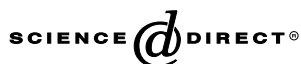


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Short communication

Phylogeny of the shanny, *Lipophrys pholis*, from the NE Atlantic using mitochondrial DNA markers

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1. Introduction

The distribution of the shanny includes the NE Atlantic from Norway to Mauritania and the Atlantic islands, with some records in the Western Mediterranean. Although several studies have been carried out on the morphology (Arruda, 1979; Bath, 1976; Lowe, 1843), ecology (Faria and Almada, 2001; Almada and Faria, 2000), ontogeny (Faria et al., 2002), and its diet composition (Maze et al., 1999) not much has been done on the genetics (Guillemaud et al., 2000).

Some authors have drawn attention to morphological differences between *Lipophrys pholis* of the European continental coasts and the Atlantic islands. One of the first studies to propose subspeciation in *L. pholis* is Arruda (1979) who identifies unique features for the Azorean shanny.

This observation led Zander (1980) to propose a model of re-colonisation of blennies for the Mediterranean after the Messinian crisis (Hsü et al., 1973). Zander suggests that as the temperature and sea level decreased, those Mediterranean blennies moved towards Atlantic islands. One of these blennies could have been an ancestor of *L. trigloides* which probably gave rise to an ancestor similar to *L. pholis*. At the end of the glaciation, this “new” species invaded the African and then the European coasts. Finally, Zander justifies the disjunct distribution of the shanny by differences in competition

pressures that lead to the giant race in Madeira (*Blennius bufo*) and a northern European shanny (*B. pholis*).

In the present study, we use molecular tools along with meristic counts with the aim of resolving the phylogeny of the shanny. Phylogenetic inferences are based on the analysis of 12S, 16S, and partial control region (CR) sequences of the mtDNA from three localities along NE Atlantic coast and two Atlantic islands.

2. Materials and methods

2.1. Morphology

A total of 283 specimens were analysed from the collections listed in Appendix A.

The specimens from “LiphAz1” to “LiphAz5” as well as the larger individual from MMF 24241 were preserved in ethanol therefore they have also been used for the molecular studies.

In addition to meristic counts, for samples from Azores, Portugal mainland, and France, the number of cephalic pores in the nasal area has also been investigated, since their absence was characterising the Azorean population (Arruda, 1979).

2.2. Molecular analysis

A total of 28 shannies were included in the comparison of the control region (CR), 12S, and 16S rDNA. Sequences were analysed separately including 20 individuals for the CR and nine for the 12S and 16S (one

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individual was shared in all genes studied). Samples were collected from the United Kingdom (Oban and Plymouth), mainland Portugal (Gelfa, Avencas, Amoreira, and Lagos), and two Atlantic islands: Azores (Salão in Fayal) and Madeira (Ponta Delgada). As the projects were carried out at different time, amplifications of CR, 12S, and 16S were not all done on the same fishes.

In addition, the sequences of *Lipophrys trigloides* were included as outgroups.

All fish were fixed in 95% ethanol before being stored at -20°C . mtDNA was extracted from white muscle following the procedure of Sambrook et al. (1989), with slight modifications (Stefanni, 2000). Fragments of the mtDNA including partial CR were amplified using the L-Pro1 and H-DL1 primers (Ostellari et al., 1996), while for 12S and 16S the primers used were 12SF, 12SR, 16SF, and 16SR (Henriques et al., 2002), respectively. The same primers were also used for sequencing, as described in Stefanni (2000).

2.3. Sequence alignment and phylogenetic analyses

All sequences were aligned using SEAVIEW (Galtier et al., 1996) and CLUSTAL X (Thompson et al., 1997). Character congruence between 12S and 16S was tested using the incongruence-length difference test (ILD; Farris et al., 1995) available in PAUP 4.0 (Swofford, 1999). 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.

Hierarchical series of likelihood ratio tests (Huelssenbeck and Rannala, 1997), implemented using MODELTEST 3.06 (Posada and Crandall, 1998) were used to identify the appropriate nucleotide substitution models. Likelihood ratio tests of the model's fit with and without the assumption of a molecular clock were conducted.

