SCI. MAR., 69 (4): 519-523 SCIENTIA MARINA 2005

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

FREDERICO ALMADA [1, 2], VITOR CARVALHO ALMADA [1], VERA DOMINGUES [1], ALBERTO BRITO [3] and RICARDO SERRÃO SANTOS [4]

[1] Unidade de Eco-Etologia, Instituto Superior de Psicologia Aplicada, R. Jardim do Tabaco, 34, 1149-041 Lisboa, Portugal. E-mail: falmada@netcabo.pt
[2] Instituto de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.
[3] University of La Laguna, Dpto. Biologia Animal (Ciencias Marinas), Calle Astrofísico Francisco Sánchez s/n, 38206, La Laguna, Tenerife, Islas Canarias.
[4] IMAR and Departamento de Oceanografia e Pescas da Universidade dos Açores. 9901-862 Horta, Açores, Portugal.

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. sanguinolentus* 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

*Received January 13, 2004. Accepted September 15, 2004.

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
P. sanguinolentus	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
P. parvicornis	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
P. gattorugine	Greece	AF414715	1	AY098835	1
P. ruber	Azores	AF414716	1	AY098834	1
P. incognitus	Azores	AY098788	1	AY098829	1
P. pilicornis	Mainland Portugal	AY098795	1	AY098831	1
T. delaisi	Madeira	AY098812	1	AY098850	1
L. nuchipinnis	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 ml, with: 1.5 mM $MgCl_2$, 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 94ºC and 30 cycles of 1 minute at 94ºC, 1 minute at 55ºC and 1 minute at 72ºC. After this sequence, these products where kept at 72ºC 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 ACCTRAN 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 (34ºN), a conclusion supported by Brownell (1978). *P. parvicornis* was never collected north of Cape Blanc (21º 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 13º of latitude.

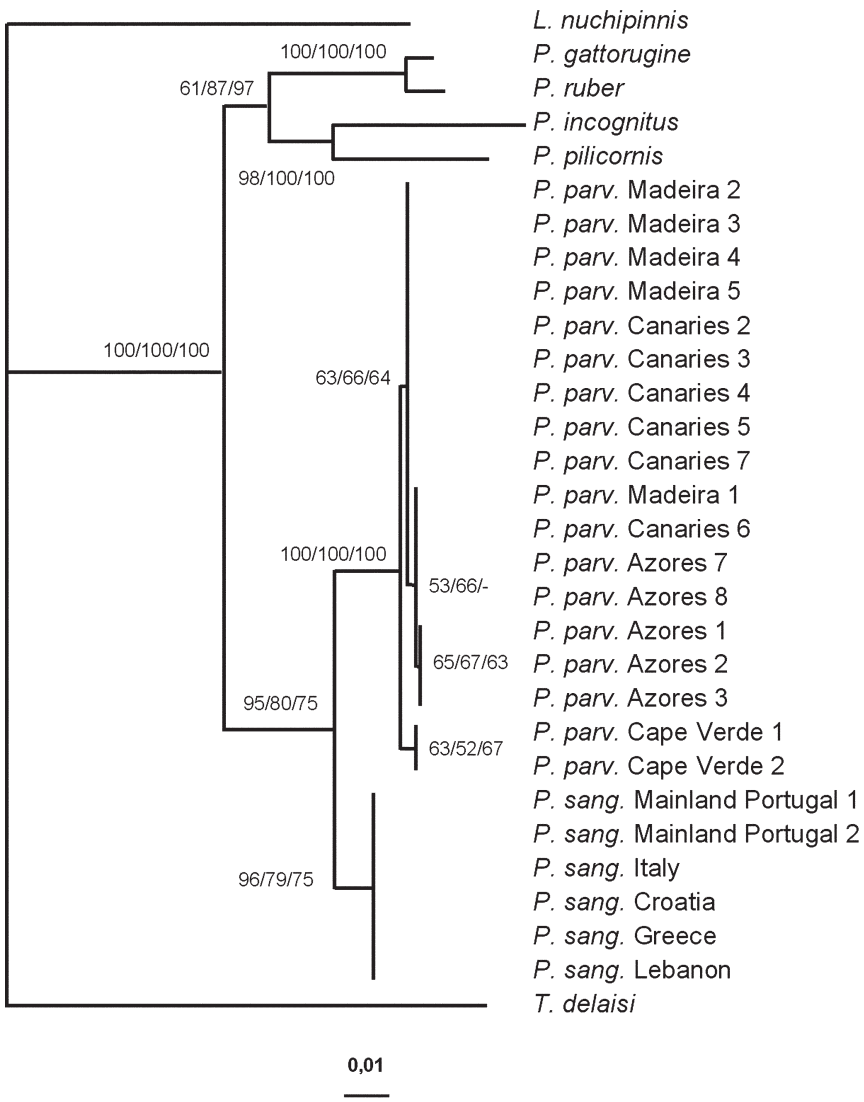

FIG. 1. – Phylogenetic tree obtained for the combined 12S-16S rDNA sequences. *L. nuchipinnis* and *T. delaisi* have been used as outgroups. Bootstrap values for each node are shown as percentages of maximum-parsimony (tree length = 421; consistency index (CI) = 0.82; homoplasy index (HI) = 0.18; retention 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.

ACKNOWLEDGEMENTS

We would like to thank Dr. R. Malhó and his team (FCUL), Dr. M. B. Rasotto, C. Mazzoldi, M. De Girolamo (Chioggia Hydrobiological Station), M. Henriques (PNA/ICN), E. Gonçalves, C. Santos, J. Robalo (ISPA), H. Cabral (FCUL) and S. Stefanni (DOP-UA) for their help. We would also like to

thank Dr. P. Wirtz who provided some samples, Dr. E. Macpherson for his editorial work and two anonymous reviewers for their criticisms and comments. This study was partially supported by the PhD Grant PRAXIS XXI/BD/11178/97 (to F.A.), the FCT Pluriannual Program UI&D 331/94 and the Research Grants PRAXIS/3/3.2/EMG/1957/95 and PNAT/1999/BIA/15017.

