

ON THE PHYLOGENETIC AFFINITIES OF *Centrolabrus trutta* AND *Centrolabrus caeruleus* (PERCIFORMES: LABRIDAE) TO THE GENUS *Symphodus*: MOLECULAR, MERISTIC AND BEHAVIOURAL EVIDENCES

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Phylogenetic analysis of partial 12S and 16S mitochondrial rDNA regions, together with meristic and ethological data, has revealed that *Centrolabrus trutta* (Lowe, 1833), a species occurring in the Canaries and Madeira, and its close relative *Centrolabrus caeruleus* (Azevedo, 1999) from the Azores, are more closely related to most species of the genus *Symphodus* than to *Centrolabrus exoletus* (Linnaeus, 1758). Underwater behavioural observations showed that *C. trutta*, like *C. caeruleus*, shares with most *Symphodus* species sexual dichromatism, male nest building and male parental care of the eggs, traits that are absent in *C. exoletus*. The low number of base substitutions found between *C. trutta* and *C. caeruleus* indicates that these species are very closely related. The 16S rDNA data suggest that *C. trutta*, *C. caeruleus* and *S. roissali* form a monophyletic group. The analysis of the 16S rDNA data also showed that *S. melanocercus* is the sister species of *C. exoletus* and it is not closely related to the species of *Symphodus* included in this study. Lack of nest building and parental behaviour in *S. melanocercus*, and its marked divergence to other members of the genus *Symphodus* in meristic characters, also stress its affinity with *C. exoletus* and its separation from the remaining species of *Symphodus*. Our data and the evidence available in the literature led us to propose the inclusion of *C. trutta* and *C. caeruleus* in the genus *Symphodus*, while *S. melanocercus* must be transferred to the genus *Centrolabrus*.

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INTRODUCTION

GÜNTHER (1861), in a revision of the family Labridae, proposed the division of the genus *Crenilabrus* Oken, 1817, into two genera: *Crenilabrus*, with 3 spines in anal fin; and *Centrolabrus* (Günther, 1861), with more than 3

spines. Presently, the species included in *Crenilabrus* are assigned to the genus *Symphodus* Rafinesque, 1810 which had precedence over *Crenilabrus* (e.g. Jordan, 1891; Bauchot, 1963). *Centrolabrus*, initially defined based on *Centrolabrus exoletus* (Linnaeus, 1758), also includes two endemic species from north Atlantic

islands: *Centrolabrus trutta* Lowe, 1833 from the Canaries and Madeira, and *Centrolabrus caeruleus* (Azevedo, 1999) recently described for the Azores, after re-examination of what had been supposed to be populations of *C. trutta*. QUIGNARD (1966) and QUIGNARD & PRAS (1986), present extensive information on morphological and meristic differences between *C. exoletus* and the species of the genus *Symphodus*. Field observations at Madeira and Canaries for *C. trutta* and those of AZEVEDO et al. (1999) at Azores for *C. caeruleus* has revealed that both species exhibit nest building, paternal care of the eggs and sexual dichromatism, quite similar to those found in most *Symphodus* species. These traits are absent in *C. exoletus*.

In a recent paper (HANEL et al. 2002) examined the phylogenetic relationships within the Labrini based on a fragment of the 16S rDNA and another of the control region. These authors found a very close relationship between *C. exoletus* and *Symphodus melanocercus*. They also found that *C. trutta* and *C. caeruleus* formed a monophyletic clade with all *Symphodus* species excluding *S. melanocercus*.

In this paper we re-examine the relationships of *C. trutta* and *C. caeruleus* with *C. exoletus* and species of the genus *Symphodus* based on sequence analysis of additional 12S and 16S rDNA and meristic and ethological data found in the literature.

METHODS

Scuba-diving observation and sampling of specimens listed in table 1 took place between 1997 and 2001 at the Azores, Madeira, Canaries and on the west coast of Portugal. Samples were preserved in 96% ethanol. *C. trutta* and *C. caeruleus* were compared with *C. exoletus* and with six out of the ten species of the genus *Symphodus*, including representatives of the two sub-genera recognized by QUIGNARD (1966). In an attempt to place the species analysed into a broader phylogenetic perspective, DNA sequences of most genera of north-eastern Atlantic Labrinae were also included in the analysis. We could not find biological material from *Lapanella fasciata*. As outgroups we chose two species belonging to two different

subfamilies, *Pseudolepidaplois scrofa* (Bodianinae) and *Coris julis* (Corinae). A summary of the numbers of individuals analysed, the numbers of haplotypes identified and the Genbank accession numbers is presented in table 1. In addition to our samples a sequence of the 16S rDNA belonging to *Symphodus melanocercus* was retrieved from Genbank (accession number AF517595) (HANEL et al. 2002).

