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Molecular Phylogenetics and Evolution 46 (2008) 1151–1156

MOLECULAR PHYLOGENETICS AND EVOLUTION

www.elsevier.com/locate/ympev

Short Communication

Reclassification of *Lepadogaster candollei* based on molecular and meristic evidence with a redefinition of the genus *Lepadogaster*

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Received 24 January 2007; revised 11 May 2007; accepted 22 May 2007

1. Introduction

Several characteristics of the Gobiesocidae, such as the fact that they are small cryptic fishes, could explain the scarcity of studies on these species when compared with other rocky littoral fish families. In the last few years different authors described several new gobiesocid species (Hutchins, 1991; Briggs, 1993, 2001; Hofrichter and Patzner, 1997). Additionally, some ecological and behavioural studies have also been published (Gonçalves et al., 1998; Hofrichter and Patzner, 2000). However, the taxonomy of the subfamily Lepadogastrinae, which is restricted to the Eastern Atlantic, Mediterranean and the Black Sea, is still based on the works of Canestrini (1864), Ninni (1933), and Briggs (1955). Canestrini (1864) divided these fishes in three genera: *Gouania*, *Lepadogaster*, and *Mirbelia*. He distinguished these genera according to the fact that the dorsal and anal fins are separated from the caudal fin by a distinct gap in *Mirbelia*, while they are broadly connected in *Lepadogaster* and continuous in *Gouania*. In other words species such as *Lepadogaster candollei* (Risso, 1810), *Diplecogaster bimaculata* (Bonnaterre, 1788) and *Opeatogenys gracilis* (Canestrini, 1864) were included in the genus *Mirbelia*.

Chambanaud (1925) and Ninni (1933), based on a continuous or non-continuous fin criterion reclassified the Lepadogastrinae as *Gouania* or *Lepadogaster*, respectively, placing *Mirbelia* as a synonym of *Lepadogaster*.

Subsequently Fraser-Brunner (1938) described the genus *Diplecogaster* and Briggs (1955) maintained the genus *Mirbelia* as a synonym of *Lepadogaster*.

Briggs (1955) also provided an extensive revision of the phylogeny and biogeography of the family Gobiesocidae and he clearly admitted that the relationships within the Gobiesocidae subfamilies are more difficult to assess than the relationships among subfamilies. Nelson (2006) did not consider tribes or subfamilies within the gobiesocids.

Traditionally, according to Briggs (1955, 1957, 1986, 1990) and Hofrichter and Patzner (1997), the subfamily Lepadogastrinae comprises six genera and thirteen species: *Apletodon dentatus*, *A. incognitus*, *A. pellegrini*, *Diplecogaster bimaculata*, *D. megalops*, *D. ctenocrypta*, *Gouania willdenowi*, *Lecanogaster chrysea*, *L. candollei*, *L. lepadogaster* (with two subspecies: *L. l. lepadogaster* and *L. l. purpurea*), *L. zebrina*, *Opeatogenys gracilis* and *O. cadenati*. However, the species *D. ctenocrypta* is based on the description of a single specimen (Briggs, 1955) and should therefore be considered with caution.

Henriques et al. (2002) showed recently that *L. zebrina* is a population of *L. lepadogaster* from Madeira Archipelago. These authors also concluded, based on molecular, morphological and ecological data, that *L. l. lepadogaster* and *L. l. purpurea* should be considered two different species and not subspecies as was frequently proposed (e.g. Briggs, 1986). They also noted that *L. purpurea* and *L. lepadogaster* form a well supported monophyletic group markedly divergent from *L. candollei*, an observation that had already been advanced by Briggs (1955). Ecological and behavioural differences between *L. candollei* and the remaining *Lepadogaster* were also stressed by other authors (Gonçalves et al., 1998; Hofrichter and Patzner, 2000). These findings led us to reassess the taxonomic status of *L. candollei*.

