Phylogenetic analysis of Peri-Mediterranean blennies of the genus *Salaria*: Molecular insights on the colonization of freshwaters

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A R T I C L E   I N F O

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A B S T R A C T

In this paper, the phylogenetic relationships of the marine blenny *Salaria pavo* and the freshwater *S. fluviatilis* and *S. economidisi* were analyzed using four molecular markers: the mitochondrial 12S rRNA, 16S rRNA, and the control region and the nuclear first intron of the S7 ribosomal protein. The monophyly of *Salaria* is supported, as well as that of *S. pavo* and that of all the freshwater members of *Salaria*. Thus, the present results support a single origin for all freshwater Mediterranean blenniids. Our results reject the placement of the species of *Salaria* in the genus *Lipophrys* as proposed in previous studies. Using a molecular clock calibrated with trans-Isthmian geminate blenniid species, the split between the ancestor of the freshwater lineage and the ancestor of *S. pavo* is tentatively placed in the Middle Miocene (well before the Messinian). The marine *S. pavo* displays a very low level of intraspecific sequence divergence consistent with a Pleistocene bottleneck. *S. fluviatilis* is a paraphyletic entity with *S. economidisi* nested within it. A Moroccan population of *S. fluviatilis* is more divergent than *S. economidisi*, both in nuclear and mitochondrial genes. Fish from Israel together with some Turkish samples represent the second oldest split. It is argued that these populations may represent cryptic species. Thus, further studies on the taxonomy of these freshwater blennies are urgently needed.

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1. Introduction

The teleost fish family Blenniidae includes about 387 species which are typically small marine benthic fishes inhabiting hard inshore substrata of tropical and temperate waters (Nelson, 2006). Among these three or four species occur in freshwater. In the Mediterranean region, *Salaria fluviatilis* (Asso, 1801) lives in rivers and lakes from Israel to the Iberian Peninsula, where it reaches some Atlantic rivers like the Guadiana. It is also present in Morocco, Algeria and many Mediterranean islands as Sardinia and Crete (e.g., Kottelat and Freyhof, 2007). Recently, Kottelat (2004) considered the population from lake Trichonis, in Greece, sufficiently distinct to be assigned to a new species *Salaria economidisi* (Kottelat, 2004). This finding raised the possibility that the widely distributed *S. fluviatilis* may be paraphyletic and that other cryptic species might be present within it.

Freshwater blennies of the genus *Salaria* have long been considered closely related to a marine inshore species, *Salaria pavo* (Risso, 1810) which also occurs around the Mediterranean, with a small extension in the adjacent Atlantic, from the bay of Biscay south to the Canaries (Zander, 1986). *Salaria pavo* occurs both on rocky habitats and coastal lagoons, in the intertidal, or in the first meters of the subtidal. Another species of the genus, *Salaria basilsca* (Valenciennes, 1836), is rarely found and was described as living on sea grass beds, also within the Mediterranean (Heymer, 1985).

The taxonomic history of the *Salaria* species has been complex. They were merged for a long time in the genus *Blennius* (Norman, 1943), which included all north eastern Atlantic blenniids except for *Coryphoblennius galerita* (Linnaeus, 1758). This heterogeneous assemblage which encompassed more than 20 species was subsequently split, giving rise to several genera. Bath (1976), Almada et al. (2001), and Almada et al. (2005) argued for the validity of *Salaria Forskål, 1775* (including *S. fluviatilis*, *S. pavo* and *S. basilsca* (Valenciennes, 1836)) although the last authors, using mitochondrial markers, could not confirm unequivocally the monophyly of the genus. Bock and Zander (1986) argued for the inclusion of *Salaria* in the genus *Lipophrys* Gill, 1896, a group of species morphologically very distinct and which currently comprises only *L. pholis* (Linnaeus, 1758) and *L. trigloides* (Valenciennes, 1836). For a review of the phylogeny of the north eastern Atlantic blenniids and its taxonomic implications see Almada et al. (2005). For the blenniids as a whole see (Almada et al., 2009).
The morphological similarities between the Mediterranean freshwater blennies and *S. pavo*, and the observation that both *S. pavo* and *S. fluviatilis* can tolerate wide ranges of salinity (Plaút, 1998) led to a general acceptance that the freshwater members of *Salaria*, must have derived from *S. pavo* (Kooswijk, 1967) or at least from a marine ancestor closely related to *S. pavo* (Zander, 1972; Perdices et al., 2000).