Phylogenetic trees of the haplotypes were constructed using PAUP. The trees were rooted with the *L. trigloides* haplotypes as outgroup. The neighbor-joining (NJ) method (Saitou and Nei, 1987) was used to construct a phylogenetic tree from the maximum likelihood (ML) distances estimated under the selected models. The support for internal branches within the NJ tree was assessed using the bootstrap (Felsenstein, 1985) with 1000 replicates. A maximum parsimony (MP) analysis was performed using the default options for heuristic search to find the best MP tree(s). The length (L), consistency index (CI), and retention index (RI) of the MP tree(s) were calculated with parsimony-uninformative sites excluded.

The degree of correlation between geographical distances and genetic differentiation was estimated by regression of a matrix of pairwise linearised Φ_{ST} values (Slatkin, 1993), calculated using the program ARLEQUIN 2.000 (Schneider et al., 2000), against a matrix of spatial distribution of the sampling localities. Geographical distances were calculated following the coastal line and for the islands as the shortest distance between one and the other according to the isolation by distance (IBD) model.

3. Results

3.1. Morphology

3.1.1. Meristics

For the mainland samples, frequency distributions of ray counts in the second dorsal (D2) and anal (A) fins show a shift towards higher numbers at higher latitudes (Table 1). Counts of the rays of the first dorsal fins (D1) are stable at 12 with rare exceptions of 11 (Table 1). Counts for rays of the second dorsal and anal fins show high frequencies of 18 and 19. Towards the north the frequency of fishes with 19 fin rays increases, with some individuals in the north having 20

Table 1
Meristics of *L. pholis*

Location	D1		D2				A				N
	XI	XII	17	18	19	20	II + 17	II + 18	II + 19	II + 20	
<i>Mainland</i>											
UK	2	17	0	46	69	4	1	27	83	8	119
France	0	15	1	6	8	0	0	4	10	1	15
Portugal	3	11	0	8	6	0	0	4	10	0	14
Morocco	1	69	0	48	22	0	0	32	38	0	70
<i>Islands</i>											
Azores ^a	3	26	0	1	26	27	0	0	19	35	54
Madeira	0	5	0	2	3	0	0	2	3	0	5
Savages	0	2	0	0	2	0	0	0	2	0	2
Canaries	0	1	0	0	1	0	0	0	1	0	1

D1, first dorsal fin; D2, second dorsal fin; A, anal fin; N, total number of specimens.

^a Ray counts for D1 do not include the specimens from the islands of Santa Maria.

rays. In contrast to the mainland, the Azores population is mainly characterised by 19 and 20 fin rays in both the second part of the dorsal fin and the anal fin (Table 1). At the other oceanic islands (Madeira, Savages, and Canaries) we did not find individuals with 20 fin rays in the second dorsal or anal fin, but samples were too small for statistical comparisons (Table 1). Frequency distributions of fin rays among the other five populations (four mainland and Azores) differ significantly (Kruskal–Wallis test, $p < 0.001$ for both D2 and A). The method of multiple comparison (Siegel and Castellan, 1988) revealed that the Azores were different from all other populations ($p < 0.05$ for both D2 and A). Furthermore, Morocco differed from the UK in one comparison (D2: $p < 0.05$), which we interpret as a result of the shift towards higher fin ray counts mentioned above.

3.1.2. Sensory pores

The number of sensory pores in the inter-orbital nasal area was identical in all fishes examined. Thus, the extra pore that Arruda (1979) described as a unique feature for the Azorean population was found in mainland populations as well.

3.2. Genetic analysis

3.2.1. CR

All shannies were successfully sequenced and the 20 sequences define 17 haplotypes (GenBank Accession Nos. AY966017–33). All fishes sharing the same haplotype are from the same sampling locality. All sequences correspond to the first hypervariable and part of the central domains of the control region with a length ranging from 450 bp (Oban, UK) to 454 bp (Azores). Five indels are required to align the sequences, giving a total length of 455 bp for all the sequences. Two indels partition the Azorean shanny haplotypes from the others, two partition the Azorean with two and three mainland Portugal haplotypes, respectively, and one is affecting the two Scottish and three Portuguese shannies haplotypes.