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doi:10.1016/j.ympev.2007.05.021

Table 1
Taxa analysed in this study, geographical origin of the samples and GenBank accession numbers

Family (subfamily *sensu* Briggs, 1955)	Species	Origin of samples	12S rDNA	16S rDNA
	Lepadogaster lepadogaster	Mainland Portugal	AY036597	AF549202
	Lepadogaster purpurea	Mainland Portugal	AY036604	AF549201
	Lepadogaster candollei	Mainland Portugal	AY036587	AF549203
		Madeira	AF549194	AF549204
Gobiesocidae				
Lepadogastrinae	*Diplecogaster bimaculata*	Mainland Portugal	AF549197	AF549205
	Gouania willdenowi	Croatia	EF363029	EF363031
	Opeatogenys gracilis	Mainland Portugal	AF549196	AF549206
	Apletodon dentatus	Mainland Portugal	AF549200	AF549207
	Apletodon incognitus	Azores	AF549198	AF549208
Aspasminae	*Aspasma minima*	Miya et al. (2003)	NC008130	NC008130
			AP004453	AP004453
Gobiesocinae	*Gobiesox barbatulus*	Brasil	EF363030	EF363032
Gobiesocinae	*Arcos* sp.	Miya et al. (2003)	AP004452	AP004452
Blenniidae	*Parablennius pilicornis*	Mainland Portugal	AY098795	AY098831

2. Materials and methods

Samples collected, their geographical origin and GenBank accession numbers are listed in Table 1. Our analysis also included *Gobiesox barbatulus*, *Aspasma minima* and one specimen identified as *Arcos* sp. available in GenBank database (Table 1). *Parablennius pilicornis* was used as outgroup according to Chen et al. (2003), who placed the Blennioidei as a sister group of the Gobiesocoidei.

DNA was extracted from fin clips preserved in ethanol, using a proteinase K/SDS based protocol (Sambrook et al., 1989). Primer sequences used to amplify a fragment 378 bp long from the 12S mitochondrial rDNA (12SFor 5′-AAC TGG GAT TAG ATA CCC CAC-3′ and 12SRev 5′-GGG AGA GTG ACG GGC GGT GTG-3′), and a fragment 445 bp long from the mitochondrial 16S rDNA (16SFor 5′-AAG CCT CGC CTG TTT ACC AA-3′ and 16SRev 5′-CTG AAC TCA GAT CAC GTA GG-3′), are described in Henriques et al. (2002). PCR conditions followed those described in Almada et al. (2005). The amplifications were performed in a Biometra thermocycler (Biometra, Trio-Thermblock, Göttingen, Germany) and were conducted as follows: 4 min at 94 °C and 30 cycles of: [94 °C (1 min), 55 °C (1 min) and 72 °C (1 min)], 10 min at 72 °C for both fragments.

All samples were sequenced in both directions and sequencing reactions were performed by Macrogen Inc. (Seoul, Republic of Korea) in a MJ research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (Applied Biosystems), following the protocol supplied by the manufacturer.

Sequences were aligned with Clustal X 1.81 (Thompson et al., 1994) and were analysed with maximum parsimony (MP), minimum evolution (neighbour-joining) (ME) and maximum likelihood (ML) methods available in PAUP 4.0 (Swofford, 1998). Bootstrapping was used to access robustness of the nodes in the trees with 1000 replicates. The molecular evolution model used for the ME and ML tree was selected according to the results of Modeltest 3.7 (Posada and Crandall, 1998) with the AIC criterion.

Dolphin et al. (2000) showed that even when ILD (Farris et al., 1995) tests reveal significant differences between two fragments it is frequently preferable to analyse them together. Thus the two fragments were combined in a single data set.