Perdices et al. (2000), using protein electrophoresis, found that there were greater differences between lake populations of *S. fluviatilis* from Spain and Greece than those found between riverine populations from both countries. Thus, they raised the hypothesis that a marine ancestor could have given rise independently and at different times to several populations of *S. fluviatilis*. This possibility is also suggested by the almost parallel geographic distributions of *S. pavo* and *S. fluviatilis*.

In this paper, we used both mitochondrial and nuclear DNA from *S. pavo* samples collected from the Levantine Mediterranean to the Atlantic shores of Portugal, and samples of the freshwater *Salaria* ranging from Turkey to Portugal and Morocco, to address the following issues: (1) Is the inclusion of the species of *Salaria* in the genus *Lipophrys* supported by genetic evidence? (2) Does *Salaria* form a monophyletic group? (3) Are the freshwater blennies of the Mediterranean region a monophyletic group or do they represent independent instances of freshwater invasion by marine ancestors related to *S. pavo*? (4) Do the colonisations of freshwaterers correspond to a narrow time interval or did they take place at different periods? (5) Is *S. fluviatilis* a sister species of the Greek *S. economidi* or is it a paraphyletic entity requiring taxonomic revision?

2. Material and methods

2.1. Sampling

Samples of *S. pavo* were obtained from 34 individuals from 16 locations along the northern and eastern Mediterranean coast, including Mediterranean Islands, and along the Atlantic coast of the Iberian Peninsula. Thirty *S. fluviatilis* were sampled from 21 rivers or lakes in Israel, Turkey, Greece, Croatia, Morocco, and, with greater incidence, in the Iberian Peninsula. For collection site locations and GenBank Accession Numbers see Fig. 1 and Table 1. Except in the few cases when not enough biological material was available, all gene fragments were sequenced for each individual. In order to investigate the relationship between *Salaria* and *Lipophrys*, the following species were also included in the analyses: *Coryphoblennius galerita* (Limnaeus, 1758); *Lipophrys pholis* (Linnaeus, 1758; type species of the genus *Lipophrys*), *Parablennius ruber* (Valenciennes, 1836), *P. salensis* Bath, 1990, and *P. sanguinolentus* (Pallas, 1814). *Coryphoblennius galerita* was shown in previous studies to belong to a well supported clade with the *Lipophrys* species (Almada et al., 2005) while the remaining taxa added tended to be closer to the species of *Salaria* (Almada et al., 2009). *Salarias fasciatus* (Bloch, 1786) was used as outgroup in all analysis because its Indo-Pacific distribution likely implies that it diverged from the remaining species, all with Atlantic-Mediterranean distributions, prior to the closure of the Tethys Sea (around 20 million years ago, Briggs, 1995).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from fin or muscle samples preserved in 96% ethanol with the REDExtract-N-Amp kit (Sigma-Aldrich) following the manufacturers instructions. Voucher specimens are deposited in ISPA and MNCN collections (ethanol preserved tissues). PCR amplification of mitochondrial fragments and the first intron of the nuclear S7 ribosomal protein gene, were performed with the following pairs of primers: control region – L-Pro1 and H-DL1 (Ostelari et al., 1996); 12s rDNA – 12S For and 12SRev (Almada et al., 2005); 16s tDNA – 16SFor and 16SRev (Almada et al., 2005); and the first intron of the S7 ribosomal protein gene — S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998). Further details on primers and PCR conditions are summarized in Supplementary material. For all genes, each sample was sequenced in both directions using the same PCR primers. Sequencing reactions were performed by Stabvida (Oeiras, Portugal; http://www.stabvida.com).

