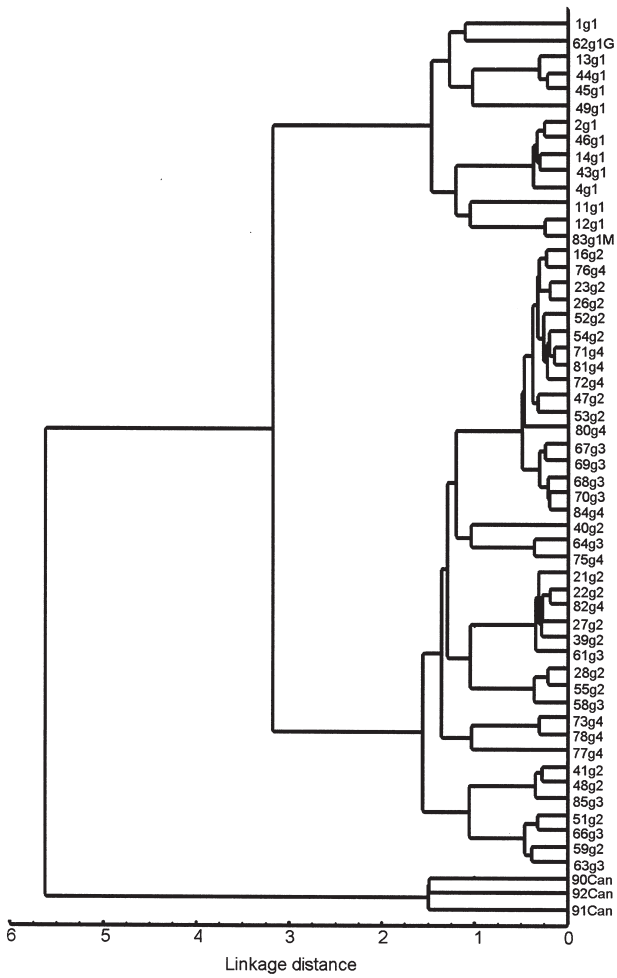

Figure 1. Cluster analysis based on the morphometric data, unweighted pair-group average with Euclidean distances. Each specimen are designated by it serial number plus the group considered (g1 to g4) or 'Can' for *L. candollei* specimens. Those of group 1 (*L. l. purpurea*) from Genoa and Madeira have the extra code G or M, respectively.

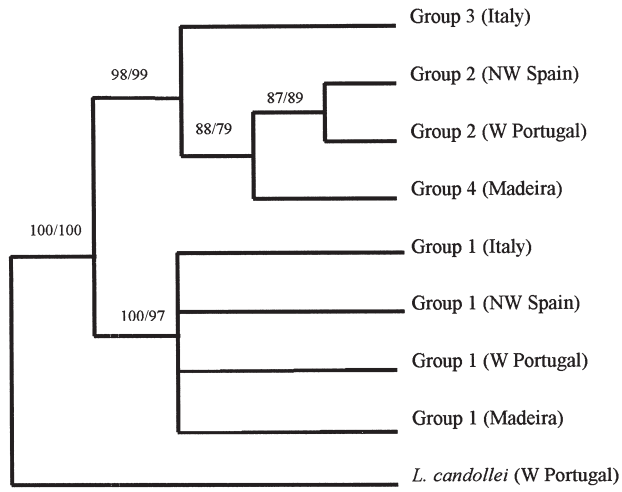

Figure 2. Phylogenetic tree obtained by maximum parsimony and neighbour joining analysis from the genetic data. Bootstrap values (parsimony/neighbour-joining) based on 1000 simulations are shown as percentages. Only values above 70% are presented in the tree.

regardless of their geographical origin; the other includes the remaining fish (groups 2, 3 and 4). The only misclassification in the discriminant analysis was one specimen of *L. zebrina* that was classified as *L. l. lepadogaster* (group 3). The cladistic analysis of morphological data is not shown because basically it repeats the information already present in Figures 1 and 2: after the separation of *L. candollei* with a bootstrap of 100%, all *L. l. purpurea* are separated from the remaining specimens with a bootstrap of 73%; again, the specimens of groups 2, 3 and 4 did not form well differentiated entities.

