

Molecular validation of the specific status of *Parablennius sanguinolentus* and *Parablennius parvicornis* (Pisces: Blenniidae)*

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SUMMARY: *Parablennius sanguinolentus* and *P. parvicornis* have been classified as either two distinct species or as two sub-species depending on the different criteria used to classify them. An analysis of fragments of mitochondrial 12S and 16S rDNA showed that the genetic distance between samples of *P. sanguinolentus* and *P. parvicornis* is similar or higher than those found for other blenniids that are widely recognized as distinct species. These results, together with the distinct geographical distributions and meristic differences, support the conclusion that *P. sanguinolentus* and *P. parvicornis* should be considered as two different species.

Keywords: speciation, 12S mitochondrial rDNA, 16S mitochondrial rDNA, glaciations, NE Atlantic, Mediterranean.

RESUMEN: CONFIRMACIÓN CON DATOS MOLECULARES DEL ESTATUS ESPECÍFICO DE *PARABLENNIUS SANGUINOLENTUS* Y *PARABLENNIUS PARVICORNIS* (PISCES: BLENNIIDAE). – *Parablennius sanguinolentus* y *P. parvicornis* han sido clasificados por diferentes autores como especies distintas o bien como subespecies. El análisis de fragmentos de las subunidades 12S y 16S del ADN ribosómico de la mitocondria muestra que la distancia genética entre los dos taxones es comparable o bien superior a las distancias entre especies de blénidos claramente reconocidas como válidas. Este resultado, sumado a las ligeras diferencias merísticas y a distribuciones geográficas bien distintas, soporta el reconocimiento de estos taxa como especies válidas.

Palabras clave: especiación, 12S ADNr, 16S ADNr, glaciaciones, Atlántico Nororiental, Mediterráneo.

INTRODUCTION

There has been considerable controversy over the taxonomic status of the blenniids *Parablennius sanguinolentus* (Pallas, 1811) and *Parablennius parvicornis* (Valenciennes, 1836). Several authors have used different criteria to classify *P. sanguino-*

lentus and *P. parvicornis* as two different species (see Bath, 1977; Bath, 1990; Santos *et al.*, 1997), or as two sub-species of *P. sanguinolentus* (see Arruda, 1979; Zander, 1980; Almeida and Harmelin-Vivien, 1983).

The only notable characters that distinguish these taxa from each other are: an additional dorsal spine in *P. sanguinolentus*, small teeth anteriorly to the canines in the upper jaw in *P. parvicornis*, which

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TABLE 1. – List of Blenniidae species and outgroup taxa included in the phylogenetic analysis, sample localities, number of specimens and corresponding Genbank accession numbers.

	Sampling localities	Genbank accession number			
		12S rDNA	N	16S rDNA	N
<i>P. sanguinolentus</i>	Lebanon	AF414697, AF414698	2	AF428242	1
	Greece	AF414700	1	AY345188	1
	Croatia	AF414699	1	AY345187	1
	Italy	AF414701, AF414702	2	AY345189	1
	Mainland Portugal	AF414703, AF414704, AF414705	3	AF428241, AY098837	2
<i>P. parvicornis</i>	Cape Verde	AF414712, AY345216	2	AY345190, AY345191	2
	Canaries	AY345210, AY345211, AY345212, AY345213, AY345214, AY345215	6	AY345200, AY345201, AY345202, AY345203, 345204, AY345205	6
	Madeira	AF414706, AF414707, AF414708, AY345206, AY345207	5	AYAF428239, AF428240, AY345192, AY345193, AY345194	5
	Azores	AF414709, AF414710, AF414711, AY345208, AY345209	5	AF428238, AY345196, AY345197, AY345198, AY345199	5
	Greece	AF414715	1	AY098835	1
<i>P. ruber</i>	Azores	AF414716	1	AY098834	1
<i>P. incognitus</i>	Azores	AY098788	1	AY098829	1
<i>P. pilicornis</i>	Mainland Portugal	AY098795	1	AY098831	1
<i>T. delaisi</i>	Madeira	AY098812	1	AY098850	1
<i>L. nuchipinnis</i>	Cape Verde	AY098807	1	AY098847	1

are absent in *P. sanguinolentus* (Bauchot, 1966), and slight differences in pigmentation (Zander, 1979).