REFERENCES

Almada F., V.C. Almada, T. Guillemaud and P. Wirtz. – 2005. Phylogenetic relationships of the North-Eastern Atlantic and Mediterranean blenniids. *Biol. J. Linn. Soc.,* 86(3): 283-295.

Almeida, A.J. and M. Harmelin-Vivien. – 1983. Quelques notes sur des blenniidés observés et capturés aux Açores en 1979 (Pisces: Blenniidae). *Cybium*, 7: 39-45.

Arruda, L.M. – 1979. On the study of a sample of fish captured on the tidal range at Azores. *Bol. Soc. Port. Ciênc. Nat.*, 19: 5-36.

Bath, H. – 1977. Revision der Blenniini (Pisces: Blenniidae). *Senckenberg. Biol.*, 57: 167-234.

Bath, H. – 1990. Blenniidae. In: J. Quéro, J. Hureau, C. Karrer, A. Post and L. Saldanha (eds.), *Check-List of the Fishes of the Eastern Tropical Atlantic* Vol. 2, pp. 905-915. UNESCO, Lisbon.

Bath H. and P. Wirtz. – 1992. On a collection of blenniid fishes from Mauritania, with a redescription of *Spaniblennius riodourensis* (Metzelaar, 1919). *Zool. Meded.*, 66: 265-276.

Bauchot, M.L. – 1966. Poissons marins de L'Est Atlantique Tropical. Téléostéens. Perciforms, V – Blennioidei. *Atlantide Repport*, 9: 63-91.

Brownell, C.L. – 1978. Sur quelques collections de poissons littoraux de l'Atlantique Marocain. *Bull. Inst. Pêches Mar.*, 23: 111-133.

Dolphin, K., R. Belshaw, C.D.L. Orme, D.L.J. Quicke. – 2000. Noise and incongruence: interpreting results of the incongruence length difference test. *Mol. Phylogen. Evol.*, 17: 401-406.

Farris, J.S., M. Källersjö, A.G. Kluge and C. Bult. – 1995. Testing significance of incongruence. *Cladistics*, 10: 315-319.

Felsenstein, J. – 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39: 783-781.

Gonçalves, E.J., V.C. Almada., A.J. Almeida and R.F. Oliveira. – 1993. On the occurrence of *Parablennius sanguinolentus* (Pisces: Blenniidae) on the Portuguese coast. *J. Mar. Biol. Assoc. UK*, 73: 465-467.

Henriques, M., R. Lourenço, F. Almada, G. Calado, D. Gonçalves, T. Guillemaud, M.L. Cancela and V.C. Almada. – 2002. A revision of the status of *Lepadogaster lepadogaster* (Teleostei: Gobiesocidae): sympatric subspecies or a long misunderstood blend of species? *Biol. J. Lin. Soc.*, 76: 327-338.

Maniatis, T., E.F. Fritsch and J. Sambrook. – 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

Oliveira, R.F., V.C. Almada, A.J. Almeida, R.S. Santos and E.J. Gonçalves. – 1992. A checklist of the blennioid fishes (Teleostei, Blenniioidei) occurring in Portuguese waters. *Arquipel. Life Mar. Sci.*, 10: 23-37.

Posada, D. and K. Crandall. – 1998. Modeltest: testing the model of DNA substitution. *Bioinf. Applicat. Note*, 14: 817-818.

Saitou, N. and M. Nei. – 1987. The Neighbor-Joining Method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4: 406-425.

Santos, R.S., S.J. Hawkins, L.R. Monteiro, M. Alves and E.J. Isidro. – 1995. Marine research, resources and conservation in the Azores. *Aquat. Conserv.: Marine and freshwater ecosystems*, 5: 311-354.

Santos, R.S., F.M. Porteiro and J.P. Barreiros. – 1997. Marine fishes of the Azores: Annotated check-list and bibliography. *Arquipel. Life Mar. Sci.*, 1: 1-242.

Stepien C.A., A.K. Dillon, M.J. Brooks, K.L. Chase and A.N. Hubers. – 1997. The Evolution of Blennioid Fishes Based on an Analysis of Mitochondrial 12S rDNA. In: Kocher T., Stepien C., eds. *Molecular Systematics of Fishes*. New York: Academic Press, 245-269.

Swofford, D.L. – 2002. PAUP* Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland, Massachussets.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. – 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 24: 4876-4882.

Zander, C.D. – 1979. Morphologische und ökologische untersuchung der schleimfische *Parablennius sanguinolentus* (Pallas, 1811) und *Parablennius parvicornis* (Valenciennes, 1836) (Perciformes, Blenniidae). *Mitt. Hamb. Zool. Mus. Inst.*, 76: 469-474.

Zander, C.D. – 1980. Zoogeography and speciation of Mediterranean blennioids (Perciformes, Pisces). In *Journées de Études Systematiques et Biogéographiques de Mediterraneen* - C.I.E.S.M., pp. 13-38, *Cagliari*.

Zander, C.D. – 1986. Blenniidae. In: P. Whitehead, M. Bauchot, L. Hureau, J. Nielsen and E. Tortonese (eds.), *Fishes of the North-Eastern Atlantic and the Mediterranean*, Vol. 3, pp. 1096-1112. UNESCO, Paris.

Scient. ed.: E. Macpherson