This means that the specimens of *L. zebrina* (group 4) occur in the same cluster as all fish that could be classified as *L. l. lepadogaster* (groups 2 and 3). No recognizable subgroups were identified within this cluster so the material from the Mediterranean, the Atlantic Iberian shores and Madeira cannot be distinguished. Additionally, the only specimen of group 1 (*L. purpurea*) coming from Madeira and that from Italy are also mixed with the other members of their respective cluster. Thus, the two forms distinguished by these analysis are in sympatry at least from northwestern Spain to the Mediterranean coast of Italy and Madeira.

Partial sequences of the third domain of 12S rDNA were folded into a secondary structure following the model of Ortí *et al.* (1996). Variation among sequences occurred mainly, although not exclusively, in loop regions. No transitional saturation was detected by plotting transitions and transversions vs. Kimura 2-parameter distances and all three methods of phylogenetic inference recovered the same topology, independently of the weighting schemes used for transitions/transversions and for stems/loops. The only gap opening in the DNA sequences alignment corresponded to the six specimens from group 2 and was one base-long. A total of 51 base differences (12.8% of the DNA fragment sequenced) separated *L. candollei* from the ingroup (groups 1–4).

Genetic analysis revealed that *L. zebrina* could not be differentiated from the other fish since no synapomorphies were found for group 4. Group 1 (*L. l. purpurea*) is monophyletic the same being true for groups 2, 3 and 4 combined (Fig. 2). Ten synapomorphies (2,3% of the DNA fragment sequenced) separated *L. l. purpurea* from the clade *L. l. lepadogaster*/*L. zebrina* (groups 2, 3 and 4). *L. zebrina* is as distantly related

Table 3. Standard discriminant analysis: Wilks' Lambda 0.001, F (96, 113) = 10.983, $p < 0.001$; 55 out of 56 fish were correctly classified. A, Mahalanobis distances (for all distances p < 0.001). B, Canonical variables. Cumulative proportion of the variance explained

A-	*purpurea*(1)	*lepadogaster*(2)	*lepadogaster*(3)	*zebrina*(4)
purpurea(1)	–			
lepadogaster(2)	95.1	–		
lepadogaster(3)	97.3	24.6	–	
zebrina(4)	88.1	18.9	28.5	–
candollei	1204.0	1411.8	1237.0	1327.0
B-	Root 1	Root 2	Root 3	Root 4
Eigenval	67.45	14.52	2.36	1.88
Cum. Prop.	0.78	0.95	0.98	1.0
purpurea(1)	–0.37	6.28	0.16	–0.07
lepadogaster(2)	3.53	–2.01	0.49	1.51
lepadogaster(3)	1.06	–2.58	2.03	–2.18
zebrina(4)	2.33	–1.74	–2.66	–0.90
candollei	–32.31	–2.45	–0.17	0.59

Table 4. Kimura two-parameter genetic distance and mutation percentages between *L. l. purpurea* (group 1), *L. l. lepadogaster* (group 2—Atlantic populations, and group 3—Mediterranean population), *L. zebrina* (group 4) and the outgroup *L. candollei*

Kimura-2	*purpurea*(1)	*lepadogaster*(2)	*lepadogaster*(3)	*zebrina*(4)	*candollei*
purpurea(1)	0–0.01	–	–	–	–
lepadogaster(2)	0.04	0–0.003	–	–	–
lepadogaster(3)	0.03	0.008–0.01	0	–	–
zebrina(4)	0.03–0.04	0.005–0.01	0.008–0.01	0–0.003	–
candollei	0.16–0.17	0.17–0.18	0.16–0.17	0.17	0–0.003
% mutations	*purpurea*(1)	*lepadogaster*(2)	*lepadogaster*(3)	*zebrina*(4)	*candollei*
purpurea(1)	0–0.99	–	–	–	–
lepadogaster(2)	3.72–4.47	0–0.25	–	–	–
lepadogaster(3)	2.73–3.23	0.99–1.24	0	–	–
zebrina(4)	3.23–3.97	0.74–1.24	0.74–0.99	0–0.25	–
candollei	14.4–15.4	14.89–16.13	14.39–14.89	14.64–15.38	0–0.25

to *L. l. lepadogaster* (Kimura 2-parameter genetic distance 0.005–0.01) as are the Madeiran and continental populations of *L. l. purpurea* (0.005–0.01) or *L. candollei* (0.005–0.008—GenBank accession number for Madeiran *L. candollei* AF388176). Finally, within the clade *L. l. lepadogaster*/*L. zebrina* the most divergent specimen was from the Italian population sample and not from the putative Madeiran species *L. zebrina* (Fig. 2).