According to Zander (1986), Bath (1990), Oliveira *et al.* (1992), Gonçalves *et al.* (1993), and Santos *et al.* (1997), the distribution area of *P. sanguinolentus* includes the Mediterranean and the Atlantic coast between France (Gulf of Biscay) and Morocco, although Bath (1990), did not list *P. sanguinolentus* as inhabiting African shorelines. The distribution of *P. parvicornis* includes the West African coast, from Morocco or Mauritania to the Congo River including the NE Atlantic archipelagos of Azores, Madeira and the Canaries and Cape Verde islands.

In this paper we discuss the taxonomic status of *P. sanguinolentus* and *P. parvicornis* using mitochondrial rDNA data.

MATERIALS AND METHODS

The species and outgroup taxa included in the phylogenetic analysis, their sample localities, number of specimens and corresponding Genbank accession numbers are listed in Table 1.

The blenniids *Parablennius gattorugine* (Brünnich, 1768), *Parablennius ruber* (Valenciennes, 1836), *Parablennius pilicornis* (Cuvier, 1829) and *Parablennius incognitus* (Bath, 1968), were also analysed to provide a wider frame of reference to clarify the relationships between the populations that are morphologically classified as *Parablennius sanguinolentus* and *Parablennius parvicornis*. *Tripterygion delaisi* (Cadenat and Blanche,

1971) (Tripterygiidae) and *Labrisomus nuchipinnis* (Quoy and Gaimard, 1824) (Labrisomidae), which represent families that are closely related to the blenniids (Stepien *et al.*, 1997), were used as outgroups.

Samples were collected underwater and in tide pools and fixed in 96% ethanol. Total genomic DNA was extracted either from muscle tissue or from fin-rays using a proteinase K/SDS based extraction buffer and phenol/chloroform with ethanol precipitation (Maniatis *et al.*, 1982).

The following primer sequences were used to amplify two fragments of the mitochondrial DNA: for 12S rDNA, 12SFor 5'-AAC TGG GAT TAG ATA CCC CAC-3' and 12SRev 5'-GGG AGA GTG ACG GGC GGT GTG-3'; for 16S rDNA, 16SFor 5'-AAG CCT CGC CTG TTT ACC AA-3' and 16SRev 5'-CTG AAC TCA GAT CAC GTA GG-3'. These primers were designed in our laboratory and have already been used in previous studies (e.g. Henriques, *et al.*, 2002).

For both fragments, PCR mixtures were prepared with a total volume of 20 µl, with: 1.5 mM MgCl₂, 200 mM of each dNTP, 0.5 mM of 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. The amplification process was performed in a Biorad Gene-Cycler™ as follows: 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. After this sequence, these products were kept at 72°C for 10 minutes. PCR products were purified with a GFX

PCR DNA purification kit (Amersham-Pharmacia), following the manufacturer's recommendations. Automatic sequencing of PCR purified products was performed with a CEQ 2000 XL, Beckman Coulter, with the same primers.

Alignments were made using Clustal X 1.81 (Thompson *et al.*, 1997), with default settings. Character congruence between the two fragments was tested using the incongruence-length difference test (ILD) (Farris *et al.*, 1995), available in PAUP 4.0b10 Win (Swofford, 2002). The null hypothesis of congruence between the two data sets was not rejected ($P=1$). Therefore, we analysed the 12S and 16S rDNA sequences combined in one single fragment (but see Dolphin *et al.*, 2000). Regions where the alignment was ambiguous were removed from the analysis, which resulted in a fragment with 847 bp. The degree of saturation was assessed by plotting transitions and transversions against uncorrected p -distances.