The appropriate model of nucleotide substitution was the HKY (Hasegawa et al., 1985) model with invariable sites (I), rate heterogeneity (G), and no clock. The transition/transversion (ti/tv) ratio, proportion of invariable sites (i), and γ shape parameter (α) were estimated to be ti/tv = 2.9294, $i = 0$, and $\alpha = 0.3877$, respectively. The base frequencies were estimated to be A = 0.3601, C = 0.1874, T = 0.3099, and G = 0.1426.

3.2.2. 12S + 16S

A total of eight fish were successfully sequenced from which seven haplotypes were defined (GenBank Accession Nos. for 12S and 16S rDNA: AY097015, AY098765–7, AY098825, AY098770, AY0987012–4,

AY0987017, and AY0987019–24). The combined fragment corresponded to a total of 863 and 369 bp for the 12S and 494 bp for the 16S. No indels were required for the alignment and the appropriate model of nucleotide substitution was K80 with ti/tv = 2.

3.3. Phylogenetics

Phylogenetic analysis from CR sequences described by the neighbor-joining (NJ) tree contains 13 internal branches with bootstrap (BS) values greater than 50% (Fig. 1). The branch separating the Azorean shanny is extremely well supported (BS = 100) while the one including samples from Madeira, mainland Portugal, and Scotland has a much lower value (BS = 72). Inside the latter clade, branches with BS values >50% define small ($n = 2$ or 3) monophyletic groupings of haplotypes from different sampling localities.

Of the 455 sites considered only 79 are parsimony-informative. The maximum parsimony (MP) search retained six best MP trees ($L = 163$, CI = 0.859, and RI = 0.832). The strict component consensus tree of the MP trees, which displays all and only those groups found in all the MP trees, resembles the NJ tree. In this tree, the first split separates the Azorean shanny from the monophyletic clade composed by two other groups. One clade which contains the same three haplotypes from mainland Portugal, Avencas (Av10, Av13, and Av17) as presented in the NJ tree, and the second contains haplotypes from all the localities, except for the Azores. The patristic distance between the Azorean

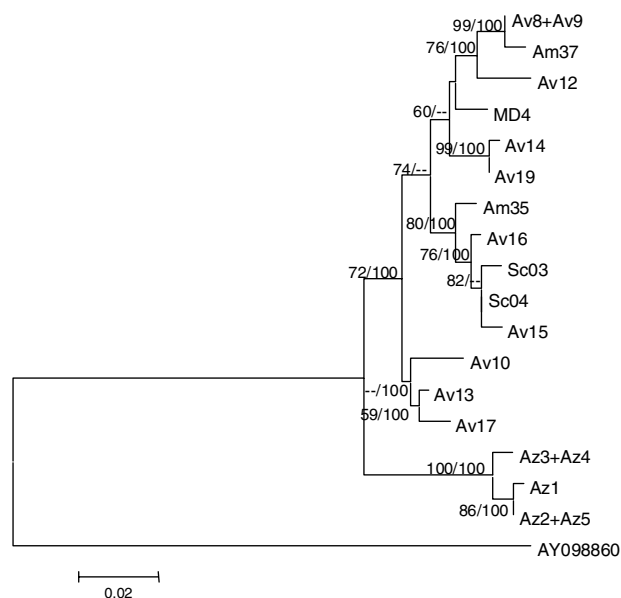


Fig. 1. Neighbor-joining tree constructed from HKY+I+G distances with ti/tv = 2.9294, $i = 0$, and $\alpha = 0.3877$. Numbers above internal branches indicate bootstrap values out of 1000 replicates (only if >50%) for NJ and MP strict consensus trees. Az, Azores; Am, Amoreira; Av, Avencas; MD, Madeira; Sc, Oban.