In conclusion, both the genetic and morphological analysis point to a much greater separation between *L. l. purpurea* (group 1) and the combined *L. l. lepadogaster* (groups 2 and 3) +*L. zebrina* (group 4). These two new entities are however, more similar than any of them is to *L. candollei*.

Briggs (1955, 1986) mentioned body depth as the only morphometric character able to discriminate between *L. zebrina* and *L. lepadogaster*, being greater in the former. We could not confirm this difference. A comparison of the residuals after log-log regression BD/SL between group 4 (*L. zebrina*) and groups 1, 2 and 3 combined (the two subspecies of *L. lepadogaster sensu* Briggs) failed to detect any significant differences (rank sum: *L. zebrina* = 277, N = 11, *L. lepadogaster* = 1493, N = 48; U = 211, p = 0.30, Mann–Whitney U-test) and if anything, the value for

Table 5. Descriptive statistics for morphometric (index over SL) and meristic variables for which there were significant differences at $p < 0.01$. Wilks'Lambda = 0.096, Rao's (6, 52) = 81.18, MANOVA test. Mean values are presented for the morphometric characters SDw, Iod and Pod, median values are presented for meristic papA, papB and Dr. *For anterior disc region (papA) *L. lepadogaster* presented only one over 42 specimens with five rows of papillae

		SDw	Iod	Pod	papA	papB	Dr
L. lepadogaster n = 42	Mean/median	0.27	0.06	0.15	4	3	17
	Minimum	0.23	0.05	0.13	3	3	16
	Maximum	0.30	0.07	0.17	4(5*)	4	18
	SD	0.018	0.005	0.009	0.5	0.4	0.6
L. purpurea n = 17	Mean/median	0.25	0.07	0.14	5	5	18
	Minimum	0.22	0.06	0.12	5	5	18
	Maximum	0.29	0.09	0.15	6	6	19
	SD	0.018	0.008	0.012	0.5	0.5	0.5

L. lepadogaster was slightly greater (median BD/SL = 0.142 for *L. zebrina* and 0.149 for *L. lepadogaster*).

The small sample sizes did not allow a comparison of all morphometric variables in a single MANOVA test. To avoid retesting errors, a preliminary exploration of the residuals of the variables between *L. l. purpurea* and the remaining groups combined (*L. zebrina +L. l. lepadogaster*) were performed with the Mann–Whitney *U*-test. Only variables for which there were differences significant at $p < 0.01$ were retained and subsequently used in a MANOVA. Although the MANOVA was performed on the residuals of the regressions between each morphometric characteristic and SL, the descriptive statistics for the indexes of these variables over SL are presented in Table 5, since these indexes are more informative than residuals.

In addition to the morphometric and meristic measures considered above, there was a consistent difference in head colour pattern between group 1 (*L. purpurea*) and the other groups (*L. zebrina +L. lepadogaster*). Fish of group 1 presented a pair of *ocelli* on the head behind the eyes, each with a central blue region surrounded by a brown ring. A blue oval line outlines each *ocellus*.

Fish of groups 2, 3 and 4 have a single blue line over the head that is not divided in two *ocelli*, although a central constriction varying in prominence is present. This crescent shape area, together with the white lines that run between the eyes in all fish, are in the origin of the so-called 'brown crescents' of the old descriptions found in the literature for some fish (e.g. Lacépède, 1800). Although two small blue marks may be present, they never show the well define round shapes and are not surrounded by the brown rings (see Fig. 3).

Body colour pattern is more variable. It is formed by light brown or purple spots in *L. l. purpurea* while in the Atlantic specimens of *L. l. lepadogaster* and *L. zebrina* there are dark vertical oval shapes that in many individuals are so stretched in length that they became vertical dark bands on the sides with dots on the back. However, in *L. l. lepadogaster* specimens from the Mediterranean, only dark spotting was observed.