Sequences were analysed with three methods of phylogenetic inference: maximum-parsimony (MP), maximum-likelihood (ML) and minimum evolution (neighbour-joining - NJ) (Saitou and Nei, 1987). The phylogenetic analysis was performed with PAUP 4.0b10 Win (Swofford, 2002). Bootstrapping (Felsenstein, 1985), was used to assess 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 inference methods. MP analysis was conducted with the ACC-TRAN option.

In order to choose the model of molecular evolution that best fitted our data we used the program Modeltest 3.06 (Posada and Crandall, 1998). For the combined 12S-16S rDNA dataset the ML settings selected, according to the results of the Modeltest, corresponded to the general time reversible model (GTR+G) with rate heterogeneity. The distribution of rates at the variable sites was assumed to follow a gamma distribution with a shape parameter equal to 0.2668. NJ was based on the distance estimator derived from the ML settings selected for the combined fragment.

RESULTS AND DISCUSSION

We analysed a total of 367 bp of the mitochondrial 12S rDNA and 480 bp of the mitochondrial 16S

rDNA, which makes a combined sequence of 847 bp. Of these, 267 sites were variable and 137 sites were parsimony informative. The TS/TV ratio was 1.53. The base frequencies were: A=0,2950; C=0,2501; G=0,2289 and T=0,2260. There was no evidence of saturation either for transitions or transversions.

The three methods of phylogenetic inference converged into the same topology represented in Figure 1.

All samples of *P. sanguinolentus* and *P. parvicornis* formed a well supported monophyletic group that was well differentiated from the remaining species of *Parablennius*. Within this group, all the samples of *P. sanguinolentus* formed a well supported clade as did those of *P. parvicornis*. It is interesting to note that all samples of *P. sanguinolentus* from Mainland Portugal to Lebanon are represented by the same haplotype. The genetic distance (p -distance) between *P. sanguinolentus* and *P. parvicornis* was 2.43% (SD = 0.07; Min = 2.40; Max = 2.64). The p -distance in relation to the variation within the *P. parvicornis* clade was 0.17% (SD = 0.14; Min = 0; Max = 0.60). The highest values were found between fish from Cape Verde and some Azorean samples, which are the more peripheral and distant localities within our study area.

The results of this study unambiguously confirm the distinctiveness of *P. sanguinolentus* and *P. parvicornis* which has been suggested by previous morphological studies (e.g. Bauchot, 1966). Furthermore, the genetic divergence between *P. sanguinolentus* and *P. parvicornis* (2.4-2.6%) is markedly higher than the values found for other pairs of blenniids widely recognized as distinct species (e.g. *Parablennius gattorugine* / *Parablennius ruber*: p -distance = 1.6%, present study; *Lipophrys canevai* / *Lipophrys nigriceps*: p -distance = 1.3%, Almanda *et al.*, 2005).

One of the authors (A. Brito) conducted three expeditions along the African coast (in 1986, 1988 and 1998), in order to examine the distribution of *P. sanguinolentus* and *P. parvicornis* by sampling the intertidal fish fauna. *P. sanguinolentus* was not found south of the Casablanca area (34°N), a conclusion supported by Brownell (1978). *P. parvicornis* was never collected north of Cape Blanc (21°N), a finding also supported by Bath and Wirtz (1992). South of this point, on the coast of Senegal, it is a common fish. These results show that *P. sanguinolentus* and *P. parvicornis* are separated by a gap of at least 13° of latitude.