We tested the monophyly of the genus *Lepadogaster* and the subfamily Lepadogastrinae by comparing unconstrained trees and topologies constrained such that (1) *L. candollei* was forced to be the sister species of the *L. purpurea*–*L. lepadogaster* clade, and (2) the Lepadogastrinae were forced to be monophyletic. Unconstrained and constrained tree were obtained using MP, ME, and ML methods, and compared using a Kishino and Hasegawa test (1989), in the case of MP topologies, and using the Shimodaira and Hasegawa (1999) tests for ME and ML trees. The latter test adjusts the expected distribution of log likelihood differences when comparing a posteriori hypotheses or multiple topologies (Buckley et al., 2001; Felsenstein, 2004). Both tests were implemented in PAUP v.4.0b10 (Swofford, 1998).

Bayesian analysis was performed using MCMC as implemented in Mr. Bayes 3.1 (Ronquist and Huelsenbeck, 2003), with four independent runs of four Metropolis-coupled chains of 2,000,000 generations each, to estimate the posterior probability distribution. Data were partitioned by ribosomal region, and independent GTR+G+I assumed for each partition. Topologies were sampled every 100 generations, and a majority-rule consensus tree was estimated after discarding the first 1000 sampled generations.

Maximum likelihood analysis used likelihood setting estimated by Modeltest 3.7 (Posada and Crandall, 1998), a heuristic search algorithm with TBR branch swapping and 100 bootstrap replicates.

The meristic variables used in this study, follow those considered by Briggs (1955, 1957) with some additions (Hofrichter and Patzner, 1997; Henriques et al., 2002) are: number of dorsal rays; number of anal rays; number of pectoral rays; number of caudal rays; number of papillae rows in the anterior region of the pelvic disc; number of papillae rows in posterior region of the pelvic disc; presence/absence of papillae lateral rows; presence/absence of central papillae in anterior region of the pelvic disc; presence/absence of teeth dimorphism; number of rakers; presence/absence of anal papillae; united/separated dorsal–anal–caudal fins; presence/absence of opercular spine; presence/absence of sexual dimorphism; presence/absence of fleshy pad on lower pectoral base; gills three and one-half/gills three; presence/absence of double disc; maximum standard length; presence/absence of gill membrane attached to isthmus; presence/absence of elongated nostril; presence/absence head-papillae; number of vertebrae. A cluster analysis (UPGMA) of meristic characters was performed based on their percent disagreement. Statistical treatment was performed with the software package STATISTICA 5.5 (StatSoft©, Inc.).

3. Results

When plotting transitions and transversions over patristic distances (plot not shown) transitional saturation was noticeable for distances greater than 22%. The genetic distance among congeneric species was less than 4% (*p*-distance) with the exception of *L. candollei*, which showed a genetic distance of 13.9–15.0% from the remaining species of the genus *Lepadogaster*, a range of distances that is of the same order as other intergeneric distances shown in Table 2.

Table 2
Percent genetic distance (*p*-distance) between the DNA sequences of a combined fragment of the 12S and 16S rDNA

	A.d.	*A.i.*	*L.p.*	*L.l.*	*G.w.*	*L.c.*cP	*L.c.*Mad	*A.m.*	*O.g.*	*D.b.*	*G.b.*	*A.* sp.	*P.p.*
A. dentatus	—	2.78	21.03	22.43	21.52	18.72	19.08	21.36	19.70	19.57	23.25	20.88	25.13
A. incognitus		—	20.65	21.80	22.54	19.99	20.22	22.24	20.72	20.59	23.11	20.37	24.86
L. purpurea			—	3.26	14.95	13.86	14.35	18.66	15.50	14.48	18.87	18.19	23.30
L. lepadogaster				—	14.18	14.85	14.97	17.78	16.24	15.11	20.22	17.66	23.18
G. willdenowi					—	15.27	15.25	19.23	16.25	15.79	20.72	18.42	24.15
L. candollei cP						—	1.38	15.87	13.01	13.27	18.53	17.98	22.38
L. candollei Mad							—	16.12	13.38	13.76	19.02	18.48	22.61
A. minima								—	18.94	18.55	22.69	19.99	23.86
O. gracilis									—	14.66	20.92	18.62	23.24
D. bimaculata										—	18.89	16.97	22.57
G. barbatulus											—	19.45	24.18
Arcos sp.												—	17.99
P. pilicornis													—