Breeding seasons and microhabitats

In continental Portugal, group 1 males (*L. purpurea*) were found guarding eggs from November 1998 to April 1999, starting one month earlier in the adjacent subtidal habitat, and from October 1999 to the end of March 2000 with no differences between intertidal and subtidal habitats. Group 2 males (*L. lepadogaster*) were found guarding eggs from March to June in 1999 and from March to the end of July in 2000, both in intertidal and subtidal habitats. Thus, the period where the two forms are found breeding simultaneously is very short with most of their breeding seasons out of phase. *L. candollei* was found guarding eggs from March to late August mainly in subtidal habitat.

Concerning the microhabitat of the two forms it was found that the two sections of the beach (see Methods) differed consistently in their use by the fish. Group 1 (*L. purpurea*) was more abundant in section A ($Z = 3.18$, $n = 13$, $p < 0.001$, Wilcoxon matched pairs test) and Group 2 (*L. lepadogaster*) was more abundant in section B ($Z = 3.18$, $n = 13$, $p < 0.001$, Wilcoxon matched pairs test).

With regard to boulder characteristics, there were significant differences between the two sections (Wilks' Lambda 0.36, Rao'R (3420) = 254.42, $p < 0.001$, MANOVA test). Stones in section A are larger (mean area 661 cm^2) than in section B (mean area 477 cm^2). Diversity and roughness of the underside surface decreased from section A to B (mean diversity = 2.99

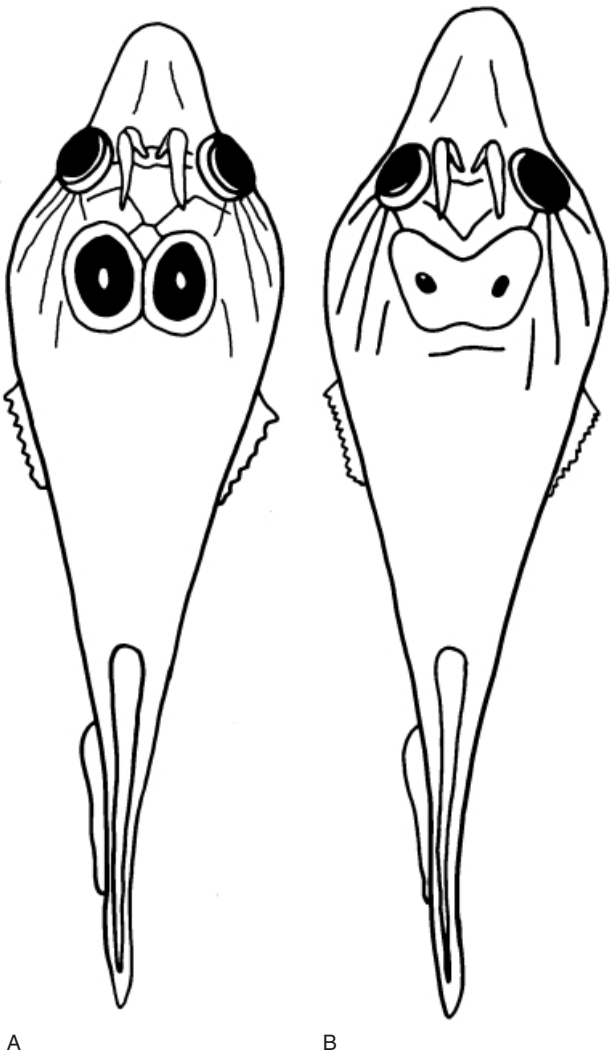

Figure 3. Head marks: A, *L. purpurea*; B, *L. lepadogaster*.

groups of organisms for section A and 1.58 for section B and mean roughness index = 2.69 for section A and 1.27 for section B).

Throughout the study period, regular diving at these sites indicated that both forms were found in the subtidal down to 7 m depth which is the lower limit of the stony habitat at the study area. Apparently, both forms continue to prefer different types of stones in the subtidal, with preferences similar to those found in the intertidal. No depth segregations could be detected.

DISCUSSION

The results of this study may be summarized as follows: both morphological and molecular comparisons indicate that *L. zebrina* is not more distinct from the populations of *L. l. lepadogaster* than these are among themselves. In contrast, *L. l. purpurea* emerged in all analysis as a distinct entity. *L. candollei* is strongly divergent from a monophyletic group, that in turn splits in two cohesive subgroups. These subgroups are sympatric from north-west Spain to the Mediterranean and Madeira island, their breeding seasons are out of phase and they differ in microhabitat choice. Examination of material belonging to the Oceanographic Museum of Arrábida Nature Park (Portugal) collected in 1905 in the study area, revealed that the two forms were already sympatric on the Portuguese shore at that time.