Total genomic DNA was extracted from muscle tissue using a proteinase K/SDS based extraction buffer and phenol/chloroform purification with ethanol precipitation (Maniatis et al. 1982). Primer sequences used to amplify one segment 392 bases long from the 12S rDNA were 12SFor 5'-AAC TGG GAT TAG ATA CCC CAC-3' and 12SRev 5'-GGG AGA GTG ACG GGC GGT GTG-3'. Primers for a fragment 577 bp long from the 16S rDNA were 16SFor 5'-AAG CCT CGC CTG TTT ACC AA-3' and 16SRev 5'-CTG AAC TCA GAT CAC GTA GG-3'. PCR reactions were performed with: 1.5 µM MgCl₂, 200 µM each dNTP, 0.5 µM each primer, 0.5 units of Taq polymerase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD), 1x buffer supplied by the manufacturer and approximately 20 ng of genomic DNA in a volume of 20 µl, for both molecules. The amplifications in a Biorad Gene-Cycler™ consisted of 4 minutes at 94°C, and 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C and 10 minutes at 72°C. PCR products were purified with GFX PCR DNA and gel band purification kit Amersham-Pharmacia following the recommendations of the manufacturer. Automatic sequencing of the purified PCR products was performed on a CEQ 2000 XL, Beckman Coulter with the same primers. Alignments were made using ClustalX (THOMPSON et al. 1997) with default settings.

Both the 12S and the 16S rDNA data sets were analysed with three methods of phylogenetic inference: maximum parsimony (MP), maximum-likelihood (ML) and neighbour-joining (NJ) (SAITOU & NEI 1987). The phylogenetic analysis was performed with PAUP 4.0b Win (SWOFFORD 1998). Bootstrapping (FELSENSTEIN 1985) was used to access robustness of the nodes in the trees with 1000 replicates for MP and NJ and 100

replicates for ML. The heuristic search option “random addition of taxa” and tree bisection reconnection (TBR), with the MULPARS option

in effect, was used with the three methods of inference. MP analysis was conducted with the ACCTRAN option.

Table 1
Summary of the putative taxonomy of the species sequenced, geographical origin of samples and Genbank accession numbers.

Genera	Species	Number of samples sequenced		Origin of samples	Genbank accession number
		12S rDNA	16S rDNA		
<i>Centrolabrus</i>	<i>C. exoletus</i>	1	1	Arrábida W Cont. Portugal	AF414200, AY0092041
	<i>C. trutta</i>	2	1	Madeira	AF414195, AF414196, AY092035,
	<i>C. caeruleus</i>	2	1	Canaries Azores	AY092045, AY092046 AF414192, AF414193, AF414194, AY092034
<i>Symphodus</i>	<i>S. rostratus</i>	2	1	Arrábida W Cont. Portugal	AF414198, AF414199, AY092040
	<i>S. melops</i>	2	1	Arrábida W Cont. Portugal	AF414197, AY092038, AY092049
	<i>S. cinereus</i>	2	1	Arrábida W Cont. Portugal	AY092036, AY092047, AY092048
	<i>S. roissali</i>	2	1	Arrábida W Cont. Portugal	AY092039, AY092050, AY092051
	<i>S. mediterraneus</i>	2	2	Arrábida W Cont. Portugal	Submitted
	<i>S. bailloni</i>	2	1	Arrábida W Cont. Portugal	AY092052, AY092053, AY092037
<i>Labrus</i>	<i>L. bergylta</i>	1	1	Costa Galé W Cont. Portugal	AF414201, Submitted
<i>Acantholabrus</i>	<i>A. palloni</i>	1	1	Arrábida W Cont. Portugal	Submitted
<i>Ctenolabrus</i>	<i>C. rupestris</i>	1	1	Arrábida W Cont. Portugal	AF414202, Submitted
<i>Pseudolepidaplois</i>	<i>P. scrofa</i>	1	1	Azores	Submitted
<i>Coris</i>	<i>C. julis</i>	1	1	Arrábida W Cont. Portugal	AY092054, AY092042

In order to choose the model of evolution that best fitted our data we used the program Modeltest 3.06 (POSADA & CRANDALL 1998). For the 12S rDNA the ML settings selected, according to the results of Modeltest, corresponded to the general time reversible model (GTR) with rate heterogeneity. The rates were assumed to follow a gamma distribution with shape parameter equal to 0.2578 and equal base frequencies. For the 16S rDNA the ML settings selected corresponded to the KIMURA (1980) two parameter model (K2P) with rate heterogeneity.