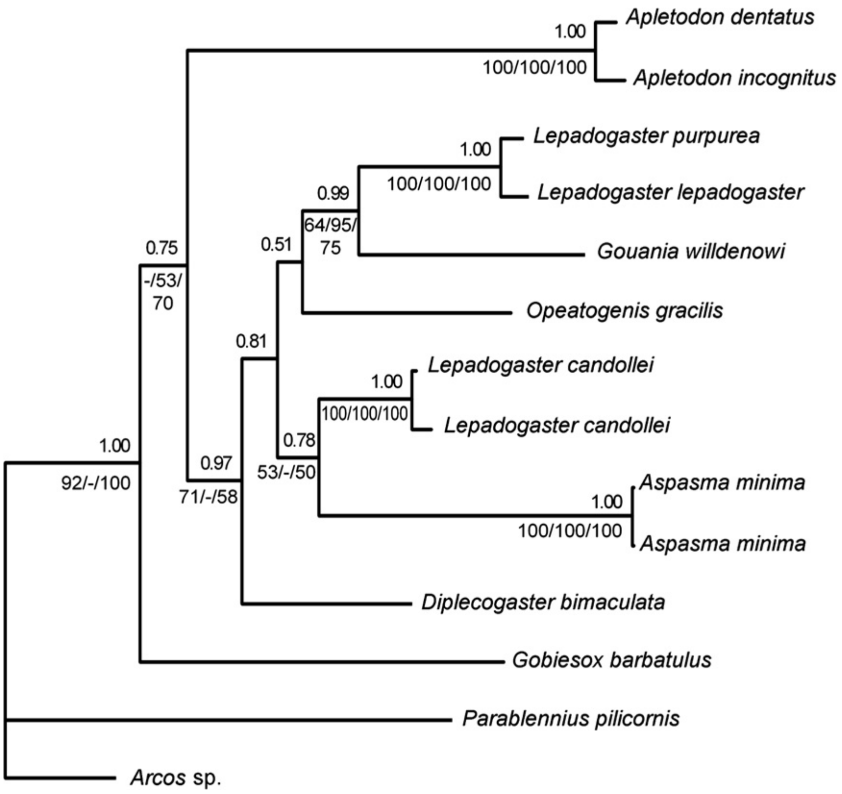

Fig. 1. Phylogenetic relationships of the Atlanto-Mediterranean gobiesocids (Lepadogastrinae) obtained from molecular data. Clingfishes from other subfamilies are also shown (*A. minima*—Aspasminae; *G. barbatulus*, *Arcos* sp.—Gobiesocinae). *Parablennius pilicornis* (Blenniidae) was used as outgroup. Bayesian posterior probabilities and MP (CI = 0.63, RI = 0.59, TL = 906), ME and ML bootstrap support are shown above and below each node, respectively. Although with a lower phylogenetic resolution, MP, ME, and ML inference methods never supported a different topology when compared with Bayesian trees.

Concerning the phylogenetic analysis (Fig. 1), all inference methods recovered the following features:

(1) The traditionally accepted genus *Lepadogaster* is polyphyletic since *L. lepadogaster* and *L. purpurea* were never recovered forming a monophyletic clade with *L. candollei*. Instead *L. lepadogaster* and *L. purpurea* form a clade that is sister to *G. willdenowi*. When we constrained trees to place *L. candollei* to be sister to the clade *L. lepadogaster*/*L. purpurea* the MP tree was longer (918 versus 906 steps) than the unconstrained tree although the difference was non significant (K–H test $t = 1.635$; $\text{SD} = 7.341$ and $p = 0.103$). Topologies unconstrained by a monophyletic *Lepadogaster* genus had significantly better likelihoods than constrained topologies (Shimodaira–Hasegawa one-tailed tests for ME and ML, respectively; difference in $-\ln L = 10.919$, $p = 0.042$; difference in $-\ln L = 10.635$, $p = 0.027$);