These findings lead us to conclude that we are in the presence of two valid species: one including the fish that traditionally were ascribed to *L. l. purpurea* (group 1) and another including the fish of groups 2, 3 and 4, traditionally ascribed to *L. zebrina* and *L. l. lepadogaster*. With the available evidence, we think that it is more parsimonious to consider two species (*L. purpurea* and *L. lepadogaster*) than to raise to specific status all subgroups that could be detected. It is important to stress again that the difference pointed by Briggs (1955, 1986) as the distinctive criterion to separate *L. zebrina* and *L. l. lepadogaster* was not supported by our results. Indeed, *L. zebrina* is as distantly related to *L. lepadogaster* as are the Madeiran populations of *L. purpurea* and *L. candollei* from their respective continental counterparts. According to the priority principle of the zoological nomenclature, the first species must be called *L. purpurea* (Bonnaterre, 1788), and the second *L. lepadogaster* (Bonnaterre, 1788). Indeed, this author was the first to use these names for fish caught in England and in the Mediterranean, respectively. His drawings and descriptions clearly correspond to the two species proposed in our study. A list of distinctive characters and a summary of synonyms, are presented in appendices A and B.

Because of the general loss of type material and the vast sympatry of these two species, one neotype for each species will be deposited at the Muséum Nationale d'Histoire Naturelle, Paris, with MNHN numbers: 2001–1240 (*L. lepadogaster*) and 2001–1241 (*L. purpurea*).

L. purpurea now has the following distribution: from Scotland to Senegal, the Canary islands and Madeira islands and the Mediterranean, at least as far east as Genoa (Italy). The distribution of *L. lepadogaster* ranges at least as far north as the extreme north-west of Galiza, south to north-west Africa, the Canary islands and Madeira islands and also the Mediterranean. The Black Sea specimens could belong to either species according to the work of Murgoci (1964) who identified in that region the *purpurea* subspecies described by Briggs (1986, 1990). Finally, the population from Morocco, that Brownell (1978) found to have intermediate distribution of meristic counts, could in fact contain specimens from both species.

It may seem hard to explain how such an extensive sympatry between *L. purpurea* and *L. lepadogaster* could go unnoticed for such a long period. In our view, it is likely that the explanation lie in the cryptic behaviour of these fish. They are very small and stay almost always under boulders, which makes them very hard to detect in conventional ichthyofaunal surveys. As *L. purpurea* is associated with boulders that are of an unusually large size, rare in most shores, this species is still more easily overlooked. In scuba surveys at Madeira (Funchal) and southern France (Cape Roux), where only the type of stones preferred by *L. lepadogaster* was available, only this species was collected. In addition, variation in trunk colour pattern between populations of *L. lepadogaster* made the situation more difficult to clarify.

There is no calibration available to use the rate of divergence of the DNA fragment studied as the basis for a molecular clock in gobiesocid evolution. Estimates of base substitution rate in mitochondrial DNA in ectotherms and for a variety of mitochondrial genes, usually range from 1 to 2% substitutions per million years (myr) (Avise, 1994). The percentage of divergence between *L. lepadogaster* and *L. purpurea* in our study ranged from 2.73% to 4.47%, as shown in Table 4. Assuming that the figures for base substitution rates are applicable to this group and to the specific fragment of DNA analysed, the higher values would point to a divergence time of more than 1 myr. Using the most conservative estimates the divergence time would point to more than 4 myr. This observation means that the speciation event that separated *L. lepadogaster* from *L. purpurea* is probably not a recent event and may have occurred prior to the beginning of the Pleistocene. The divergence of about 1% between haplotypes from populations of each species provide additional evidence in favour of a pre-Pleistocenic timing for the speciation event that separated the two species. The large extent of their sympatry is also suggestive of a considerable long history as separate species.

L. lepadogaster and *L. purpurea* are very similar in morphology and both are cryptic species that breed, feed and hide under boulders. However, they select boulders of very different sizes which means that it is unlikely that they actually compete for breeding or shelter sites. The large boulders inhabited by *L. purpurea* harbour a much higher diversity of organisms and form the basis of a more stable community. Thus, we suggest that the two species, although sympatric in most of their range, must display low levels of interspecific competition.

