Journal of Fish Biology (2007) **70** (Supplement B), 248–254
doi:10.1111/j.1095-8649.2007.01397.x, available online at http://www.blackwell-synergy.com

BRIEF COMMUNICATIONS

Molecular data confirm the validity of the Portuguese blenny (*Parablennius ruber*, Valenciennes, 1836) and its presence in Western Europe

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(Received 31 January 2006, Accepted 22 September 2006)

DNA sequence analysis confirms the distinction between *Parablennius ruber* and *Parablennius gattorugine*, simultaneously validating the presence of the former species in Western Europe where it has been reported for >150 years. A possible scenario involving speciation of *P. ruber* at the Azores and subsequent transport of larvae to Europe, a process that may be still occurring nowadays, could explain this pattern of occurrence.

Key words: Northeastern Atlantic; biogeography; Blenniidae; mitochondrial ribosomal DNA; speciation.

The Portuguese blenny (*Parablennius ruber*, Valenciennes, 1836) is a blenniid fish, which constitutes a major element of the rocky subtidal ichthyofauna of the Azores (Santos, 1987; Azevedo & Homem, 2002). Almada *et al.* (2005) showed, based on molecular data, that *P. ruber* is a sister species of *Parablennius gattorugine* (Linnaeus, 1758), with which it forms a very differentiated clade within the genus *Parablennius*. The similarity between the two species is so strong that, although described in the 19 century (Valenciennes, 1836), for many years, the validity of *P. ruber* was questioned and it was often not distinguished from *P. gattorugine*. The species was revalidated independently by Bath (1982) and by Almeida (1982). Both the authors showed that *P. ruber* differs from *P. gattorugine* in a number of morphological traits that include the morphology

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of the lateral line system and the shape of the orbital tentacles (Fig. 1). Surprisingly, although the species was commonly viewed as a fish from the Macaronesian Islands, both the original description of Valenciennes (1836) and the revalidation by Almeida (1982) were based on material collected in the shores of mainland Europe, from the western coast of France to the southern coast of Portugal. This is remarkable because *P. gattorugine* is quite abundant in both the Atlantic and the Mediterranean shores of Europe. The finding of these specimens of *P. ruber* indicates that, at least from the time of Valenciennes (1836), *P. ruber* is sympatric with *P. gattorugine* although surveys of European littoral fishes very rarely report its presence. In recent years, the presence of this species in the shores of western Britain and Ireland became clearly noted (pictures and records available at http://www.habitas.org.uk/marinelife/species.asp?item=ZG6370; B. Picton, pers. comm.). One of these pictures was also examined by P. Wirtz (pers. comm.) who also confirmed the identification.

Two distinct issues are addressed within this note: first, using the mitochondrial 12S and 16S rDNA, Azorean *P. ruber* samples are compared with those of *P. gattorugine* from the Mediterranean and from the Atlantic shores of Europe, in order to test, using molecular data, the consistency of the distinction between the two species as suggested by their morphologies. Additionally, the DNA of fish morphologically classified as *P. ruber* and caught in Northwest Portugal was compared both with material from *P. gattorugine* and with Azorean samples of *P. ruber*. This comparison was made to test the hypothesis that the morphological similarities between continental and insular fish classified as *P. ruber* reflect true genetic affinity.

Samples of *P. ruber* were collected from the Azores (Faial 38°40′ N; 27°10′ W, eight specimens, PRAZ1–PRAZ8) and from the northwest coast of mainland Portugal (Moledo do Minho, Caminha 41°50′ N; 8°50′ W, three specimens, PRMM1–PRMM3). Samples of *P. gattorugine* were collected from mainland Portugal (S. Pedro do Estoril, Cascais 38°41′ N; 09°25′ W, eight specimens, PGSP1–PGSP8), England (Plymouth 50°25′ N; 04°05′ W, one specimen, PGEN1), the Mediterranean from Eastern Italy (Chioggia 45°13′ N; 12°17′ E, two specimens, PGIT1 and PGIT2) and Eastern Greece (Lavrio