2.3. DNA analysis

All sequences were aligned using Clustal X (Thompson et al., 1997) and the alignment was deposited in TreeBase (http://www.treebase.org, submission ID number: SN4366). Congruence among all data sets was tested by the incongruence-length difference test (ILD) (Farris et al., 1985), as implemented in Paup* v.4.0b10 (Swoford, 2003). ILD tests did not reveal significant heterogeneity neither among the mitochondrial markers (*p* = 0.85) nor between the combined mitochondrial data and the S7 intron (*p* = 0.57). Thus, all markers were combined and analyzed as a single data set. In addition, we also analyzed data sets including only the S7 nuclear region, a combined mitochondrial data set, and each mitochondrial fragment separately.

Maximum parsimony-based (MP) phylogenetic relationships were estimated using PAUP* with 100 heuristic searches using random additions of sequences and implementing the TBR algorithm. Support values for individual nodes was assessed using 1000 bootstrap resamplings (Felsenstein, 1985). We conducted phylogenetic analysis on each fragment separately, on a concatenated mitochondrial...
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Table 1

Collection sites for the specimens used in this paper.

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V.C. Almada et al. / Molecular Phylogenetics and Evolution 52 (2009) 424–431
The mitochondrial data set consisted of a total of 1512 characters (433, 604 and 475 bp for 12S, 16S and control region, respectively), including 445 parsimony informative sites and 857 invariant sites, whereas the S7 nuclear region consisted of 743 characters, including 194 parsimony informative sites and 355 invariant sites. No indication of saturation was found when plotting transitions and transversions versus uncorrected $p$ distances (data not shown). The results of the phylogenetic analyses for the entire data set are presented in Fig. 2. The results for the mitochondrial and nuclear data sets are presented as Supplementary material. For the entire data set, 1260 equally parsimonious trees of 2032 steps were obtained. Regardless of data set, no inference method yielded trees in which the Salaria species were recovered with L. pholis and C. galerita. These two species were consistently recovered in the same clade. When the consensus MP tree was compared with the one in which L. pholis, C. galerita and all members of *Salaria* were constrained to form a monophyletic group the difference was not significant (Templeton test: $p = 0.064$; K–H test: $p = 0.066$). However, the constrained ME tree had a significantly lower likelihood than the unconstrained one (S–H: $p < 0.0001$; K–H: $p < 0.0001$). On the contrary, analysis of the full data set for MP and BY analysis (see Fig. 2), MP analysis of the mitochondrial data set and ME and BY analysis of the S7 region (see Supplementary material), indicated a closer relationship between *Salaria* and *Parablennius*.

The ME and BY analysis of the full data set supported the monophyly of the genus *Salaria* (see Fig. 2). Although MP failed to recover this monophyly (Fig. 2), there was no significant difference between the constrained tree and one in which the monophyly of *Salaria* was enforced (Templeton test: $p = 0.80$; K–H test: $p = 0.80$). MP, ME and BY, when applied to the S7 region (see Supplementary material) also supported the monophyly of *Salaria*. All phylogenetic analyses, regardless of inference method, strongly supported the monophyly of both *S. pavo* and that of the freshwater blennies. This result was recovered with all inference methods using each genetic marker separately, combining all mitochondrial markers, and combining mitochondrial and nuclear markers. The freshwater blenny clade revealed greater substructure than *S. pavo*. All genetic markers and combinations of markers identified a well supported basal Moroccan clade that differed greatly from the remaining freshwater *Salaria* (average net genetic distance of concatenated mitochondrial markers between this clade and the remaining freshwater *Salaria*: 0.054; the corresponding value for S7 was 0.023). The next most basal clade, which also appeared recurrently in all analyses with significant support, comprised sequences from Israel and some from Turkey. Sister to this clade was a heterogeneous group of fish widespread across the Mediterranean. Within this group, *S. economidisi* (Lake Trichonis) emerged as sister to a clade comprising the samples from the Iberian Peninsula, Croatia, Greece and a few Turkish samples.
Overall, we found little geographic structure among samples of S. pavo. The presence of fish from Israel and Spain in the same clade (Fig. 2) indicates either the persistence of an ancient polymorphism, or a high level of gene flow throughout the Mediterranean.