L. candollei emerged in all analysis as very distinct from the monophyletic group formed by *L. lepadogaster* and *L. purpurea.* The percentage of divergence between *L. candollei* and the two other species ranged from 14.39 to 16.13%. These values point to a Miocenic timing for their separation likely prior to the Messinian crisis that affected the Mediterranean at about 5.5 myr (Briggs, 1995). *L. candollei* is also very distinct from the other species both in morphology and behaviour (Gonçalves *et al*., 1998). It is an active swimmer that feeds out of shelter and often preys the nests of other fish species (Almada *et al*., 1987). The disparity in divergence times, and all differences mentioned above between *L. candollei* and the other two *Lepadogaster* species, raise doubts about the inclusion of these species in the same genus. Further phylogenetic analysis of Lepadogastrin gobiesocids, including a broader spectrum of genera and species, is required to clarify their relationships.

Finally, we would like to mention that, previous works from some of the authors of the present paper (Gonçalves *et al*., 1996, 1998), supposedly dealing with *L. l. purpurea* are, in the light of the present findings, studies on *L. lepadogaster*.

ACKNOWLEDGEMENTS

The authors would like to thank to Estação de Biologia Marinha do Funchal and its team for field and logistic help during work on Madeira island, and to Dr Riccardo Cattaneo-Vietti, Simone Bava and Stefano Schiaparelli for collecting fish from Genoa. We also thank Emanuel Gonçalves, John C. Briggs and an anonymous referee for their helpful review comments. This study was partially supported by Fundação para a Ciência e Tecnologia (FCT) through the project PRAXIS/3/3.2/EMG/1957/95, the fellowships GGPXXI/BIC/3570/96 (to R.L); PRAXIS XXI/BPD4470/96 (to T.G); PRAXIS XXI/BD/11178/97 (to F.A) and through the Pluriannual Program (UI & D 331/94).

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APPENDIX A

Characters used to distinguish the two species of *Lepadogaster*. The distinction of the two species using live animals may be based on:

1 Head marks—Two *ocelli* in *L. purpurea* and one large crescent shape in *L. lepadogaster* (Fig. 3).

2 Papillae of sucking disc region A–5/6 rows in *L. purpurea* and 3/4 rows in *L. lepadogaster* (Fig. 4). We found one *L. lepadogaster* over 49 with 5 rows in this region.

3 Papillae of sucking disc region B–5/6 rows in *L. purpurea* and 3/4 rows in *L. lepadogaster*, being the papillae larger in the later species (Fig. 4).

4 At least out of the Mediterranean, the two species also clearly differ in body colouration pattern: spotted in *L. purpurea* and vertically striped in *L. lepadogaster*.

5 When seen side-by-side, it is clear that *L. purpurea* has a shorter nose and eyes much more separated (Fig. 3).

When dead fish are preserved items 1 and 4 rapidly became useless. Criteria 2, 3 and 5 can be used but the papillae of the sucking disc tend to be lost and even their insertion marks on the disc are not always apparent. In these cases, identification could be based on the size of the individual papillae: those of *L. purpurea* are more numerous but smaller in size than those of *L. lepadogaster* (see Fig. 4). Papillae of sucking disc region C could be in some cases useful: 5/4 rows in *L. purpurea* and 4/3 rows in *L. lepadogaster*. The same applies to the dorsal fin rays: 18/19 rays in *L. purpurea* and 16/18 rays in *L. lepadogaster*.