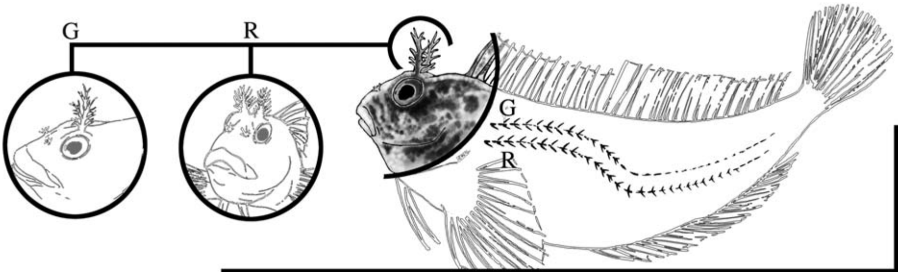

FIG. 1. Diagrammatic representation outlining the more salient differences between *Parablennius gattorugine* (G) and *Parablennius ruber* (R). The differences at the level of the orbital tentacles and the lateral line are especially conspicuous. In *P. ruber* the supraorbital tentacles tend to be bilobed, the main stem is short with many branches arising almost at the same level. In *P. gattorugine* there is a central stem along which thinner branches arise at different levels. The shape of the supraorbital tentacles is, however, quite variable in *P. gattorugine*.

37°42′ N; 24°04′ E, one specimen, PGGR1). Sequences are available at GenBank under accession numbers AY098778, AY098779, AY098834, AY098835 and DQ160198–DQ160205. The voucher specimens are deposited in the fish collection of the Eco-Ethology Research Unit at the Instituto Superior de Psicologia Aplicada, Lisbon. *Parablennius pilicornis* (Cuvier, 1829) and *Parablennius sanguinolentus* (Pallas 1814) were used as out-groups (GenBank accession numbers AY098796, AY098831, AF414700 and AY345187). Fin clips were cut immediately after collection of the individuals and stored at ambient temperature in 96% ethanol. Total genomic DNA was extracted from fin rays using a proteinase K/sodium dodecyl sulphate (SDS) based extraction buffer following the Sambrook *et al.* (1989) protocol.

Fragments of the 12S and 16S rDNA were amplified using primers described in Henriques *et al.* (2002), and polymerase chain reaction and sequencing conditions described in Almada *et al.* (2005). Sequence alignments were made using ClustalX 1.81 (Thompson *et al.*, 1997) with default settings. Character congruence between the two fragments was tested using the incongruence-length difference test (Farris *et al.*, 1995) available in PAUP (version 4.0; Swofford, 1998). The null hypothesis of congruence between the two data sets was not rejected ($P = 1$), which led us to analyse the 12S and 16S rDNA sequences combined in one single fragment.

Phylogenetic relationships were assessed using maximum parsimony (MP) and neighbour-joining (NJ) methods, implemented by the software package PAUP. Bootstrapping (Felsenstein, 1985) was used to assess robustness of the nodes in the trees with 1000 replicates. As the authors were dealing with very closely related species with very small genetic distances, patristic distances were adopted (following Nei & Kumar, 2000). An analysis of molecular variance (AMOVA; Excoffier *et al.*, 1997) was performed using ARLEQUIN 2.0 (Schneider *et al.*, 2000), to test the genetic differentiation between the samples of *P. ruber* and *P. gattorugine*.

A total of 367 bp of the mitochondrial 12S rDNA and 480 bp of the mitochondrial 16S rDNA were analysed, resulting in a combined sequence of 847 bp. In Fig. 2, the results of the phylogenetic analysis are summarized. A single phylogenetic MP tree of 139 steps was recovered and had the same topology as the NJ tree. *Parablennius ruber* and *P. gattorugine* form a monophyletic clade clearly separated from the other species of the genus *Parablennius* used as out-groups. All samples of *P. gattorugine* form a well-supported clade, which is sister to another equally well-supported clade that contains all *P. ruber* samples. The samples from mainland Portugal, group unambiguously with the Azorean samples of *P. ruber*. The mean interspecific uncorrected *p* distances are 1·67% (S.D. = 0·12%) and 1·71% (S.D. = 0·14%) for 12S and 16S, respectively. The intraspecific distances are 0·91% (S.D. = 0·17%) and 0·11% (S.D. = 0·17%) for *P. ruber* (12S and 16S, respectively), and 0% (S.D. = 0%) and 0·096% (S.D. = 0·11%) for *P. gattorugine* (12S and 16S, respectively).

The AMOVA analysis showed that 84·99% of the total genetic variation was due to interspecific differences, while the intraspecific variation accounted for 15·01%. The fixation index was highly significant ($P = 0$) for 1023 permutations.