The genetic distance among sequences was lower within S. pavo than within freshwater Salaria (average genetic distance of concatenated mitochondrial markers: 0.016 (range 0.00–0.038) and 0.068 (range 0.00–0.630), respectively. The corresponding figures for S7 were 0.002 (0.00–0.010) and 0.005 (0.00–0.029), respectively. The distance between S. pavo and the freshwater clade was an order of magnitude greater than the distances within each of these clades (average net distance were 0.332 for the concatenated mitochondrial markers and 0.120 for S7, respectively). The estimated times of divergence for the major splits in our tree are presented in Fig. 2 and summarized in Table 2. These times were obtained using only the 12S and 16S fragments because the strict molecular clock hypothesis was rejected for the control region and S7 intron. A striking feature of these results is the very sharp discrepancy between the time estimated for the divergence of S. pavo and the freshwater Salaria, and the average divergence within S. pavo. The intra-specific divergence in S. pavo, particularly in the 12S fragment, is so small that almost a single haplotype was found across its distribution, which suggests that a major bottleneck may have occurred in the recent history of this species. This low divergence means that we may be overestimating the net divergence time in comparisons which involve S. pavo and the freshwater blennies.

4. Discussion

Our results clearly reject the inclusion of the species of Salaria in the genus Lipophrys, as proposed by Bock and Zander (1986) and Zander (1986). This finding had already been suggested based on mitochondrial data only, by Almada et al. (2005). The latter authors suggested that the species of Salaria were more closely related to species of Parablennius and Scartella than to Lipophrys. Although Scartella was not included in the present study, the affinity between the species of Salaria and Parablennius was supported, although further studies including more taxa are needed to con-
Table 2

| Divergence times (millions of years ago; MYA) based on average net genetic distances among groups, based on TVM + I + G (12S) and GTR + G (16S) models, as selected by ModelTest using the AIC criterion; and Bayesian estimates (along with the 95% highest posterior density interval), assuming a strict molecular clock of 1.289% (12S) and 1.585% sequence divergence/MY (16S). Letters refer to nodes in Fig. 2. |
|---|---|
| b. mrca of the freshwater clade | 3.96 | 3.86 | 3.23 [2.17–4.32] | 2.80 [1.93–3.68] |
| c. mrca of the Israeli-Turkish clade | 1.26 | 1.04 | 1.86 [1.19–2.57] | 1.47 [0.93–2.09] |
| d. mrca of S. basilisca | 1.45 | 0.92 | 1.86 [1.19–2.37] | 0.94 [0.55–1.33] |
| e. mrca of S. pavo | 0.01 | 0.52 | 0.83 [0.53–1.39] | 1.14 [0.70–1.63] |

Our results point to the monophyly of Salaria. Almada et al. (2005), in a phylogenetic study of the north eastern Atlantic and Mediterranean blenniids, were unable to recover Salaria as a clade. Their results were, however, based on fragments of the mitochondrial ribosomal genes only. In the present study, the inclusion of the control region and S7 allowed the recovery of the monophyly of the genus for the entire data set, as well as for the control region and S7 when analyzed separately. Unfortunately, the authors were unable to get sequences of S. basilisca. Despite of intensive efforts and contacts with museums and ichthyologists working in the Mediterranean, no tissues samples adequate for genetic analysis could be found and apparently the species has not been collected in recent years. It is known to be a close relative of S. pavo, that inhabits eelgrass beds and which hybridizes naturally with S. pavo (Heymer, 1985). Because the type species of Salaria is S. basilisca, its absence from the present analysis precludes drawing definitive conclusions on the phylogeny of Salaria. However, the present paper clearly demonstrates that all the freshwater blenniids of the Mediterranean region and the marine S. pavo derived from a common ancestor.