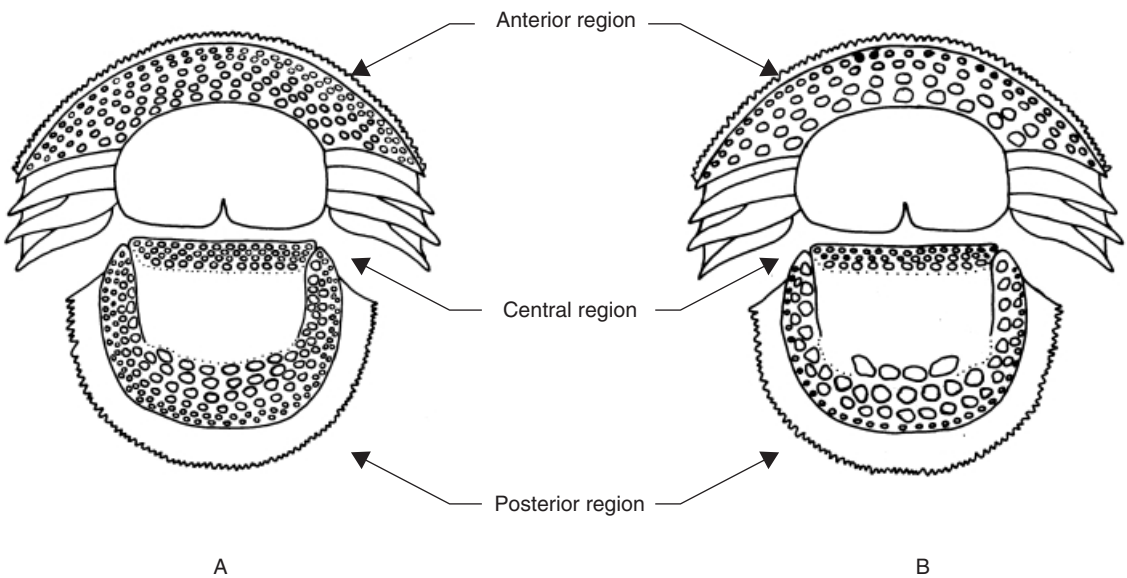

Figure 4. Sucking disc with rows of papillae: A, *L. purpurea*; B, *L. Lepadogaster.*

APPENDIX B – SYNONYMS

Although many designations are too vague to be ascribed with certainty to any of the two species, we present below a list of the synonyms that correspond unambiguously to each species, based on our own survey of the primary literature. We must mention that**,** contrary to Briggs (1986, 1990), *L. balbis* and *L. biciliatus* were found to be synonyms of *L. purpurea* based on the original descriptions of the head colour patterns.

Lepadogaster purpurea (Bonnaterre, 1788)

Cyclopterus purpureus Bonnaterre (1788) (British Isles).

Lepadogaster rostratus Bloch & Schnider (1801) (British Isles).

Cyclopterus cornubicus Shaw (1804) (British Isles).

Cyclopterus ocellatus Donovan (1806) (British Isles).

Lepadogaster balbis Risso (1810) (France, Mediterranean)

Lepadogaster biciliatus Risso (1826) (France, Mediterranean); Ninni, 1933 (Italy)

Lepadogaster cornubiensis Fleming (1828) (British Isles); Yarrel, 1836 (British Isles).

Cyclopterus spatulata Lacépède (1831) (British Isles).

Lepadogaster gouanii Couch (1877) (British Isles); Guitel, 1888 (Roscoff, France); Jenkins, 1936 (British Isles); Yonge, 1949 (British Isles).

Lepadogaster lepadogaster Fowler (1936) (Canaries); Wheeler, 1969 (British Isles); Russel, 1976 (British Isles).

Lepadogaster lepadogaster purpurea Briggs (1955), (1957), (1986), (1990) (Scotland to Dakar, Canaries, Madeira, western Mediterranean); Murgoci, 1964 (Black Sea); Brito, 1982 (Canaries).

Lepadogaster lepadogaster (Bonnaterre, 1788)

Cyclopterus lepadogaster Bonnaterre (1788) (Mediterranean).

Lepadogaster gouanii Lacépède (1800) (Mediterranean); Risso, 1810, 1826 (France, Mediterranean); Canestrini, 1864 (Italy); Ninni, 1933 (Italy); Soldjan, 1948 (Adriatic, Italy).

Pischephalus adherens Rafinesque-Schmaltz (1810) (Sicily, Italy).

Lepadogaster brownii Risso (1826) (France, Mediterranean); Canestrini, 1864 (Italy); Ninni, 1933 (Italy); Soldjan, 1948 (Adriatic, Italy).

Lepadogaster zebrinus Lowe (1839) (Madeira)

Lepadogaster acutus Canestrini (1864) (Italy); Ninni, 1933 (Italy).

Lepadogaster zebrina Briggs (1955), (1986), (1990) (Madeira, Canaries); Brito, 1982 (Canaries).

Lepadogaster lepadogaster lepadogaster Briggs (1955), (1957), (1986) (eastern Mediterranean).