The rates were assumed to follow a gamma distribution with shape parameter equal to 0.4851 and a transition/transversion ratio of 2.9985.

NJ was based on the estimator of distance derived from the ML settings selected for each fragment.

A list of meristic and behavioural characters (see Table 2) was completed with data from: QUIGNARD (1966); QUIGNARD & PRAS (1986); LOWE (1833, 1837); LEJEUNE (1985); AZEVEDO (1999) and AZEVEDO et al. (1999). A cluster analysis was performed on these characters using

Table 2

Meristic and behavioural comparison of *C. trutta* and *C. caeruleus* and the related genera *Centrolabrus*, *Symphodus*, *Labrus*, *Ctenolabrus*, *Acantholabrus* and *Pseudolepidaplois*. Key items in *Centrolabrus/Symphodus* discrimination are highlighted. * 3/10 species currently considered in the genus *Symphodus* exhibits occasional sex reversal (QUIGNARD & PRAS 1986), ** only *S. dodderleini* did not take care of the eggs; *** *L. bergylta* and *L. bimaculatus* transport some algal material to the spawning area.

	<i>Symphodus</i>		<i>Centrolabrus trutta/</i> <i>Centrolabrus caeruleus</i>		<i>Centrolabrus</i>	<i>Symphodus</i>	<i>Labrus</i>	<i>Ctenolabrus</i>	<i>Pseudolepidaplois</i>	<i>Coris</i>
	<i>exoleus</i>	<i>metanocerius</i>	<i>exoleus</i>	<i>metanocerius</i>						
Anal spines	3		4-5/5-6		3		3	3	3	3
Anal soft rays	7-12		8-9/7-9		6-8	8-11	8-13	6-9	13	11-12
Dorsal spines	12-18		16-18/17-18		18-20	15-17	16-21	16-19	12	9
Dorsal soft rays	8-12		8-9		5-7	6-10	9-14	7-10	11	12
Pectoral finrays	11-15		14-15		12-14	11-13	13-15	13-16	15	12-14
Teeth	1 row small canine		1 row small canine		1 row small canine	1 row small canine	1 row strong canine	Several rows	1 row canine	2 rows canine
Teeth sup. Maxilla	2-16		4.5/5.5		7	3-5	7-12	7-15	?	11
Teeth inf. Maxilla	5-15		8/7.5		11	6-7	7-19	11-16	?	12.5
Preoperculum serrated	Yes		Yes		Yes	Yes	No	Yes	No	No
Gill arch rakers	11-18		13-15		10-13	16	16-22	14-18	?	14-17
Scales base D, A fins	No		No		Yes	No	No	Yes	Yes	No
Scales inter-operculum	4-18		10-11		7-11	5-8	0-4	9-18	>100	No
Tempor-occipit. scales	2-5		3		5-8	3-4	>15	7-12	>50	No
LL scales	30-38		31-34/33-34		33-37	30-38	40-49	35-39	45	73-80
Scales above LL	2.5-5.5		3.5-4.5/4.5		3.5	3.5	5-8	3	7.5	4.5
Longit. brownish bands	Yes		Yes		No	No	No	No	No	No
Dark spot on caudal ped.	Yes		Yes		No	No	No	Yes	No	No
Sexual dimorphism	Yes		Yes		Yes	Yes	No (<i>L. bimacul.</i> yes)	No	Yes	Yes
Sex reversal	No *		No		No	No	Yes	No	No	Yes
Eggs	Demersal		Demersal		Demersal	Demersal	Demersal	Pelagic	Pelagic	Pelagic
Parental care	Yes **		Yes		No	No	Yes	Yes	No	No
Nest building	Yes		Yes		No	No	Yes***	No	No	No
Urogenital papilla	Yes		Yes		No	No	No	No	No	No

the euclidian distance and unweighted pair-group average method (UPGMA) with the software package STATISTICA 5.5 (StatSoft[®], Inc.).