The results presented in this study provide molecular support for the distinction between *P. ruber* and *P. gattorugine*. They also demonstrate that *P. ruber* is present both in the Azores and in the northwestern shore of mainland Portugal. This conclusion could be criticized due to the fact that the molecular markers

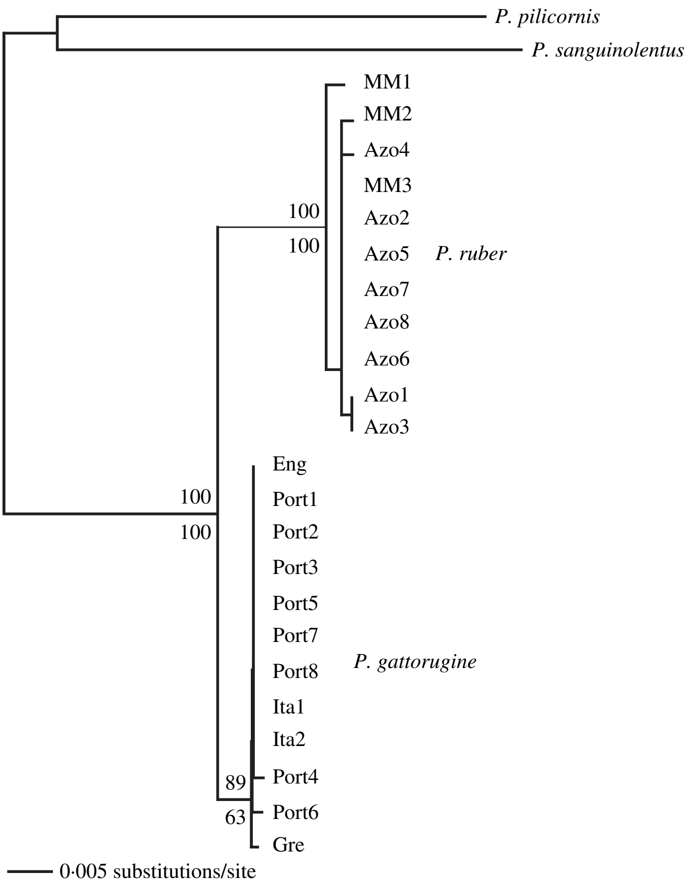

FIG. 2. Phylogenetic relationship of *Parablennius ruber* and *Parablennius gattorugine* using *Parablennius pilicornis* and *Parablennius sanguinolentus* as out-groups. A neighbour-joining tree is shown with neighbour-joining (above the nodes) and maximum parsimony (below the nodes) bootstrap support at the major nodes. Labels are: MM (Moledo do Minho, Northern Portugal), Azo (Azores), Port (Southern Portugal), Eng (Plymouth, England), Ita (Italy), Gre (Greece). The length of each branch is proportional to the number of nucleotide substitutions.

used are maternally inherited, and the presence of hybrids could not be excluded. This possibility seems unlikely as all the specimens, from both mainland Europe and Azores, all genetically classified as *P. ruber*, were unequivocally assigned to the same species based on all available morphological diagnostic criteria.

In recent years, many warm water organisms have been recorded in European waters for the first time, while the distribution of others have shifted, tracking changes in sea surface temperatures (Southward *et al.*, 1995; Kröncke *et al.*, 1998; O'Brien *et al.*, 2000). In the case of *P. ruber*, it is interesting that the original description by Valenciennes (1836) was based on specimens from the western coast of France, which means that this species already occurred in Europe more than one and a half century ago. The material included by Almeida (1982), in his revalidation of the species, was from northwest Portugal and from Algarve, in the south of the country. Bath (1982) used material from Azores and from

Madeira, implying that the species was also found at these archipelagos. Recent surveys (N. Monteiro, unpubl. data), on the shore of Madeira, could not detect *P. ruber*, suggesting that the species may now be uncommon at this archipelago.