(2) If the hypothesis that the subfamilies Aspasminae and Lepadogastrinae are not monophyletic is confirmed by a broader taxonomic analysis, it has interesting biogeographic implications. However, these results must be taken with caution as neither the K–H test nor the S–H tests for ME and ML yielded statistically significant differences (K–H test $t = 1.306$, $\text{SD} = 8.423$ and $p = 0.192$; S–H test ME diff. $-\ln L = 3.243$, $p = 0.216$; difference in $-\ln L = 6.342$, $p = 0.027$);

(3) The subfamily Gobiesocinae is also a problematic taxon. While *Gobiesox* was recovered as being sister to the Lepadogastrinae plus *Aspasma*, *Arcos*, which also belongs to the Gobiesocinae, never formed a clade with *Gobiesox*, being recovered in a very basal position;

(4) The relationships of *L. candollei* with other taxa are unresolved although moderate support exists for a sister relationship with *Aspasma*. A grouping corresponding to *Mirbelia* as defined by Canestrini (1864) including *L. candollei* and species of the genera *Diplecogaster* and *Opeatogenys* was never recovered.

The analysis of the meristic data (Fig. 2) independently supports the similarity between *L. lepadogaster*, *L. purpurea*, and *G. willdenowi* and the separation of *L. candollei* from this group. It even suggests some morphological similarities between *L. candollei*, *D. bimaculata*, and *O. gracilis*, again with *A. minima* included in the same group. In contrast to the phylogenetic results, cluster analysis grouped the genera *Gobiesox* and *Arcos*, placing the Lepadogastrinae plus the genus *Aspasma* in a distinct cluster.

4. Discussion

A major conclusion emerging from the results presented above is the need to remove *L. candollei* from the genus *Lepadogaster*. Canestrini (1864) had already felt the need to place *L. candollei* in a genus (*Mirbelia*) distinct from *Lepadogaster*. In turn Briggs (1955), in his detailed revision, although placing *L. candollei* in *Lepadogaster* had noted that: "*L. candollei* is well separated from the other three forms (of *Lepadogaster*) which are very closely related to each other". These three species included *L. zebrina* which was subsequently shown to be a synonym of *Lepadogaster lepadogaster* by Henriques et al. (2002). Gonçalves et al. (1998) and Hofrichter and Patzner (2000) showed that *L. candollei* differs very markedly both

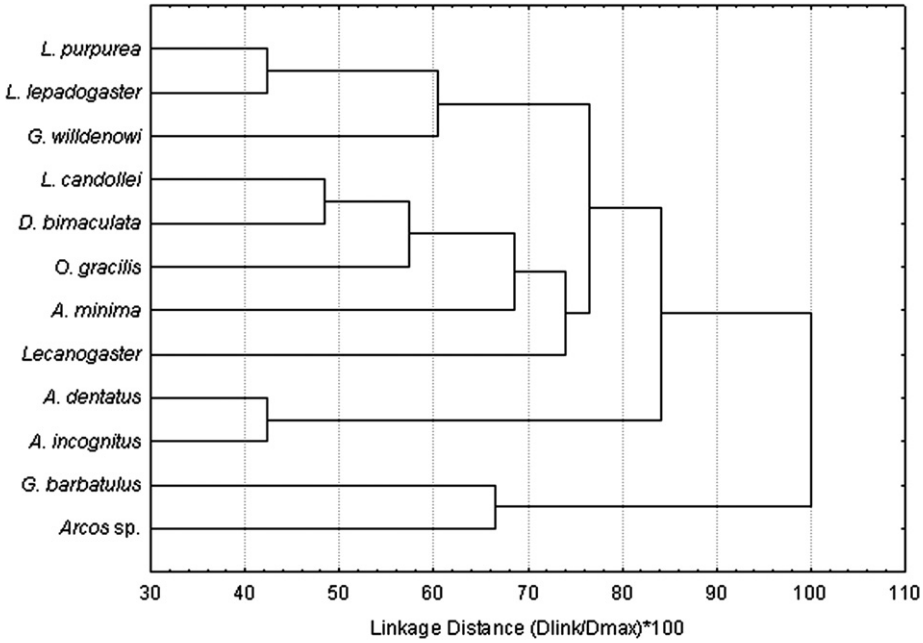

Fig. 2. Cluster analysis obtained from meristic data (UPGMA with percent disagreement distances).