RESULTS

Mitochondrial rDNA data revealed no saturation either by plotting transitions versus transversions (for 12S Pearson $R=0,94$, $p < 0,0001$, $N=78$; for 16S Pearson $R=0,91$, $p < 0,0001$, $N=78$) or by plotting each kind of substitution against the percentage of sequence divergence. By analysing the divergence percentages of both molecules for each dyad of species, it was possible to determine that the 16S rDNA is evolving almost 1.5 times faster than the 12S rDNA (mean=1,53; s.d.=1,4; n=77). Furthermore, the mean transition/transversion ratio was 2.32 (s.d.=1,9; n=63) for 12S rDNA and 2,67 (s.d.=2,0; n=76) for 16SrDNA. Nucleotide composition consisted of 29%-29% A, 27%-25% C, 23%-24% G and 21%-22% T for 12S and 16S fragments, respectively. Thus, both fragments presented similar base compositions, with a slight over-representation of adenine and an under representation of thymine, as was previously observed for mitochondrial DNA of several fish species (see MEYER 1993 and KOCHER & CARLETON 1997).

All the specimens of *C. trutta* and *C. caeruleus* possessed identical DNA sequence for the 12S rDNA fragment. Nevertheless, two transitions separated *C. trutta* and *C. caeruleus* in the 16S rDNA fragment examined. In all cases in which the DNA from more than one specimen was sequenced, no intraspecific differences were detected either in 12S or 16S rDNA.

The three methods of phylogenetic inference produced trees that unambiguously placed *C. trutta* and *C. caeruleus* in the same clade with the species of *Symphodus* (Figs 1A and 1B). Both MP and ML analysis of the 16S rDNA data indicate that *C. trutta* and *C. caeruleus* form a monophyletic clade that also includes the Atlanto-Mediterranean *S. roissali*. Although with a lower bootstrap (65%) NJ also supported this clade.

Although we did not intend to cover the whole phylogeny of the Labrinae, the available data strongly suggest that, at a higher level, the

genera *Symphodus* and *Centrolabrus* form a well supported clade.

Fig. 1. (A) Phylogenetic tree obtained from the 12S rDNA fragment sequenced; (B) Phylogenetic tree obtained from the 16S rDNA fragment sequenced. Bootstrap values are shown as percentages for maximum-parsimony, maximum-likelihood and neighbour-joining (MP/ML/NJ), respectively. *C. julis* and *P. scrofa* have been used as outgroups. Only bootstrap values above 70% are shown. Note that *S. melanocercus* was only analysed with the 16S rDNA fragment.

Concerning the 16S rDNA all the methods of phylogenetic inference recovered a clade formed by *C. exoletus* and *S. melanocercus* with very high bootstrap support. This clade is the sister group of the clade which includes all other *Symphodus* species plus *C. trutta* and *C. caeruleus*.

Inspection of Table 2 clearly revealed that from the twenty three variables considered, ten grouped *C. trutta* and *C. caeruleus* with most *Symphodus*, but only one grouped those species with *C. exoletus*. The remaining variables are inconclusive on this issue. Cluster analysis produced one tree that unambiguously placed *C. trutta* and *C. caeruleus* in the same cluster with all species of *Symphodus* excepting *S. melanocercus*. *S. melanocercus* is clearly isolated in a separate branch together with *C. exoletus* (Fig. 2). Again at a higher taxonomic level all the species of *Centrolabrus* and *Symphodus* are

included in the same cluster. Thus, the phenogram shown in Fig 2 supports the conclusions previously derived from the phylogenetic analysis of the 16S rDNA fragment (Fig. 1B).

Fig. 2. Cluster analysis based on the meristic and behavioural data, unweighted pair-group average with Euclidean distances.

Scuba-diving observations in Madeira, Canary Islands and the Azores revealed that *C. trutta* and *C. caeruleus* are similar to the majority of the species of *Symphodus*, both in ecology and behaviour. Males are larger than females, they build nests with algae and they guard the eggs. They show a typical *Symphodus* colour pattern with mottled brown body, dark longitudinal bands and a dark spot on the caudal peduncle. The reproductive males have a bluish (*C. caeruleus*) or greenish (*C. trutta*) tinge, with females, in contrast, exhibiting a brownish body. The nest building described by AZEVEDO et al. (1999) for *C. caeruleus* in the Azores fully matches the patterns described for *Symphodus melops* (Potts 1985).