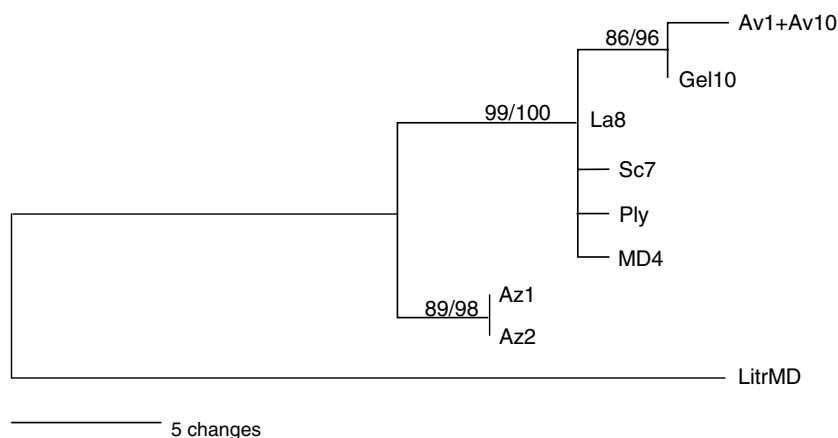


Fig. 2. Maximum parsimony tree for 12S + 16S with numbers above internal branches indicating bootstrap values out of 1000 replicates (only if >50%) for MP and NJ trees. Az, Azores; Av, Avencas; Gel, Gelfa; La, Lagos; MD, Madeira; Ply, Plymouth; Sc, Oban; Litr, *L. trigloides*.

clade and the clade containing the remaining samples is between 1.55 and 1.69%.

Not taking into account the Azorean samples, pairwise Φ_{ST} values between sites varied between 0.093 (Amoreira—Oban) and 0.935 (Oban—Madeira). The latter is of the same order of magnitude when comparing the Azorean haplotypes with the ones from the other localities. However, p values showed very high significance only when any of the sites is compared with the Azores.

The corrected sequence divergence between the Azores sequences and all the others (Madeira, mainland Portugal, and Scotland) is 4.42% while the sequence divergence within the two groups is 0.34 and 2.53%, respectively.

The estimates of the number of migrants (N_m) calculated from the Φ_{ST} values (Slatkin, 1993) are much reduced between the two most remote sites ($N_m=0.0345$) as well as between the islands of Madeira and Azores ($N_m=0.0338$). The reduction in gene flow between localities is not supported by the IBD model and the correlation between geographical distances and genetic differentiation is not significant ($p=0.076$).

For the phylogenetic analysis of the combined 12S + 16S fragment, all inference methods converged on the same tree topology (Fig. 2). MP yielded a single most parsimonious tree of 54 steps. All methods resulted in the same samples of shannies as a monophyletic group clearly divided in two clades with very high support values. One clade contains the Azorean fish while in the other clade all the remaining localities are grouped together (mainland Portugal, Madeira, and UK). The patristic distance between the Azorean clade and the one containing the remaining samples is 1.24%.

4. Discussion

The phylogenetic analyses and the population genetics strongly support the presence of two groupings of

shanny, one for the Azores and one for the mainland Europe which includes the island of Madeira. The clade constituted by the Azorean sample is highly supported by NJ and MP trees using CR and 12S + 16S. Genetics show unique haplotypes for the Azorean shanny. Further support to this finding is the substantial divergence between Azorean sample and the others. Patristic distances between these two groups are 0.77 and 1.58% for the two fragments at slower rate of evolution (12S and 16S, respectively) and 1.55–1.67% for the CR. While in the 12S the two Azorean fishes share the same haplotype, in the 16S they differ from each other, and the distances between them and the group containing the remaining shannies have an average of 0.61%.

It is important to note that the patristic distance between the Azorean shanny and those from mainland Portugal, UK, and Madeira are of the same order of magnitude (or even higher) than the values between some of recognised distinct species of blennies. For example, the p distance between *Parablennius gattorugine* and *P. ruber* is 1.6% and between *Lipophrys canevai* and *L. nigriceps* is 1.3% (Almada et al., 2005).