Concerning the presence of *P. ruber* in the Atlantic shores of Western Europe, the issue that requires clarification is the apparent contradiction between the relatively old presence of this species in Europe and its rarity or absence in most surveys of fishes of the European rocky shores. One has to admit the possibility that the species was not recorded because of insufficient sampling or inadequate identification. This seems, however, unlikely for areas like the British Isles where thorough surveys of the inshore ichthyofauna have been conducted for many years. Thus, the possibility that *P. ruber* is regularly present on the west European shores, although at low numbers should not be ruled out. If it occurs in very small numbers, it is possible that *P. ruber* may not be able to establish viable populations in Europe. The authors suggest that these populations may be, to a considerable extent, replenished by occasional larval transport from the Azores. The idea that blenniid larvae are able to undergo long distance transport was already proposed by Muss *et al.* (2001) to explain the distribution of *Ophioblennius atlanticus* (Valenciennes, 1836) across the Atlantic Ocean. Rafting on algae is another form of long distance transport that was demonstrated for blenniids. Specimens of *Hypleurochilus fissicornis* (Quoy & Gaimard, 1824), a species living in the southwestern Atlantic, were collected from a raft, off Azores (Santos *et al.*, 1997), a transport that involves a much greater distance that the one between Azores and Western Europe.

Azevedo & Homem (2002) showed that, at the Azores, *P. ruber* breeds in winter, the time when winds blowing from southwest are stronger, maximizing the likelihood of larval transport from the Azores to Western Europe. Thus, *P. ruber* may be a species conforming to the model of blenniid speciation proposed by Zander (1973, 1980). According to this model, blenniids transported from the European shores, could sometimes reach the Atlantic Islands where they could survive the glacial periods undergoing some degree of independent evolution to become incipient new species. These new species would then be transported back to Europe by the predominant currents and, if reproductive isolation was sufficiently strong, could become a new component of the European ichthyofauna, when temperatures become more favourable. The idea that the Macaronesian Islands like the Madeira and the Azores were colonized from eastern Atlantic sources is supported by the fact that from the eight blenniid species found at the Azores, six were 'Lusitanian'. They are shared with the warm temperate eastern Atlantic shores of Europe and Africa extending in most cases into the Mediterranean. One species (*O. atlanticus*) is shared with the tropical Africa and the tropical western Atlantic, and another (*Parablennius parvicornis*, Valenciennes, 1836) occurs in the Macaronesia and in the tropical Africa. The situation of the blenniid fauna of Madeira is very similar and many Azorean fish probably derived from a Madeiran source (Santos *et al.*, 1995). Thus, at least in the past, it is certain that blenniids dispersed from the eastern Atlantic shores to the Macaronesian Islands (Almada *et al.*, 2001). Such a scenario may well apply to *P. ruber*. It very likely derived from a *P. gattorugine*-like ancestor that colonized the Azores. It may have evolved there, surviving for one or more glacial periods. During glacial peeks, the Atlantic shores of

Europe became so cold, that most blenniid species were probably extirpated. Indeed, Dias *et al.* (1997) showed that at the last glacial maximum, the sea surface temperatures along the western coast of Portugal were many degrees lower than today and the polar front was located south of Lisbon. Neither *P. gattorugine* nor *P. ruber* are found in these subpolar conditions. On the contrary, in the Azores, the drop in sea surface temperature was only of *c.* 3° C, corresponding to a temperature similar to that found nowadays in Western European shores (Crowley, 1981). If the scenario outlined above applies to *P. ruber*, it may well be that the increase in occurrences reported by B. Picton (pers. comm.) for the British Isles may represent the effects of increasing water temperatures, which would favour the survival and reproduction of a species that, coming from the Azores, probably requires warmer waters than *P. gattorugine* to breed successfully. It may also represent increased transport promoted by circulation coming from the southwest. The apparent decrease, or even disappearance, of the species in lower latitudes, as in Madeira, may be related to increasing temperatures to levels above the upper range for the species. The same seems to be occurring with *Lipophrys pholis* (Linnaeus, 1758). In Madeira, and also in the Azores and in the Canaries, new additions to the marine fauna originating from southern latitudes are noticeable (Wirtz, 1998; Azevedo *et al.*, 2004).

More intensive surveys of the western European fish assemblages combined with more intensive genetic sampling are required to test this hypothesis. It is particularly necessary to clarify to what extent *P. ruber* is able to breed in European waters and the extent of its isolation from the much more abundant *P. gattorugine*.

This study was funded by Plurianual Program (Fundação para a Ciência e Tecnologia, FCT), UI&Ds 331 and 531, partially FEDER funded, and PNAT 1999/BIA/15017. V.S.D. has a PhD grant SFRH/BD/13069/2003, N.M.M. has a post-doc grant SFRH/BPD/14992/2004 and F.A. has a PhD grant PRAXIS XXI/BD/11178/97 from the Portuguese Foundation for Science and Technology. We would like to thank B. Picton and P. Wirtz for their personal communications.

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