DISCUSSION

The presence of nest-building, parental care and a pattern of sexual dichromatism in *C. trutta* and *C. caeruleus*, very similar to those found in most *Symphodus* species, contrast with the absence of these traits in *C. exoletus*. It is also interesting to note that, like *C. exoletus*, *S. melanocercus* is the

only species of *Symphodus* lacking nest building behaviour. In addition, like *C. exoletus*, *S. melanocercus* does not provide parental care.

Another similarity between *C. exoletus* and *S. melanocercus*, concerns the presence of cleaning behaviour in both species (POTTS 1968, HENRIQUES & ALMADA 1997). Indeed, HENRIQUES & ALMADA (1997) found that, on the Portuguese shore, *C. exoletus* is the only wrasse observed to clean other fishes, a role played by *S. melanocercus* in the Mediterranean. *C. exoletus* even cleans *S. melops* and other labrids which have been described as cleaners in aquaria (POTTS 1973) and have been used in aquaculture to clean cultivated fish (BJORDAL 1988).

These ethological traits reinforce the genetic and meristic findings and stress the similarities between *C. exoletus* and *S. melanocercus* and their differences to the remaining species of *Symphodus* plus *C. trutta* and *C. caeruleus*. Although these ethological similarities may be merely convergent, in our view, it seems more likely that they represent genuine homologies.

In the present study we did not attempt to address the phylogeny of the Labrinae as a whole because several taxa were lacking from the analysis. However, the inclusion of additional Labrinae provides evidence that *Centrolabrus* and *Symphodus* represent a closely related group of species, very probably monophyletic. The results presented above also indicate that *C. trutta* and *C. caeruleus* form a monophyletic group that also includes several species of the genus *Symphodus*. This conclusion is supported by the genetic, meristic and ethological data. The analysis of the 16S rDNA suggests that *S. roissali* forms a clade with *C. trutta* and *C. caeruleus* while *S. melanocercus* appears in a distinct clade being the sister species of *C. exoletus*. These findings make the genus *Symphodus* paraphyletic since it does not include all the descendants of its common ancestor. At the same time, the present results demonstrate that *Centrolabrus* does not form a monophyletic group.

By the time when the present paper was submitted, HANEL et al. (2002) published a comprehensive molecular phylogeny of the tribe Labrini that included all the species of *Symphodus* and *Centrolabrus*. They used a fragment of the 16S rDNA quite similar to the

one used in this study plus a fragment of the mitochondrial control region. Their results fully support all the conclusions presented above. In their study, the monophyly of the group *Symphodus/Centrolabrus* is clearly established. *S. melanocercus* forms a monophyletic group with *C. exoletus* while *C. trutta* and *C. caeruleus* emerged in another monophyletic group with the remaining nine species of *Symphodus*.

The combined results of these two independent studies cover all the species of the genera *Symphodus* and *Centrolabrus* regarding molecular, meristic and ethological data. In addition, for the majority of the species of the two genera, there are now three different mitochondrial DNA fragments, from the 12S rDNA, 16S rDNA and the control region. All these evidences provide strong support to the following taxonomic proposals:

- *Centrolabrus trutta* (Lowe, 1833) and *Centrolabrus caeruleus* (Azevedo, 1999) should be transferred to the genus *Symphodus* thus being re-designated as *Symphodus trutta* (Lowe, 1833) and *Symphodus caeruleus* (Azevedo, 1999). This recommendation is based on the following arguments: molecular, meristic and ethological data revealed that *S. trutta* and *S. caeruleus* form a monophyletic clade with all the species of *Symphodus* excluding *S. melanocercus*; since *Symphodus* has priority over *Centrolabrus*, the assignment of *S. trutta* and *S. caeruleus* to the genus *Symphodus* will be maintained even if the two genera are merged in the future.
- The molecular, meristic and ethological data unambiguously support the redefinition of the genus *Centrolabrus*, limiting it to the species that form a clearly monophyletic group: *C. exoletus* and *S. melanocercus* which must be designated as *Centrolabrus melanocercus* (Risso, 1810).

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