A clear result of the present study is the strong evidence supporting the monophyly of S. pavo on one hand, and that of the freshwater forms of Salaria, on the other. All markers supported this conclusion. Furthermore, the distance between the two clades is much larger than the distances within each group, suggesting that the split between S. pavo and the freshwater lineage occurred well before the diversification of the present day populations. Thus, the hypothesis suggested by Kosswig (1967) that S. fluviatilis could be a polypetty entity formed by multiple invasions of freshwater by S. pavo is not supported. The hypothesis of Perdices et al. (2000) differs from that of Kosswig in that the presumed ancestor of the freshwater blennies was not S. pavo, but a closely related marine species that would have become extinct. In any event, it is not parsimonious to assume that the ancestor of all freshwater blennies kept its saltwater preferences after splitting from S. pavo, colonizing multiple freshwater systems and becoming extinct in the sea. Thus, although the available evidence is not conclusive, we consider that a single evolutionary step from a marine form to one capable of living and breeding in brackish and freshwater is more parsimonious. This situation contrasts with that found in sticklebacks Gasterosteus aculeatus Linnaeus, 1758 complex, where freshwater populations resulted from multiple invasions from the sea (Bell and Foster, 1994). In sticklebacks, however, many individuals display active migratory behavior and move between the sea and freshwater habitats, a feature that likely facilitated the establishment of permanent freshwater populations. Blenniids, on the contrary, are benthic sedentary fish that after settlement display restricted movements and tend to stay near stones, boulders and other shelters.

The within-clade distances were much higher in the freshwater blennies than in S. pavo. This finding, although surprising at first, is to be expected, considering that the freshwater populations became isolated, increasing the likelihood that drift led different alleles to fixation in different populations, helping to preserve an overall higher level of genetic diversity (Avise, 2000). In any case, as it was already stated, the intra-specific divergence values in S. pavo are extremely low, raising the possibility of a very severe bottleneck. Salaria pavo is a fish with high thermal preferences and laboratory studies showed that development of the embryos is arrested at temperatures of 15 °C or lower (Westermar, 1983). Hayes et al. (2005) estimated that western Mediterranean sea surface temperatures in the last glacial maximum ranged from about 9 °C in winter to 13 °C in summer. These values mean that temperatures suitable for the reproduction of S. pavo were likely absent, in much of the Mediterranean. As the paleotemperatures of the north eastern Atlantic along the Iberian coast were even more severe during glacial periods (Dias et al., 1997; Loncaric et al., 1998) it is very likely that this species must have been extirpated from the Atlantic, surviving only in the warmer parts of the Mediterranean. Moreover, S. pavo occurs in a spectrum of habitats that are more restricted than those used by other Mediterranean blenniids. It requires reduced wave action and is often found in places with freshwater runoff (Zander, 1972). These restrictive habitat preferences may have aggravated the situation of the species during cold periods, when compared to other blenniids, as many of the rocky habitats available in the warmer refugia would not be suitable for S. pavo.

It could be argued that the reduced variation of S. pavo across its distribution may have resulted from high gene flow, instead of a bottleneck. High gene flow would explain the lack of a geographic pattern of variation but it would be more difficult to explain the paucity of haplotypes found in the species as a whole. Without a bottleneck, it is hard to conceive a population spread across the entire Mediterranean and adjacent Atlantic with such a small number of distinct haplotypes. Studies on the phylogeography and historical demography of S. pavo are needed to test this hypothesis.