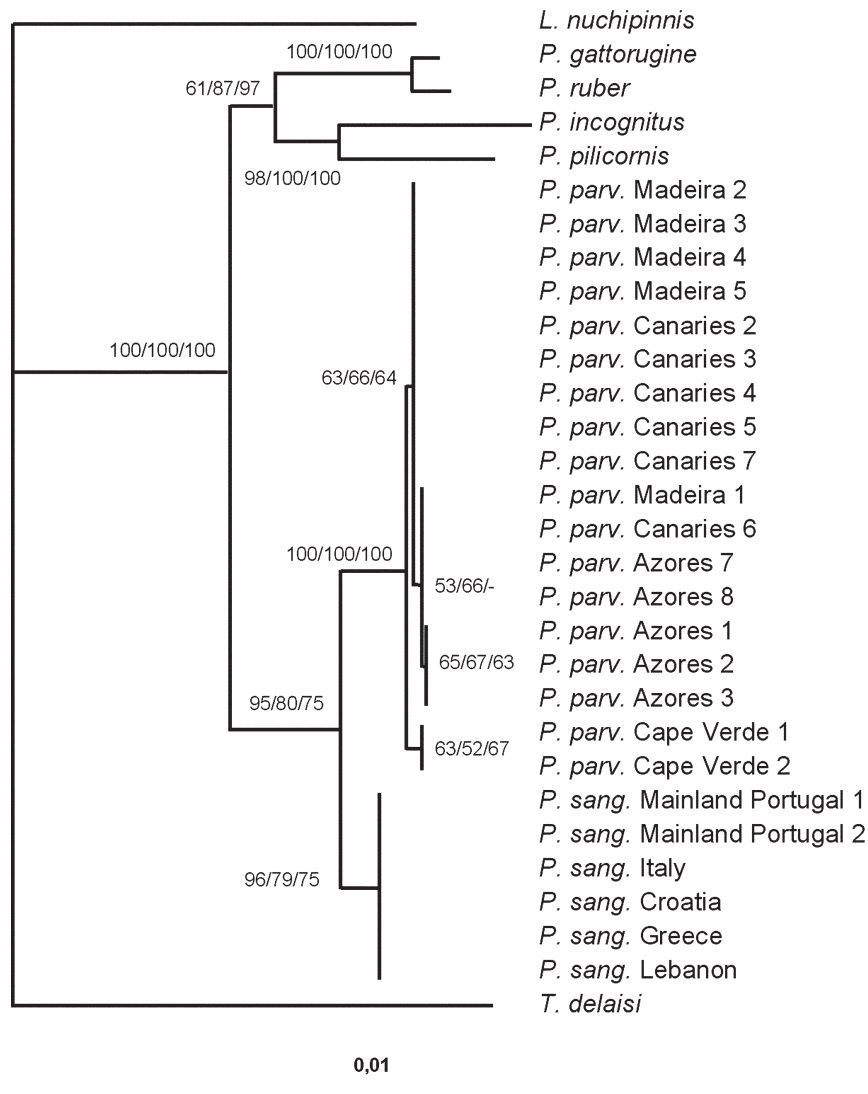


FIG. 1. – Phylogenetic tree obtained for the combined 12S-16S rDNA sequences. *L. nuchipinnis* and *T. delaisi* have been used as outgroups. Bootstrap values for each node are shown as percentages of maximum-parsimony (tree length = 421; consistency index (CI) = 0.82; homoplasy index (HI) = 0.18; retention index (RI) = 0.82), of maximum-likelihood (GTR+G model) and of neighbour-joining (distance based on ML settings) respectively (MP/ML/NJ). The topology of the trees is the same for all inference methods. Branch lengths are proportional to the genetic divergence between haplotypes.

From a biogeographical point of view it is interesting to note that the northern limit of *P. parvicornis* is much more to the south on the West African coast than it is near the Atlantic islands. Indeed, it reaches its northern limit at the Azores well to the north of the entrance to the Mediterranean. This distributional pattern compares well with the results of previous studies (e.g. Santos *et al.*, 1995 and the references therein), that have demonstrated that there are significant affinities between the ichthyofauna of the Azores and those of Madeira and the Canaries. These authors discussed the possible oceanographic processes fish of tropical African origin may use to reach the Azores via the Canaries and Madeira.

Further genetic studies using larger samples for each geographical location and rapidly evolving markers, such as the control region of the mitochondrial DNA, could help to test this biogeographical hypothesis.

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