in ecology and behaviour from the remaining species of *Lepadogaster*. While these are very cryptic species that spend most of the time hidden under boulders, and rarely swim in the water column, *L. candollei* is a more active fish that swims frequently and forages openly out of shelter. It also differs in agonistic behaviour from the other *Lepadogaster*.

Thus our analyses of molecular and meristic data are in agreement with the views of previous workers. Therefore, we propose that the genus *Lepadogaster* must be redefined to include only the species *L. lepadogaster* (Bonaterre, 1788) and *L. purpurea* (Bonaterre, 1788). Removing *L. candollei* from the genus *Lepadogaster* begs the question of its generic position. *Lepadogaster candollei* did not occur in any well supported clade that would suggest its inclusion in any particular genus.

Our analysis included small numbers of individuals of each genus and lacked representatives of *Lecanogaster*, a tropical African monospecific genus. Therefore the molecular data do not allow the resolution of the relationships of *L. candollei* except for the inadequacy of keeping it in *Lepadogaster*. The analysis of the meristic data that already includes *Lecanogaster* also failed to point to the inclusion of *L. candollei* in any other genus, while suggesting a possible association with *Diplecogaster* and *Opeatogenys*.

Canestrini (1864) included *L. candollei* with *D. bimaculata* and *O. gracilis* in the genus *Mirbelia*. We propose that for the time being the genus *Mirbelia* should be revalidated and redefined to include only the species *Mirbelia candollei* (Risso, 1810). This proposal has the advantage of calling attention to the need to separate *M. candollei* from *Lepadogaster* and to its distinctiveness from other clingfishes.

The genus *Lepadogaster*, excluding *L. candollei*, should be redefined as follows: median fins united to the caudal fin; long dorsal fin with 16–21 rays; 20–23 pectoral fin rays; anterior nostril with an appendage on its posterior margin with the form of a large cirrus extending back to the posterior margin of the eye and 3–6 rows of papillae in the posterior region of the sucking disc. The monospecific genus *Mirbelia* is characterized by the following diagnostic traits: dorsal, caudal, and anal fins clearly separated; dorsal fin long, with 13–16 rays; pectoral fins with 26–29 rays; an appendage on the posterior margin of the anterior nostril in the form of a very small dermal flap; and, the posterior region of the sucking disc with 7–9 rows of papilae.

It could be argued that because there are some signs of saturation of the molecular data our conclusions may be weak. Transitional saturation was however noticeable at distances of about 22% while the conclusions presented above are based on distances well below that value. It is urgent to perform a comprehensive phylogenetic analysis of the family Gobiesocidae with a broader taxon sampling and additional molecular data and proper mapping of the morphological characters on the new phylogeny. Indeed the present study while not intended to address this broader issue serves as a warning that the Gobiesocidae subfamilies, as currently viewed, need to be re-evaluated.

Acknowledgments

The authors thank to Marcelo Kovačić and Mauricio Hostim Silva for providing some of the samples and to Thomas Guillemaud (USVE, INRA), Leonor Cancela and her team (UA—UCTRA), and to Dr. Rui Malhó and his team (FCUL, DBV) for their help. This study was funded by Fundação para a Ciência e Tecnologia as part of the Plurianual Program UI&D 331/94. F.A. was supported by an FCT grant PRAXIS XXI/BD/11178/97 and A.L. was supported by an FCT Grant (SFRH/BPD/18067/2004).

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