The very low divergence observed in S. pavo implies that any attempt to apply a molecular clock to the present data must be subjected to a substantial level of uncertainty. Another caveat refers to the use of the timing of the formation of the Isthmus of Panama to calibrate the molecular clock. Indeed, rocky habitats and fully marine conditions may have ceased to exist considerably earlier than the final completion of the isthmus, which means that we may be underestimating the actual divergence times. Moreover, there is no publish phylogephy of the genus Hypsoblennius which implies that we can not be sure that H. inermis and H. brevipinna are true sister species. This possibility also means that the divergence between the two Hypsoblennius species may be much older than
The most recent rising of the isthmus. Unfortunately, there are no other species pairs of blenniids distributed on both sides of the isthmus to undertake a more accurate calibration. Muss et al. (2001) compared the cytochrome b of species of Ophioblennius, a genus that occurs in the tropical Atlantic and the eastern tropical Pacific. They found that even the divergence among different Atlantic populations started well before the separation of the two oceans. In view of this, these authors adopted the calibration of Bermingham et al. (1997), which is not specific for blenniids and was only based on comparisons of the cytochrome oxidase gene. Judging from the figures in Table 2, it seems likely that the cladogenic event that separated the ancestors of S. pavo from those of the freshwater blennies took place at least in the Middle Miocene or earlier, well before the Messinian salinity crisis.

Our data suggest that the split of the Moroccan lineage from the remaining freshwater Salaria may have occurred in the Pliocene. However, the upper limit of the credibility interval estimated by BEAST for this split was 4.32 MYA. As we know that our calibration provides only a lower limit, it is not unrealistic to assume that this event may have occurred 5–5.5 MYA during the Messinian Salinity Crisis (e.g. Briggs, 1995). The Messinian provided a narrow time window during which the Mediterranean was partly desiccated and partly occupied by small water bodies. Towards the final stages of the Messinian salinity crisis much of the Mediterranean basin contained water of reduced salinity (the Lago Mare phase, Briggs, 1995). Thus, the Messinian may have provided the roots through which the freshwater Salaria reached both European and African waters and the Middle East. The samples from Israel and some from Turkey form the second clade to emerge after the splitting of the Moroccan samples. Kosswig (1967) stated that the fish from the Jordan valley are isolated at least since the Pliocene. If this observation is confirmed, it means that our molecular clock is not overestimating the more basal divergences in Salaria. It would also mean that the Moroccan fish may have diverged much earlier, most likely in the Late Miocene, as suggested above.

From a biogeographic perspective it is interesting to note that in Turkey, apart from this old lineage, there are other fish that belong to a very distinct clade which also includes fish from Croatia, Greece and Spain. This observation emphasizes the very interesting position of Turkey and the Middle East, as a whole, as an area of major crossroads of distinct biogeographic influences (Durand et al., 2002; Hrbek and Meyer, 2003).

The present data show a very low divergence between populations from places as far apart as Spain, Croatia, Greece and some fish from Turkey (Fig. 2 and Table 2). Indeed, the divergence times among these samples place their separation well within the Pleistocene. Although the present paper did not support the hypothesis of Perdices et al. (2000) for the entire radiation of the freshwater Salaria, we can not rule out the possibility of some dispersal by sea as S. fluviatilis retains the ability to survive and osmoregulate efficiently in seawater for periods of at least three months (Plaut, 1998). This means that the ancestors of some populations could have dispersed via the seas, especially during the glacial periods. Although the freshwater blennies breed and develop in freshwater as may have pushed freshwater Salaria to areas of brackish or marine conditions.

The present paper highlights the need of a detailed taxonomic reassessment of the status of the freshwater populations of Salaria.

The Moroccan samples must have split more than 2 MYA. They are sister to all other freshwater Salaria. Their distinctiveness, both in the mitochondrial and nuclear genes analyzed, argues in favor of the recognition of a new species in Morocco. Salaria economidisi is nested within samples ascribed to S. fluviatilis, which makes the latter species a paraphyletic entity. Kottelat (2004) found that the morphological differences between the fish in Lake Trichonis (now S. economidisi) and other freshwater Salaria were sufficiently large to warrant specific status to that population. Our results show that there are some splits in the freshwater blennies that are of comparable or greater age, namely that of the Moroccan samples and those of Israel. It seems plausible that several, as yet unrecognized, species may be present in the freshwater clade of Salaria. Thus, a general revision of all freshwater members of this genus is needed. This task is of the highest relevance for conservation as many of these populations are threatened in various degrees.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ympev.2009.03.029.

References