As for 12S and 16S, the CR also describes unique haplotypes for the Azorean shanny. They are defined by 12 substitutions and four insertions, and are mainly encountered (more than 80% of them) in the first domain. Within the two clades, the level of nucleotide diversity is very different (Azores: $\pi=0.008$; continental Europe + Madeira: $\pi=0.056$) differences which are also reflected in the number of shared haplotypes (Azores: 40%; continental Europe + Madeira: 7%). These rough values also suggest that the population of shanny in the Azores may be very limited in size when compared to the continental one.

When all sampling localities are compared one to another, pairwise values of Φ_{ST} result statistically significant only when Azores is included. This is further supported by the lack of correlation between genetic differentiation and geographical distance.

When comparing p distances from partial CR sequences of several *Lipophrys* species (available from GenBank), the lowest value, after the 0.09 between “Azorean” *L. pholis* and “mainland Portugal” *L. pholis*, was 0.27 between *L. adriaticus* and *L. caboverdensis*, followed by a 0.32 between *L. adriaticus* and *L. dalmatinus*, and 0.41 between *L. caneveae* and *L. caboverdensis*.

Interruption of gene flow in *L. pholis* between Azores and continental Europe (most probably through Madeira and Canaries islands) might have happened more recently than the Messinian crisis (5.9 Mya) as proposed by Zander (1980). Applying mutational rates compatible with universal molecular clocks, 1% for region at slow-paced rate (as 12S and 16S), places the split between continental and Azorean shannies at 1.23 Mya, during the early Pleistocene. It is well known that CR yields a mutation rate which is several times faster compared to the rest of the mitochondrial genome (Brown et al., 1993). Taking into account that early Pleistocene is a plausible period for this separation to occur, the mutation rate affecting the CR of *L. pholis* would range between 2.9 and 5.5%, values also encountered in other fish species with similar transition/transversion rate (McMillian and Palumbi, 1997). A reliable calibration of the CR clock for teleosts is provided by the comparison between two amphipanamic geminate species of snook (Donaldson and Wilson, 1999), and using this estimate, separation between continental and Azorean shannies occurred about 1 Mya.

Early Pleistocene was characterised by an inter-glacial period with relatively high global temperature and little ice locked up in polar caps (Lambeck et al., 2002). With such warm conditions in the northern hemisphere we might expect to find the anticyclone known as the “Azores High” located further north than today. In such a scenario, we would expect that the trade winds would also be blowing at a higher latitude, maybe from the coasts of southern Europe westward. In this situation, the Azores would receive a constant supply of eggs and larvae from marine organisms breeding in Europe. Such a regular gene flow would possibly have been affected by interruptions, especially during the glaciations that were becoming progressively intense.

During the last glacial maximum, probably several times during the Pleistocene, the temperatures in the European shores would have been so low that *L. pholis* would have become extinct, re-colonizing from more southern refugia during inter-glacials. On the contrary, at the Azores, the drop in sea surface temperature was apparently much smaller, about 3 °C (Climap, 1981; Crowley, 1981). As a result, the present population of Azores would have survived there, eventually adapting to long cold periods. This difference in population histories may help to explain the genetic differentiation that we found and the contrast in patterns of meristic characters.

The strong differentiation of the Azorean shanny which is indicated by both, genetic and meristics data and the fact that this fish is extremely rare in the Azores, suggests that the taxonomic and conservation status of this species should be carefully revised.

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Appendix A. Appendix

List of collections of *L. pholis* used for morphological study: BMNH—British Museum of Natural History, London, UK; SMNS—Senckenberg Natural History Museum, Frankfurt, Germany; MMF—Museum Municipal do Funchal, Madeira, Portugal; DOP—Department of Oceanography and Fisheries, Fayal, Azores, Portugal; RMNH—State Natural History Museum, Leiden, The Netherlands. The specimens were originally collected from the following locations:

1. United Kingdom, 119 specimens: BMNH 47015; $n=11$, Devon; $n=2$, Loch Kishorn; $n=3$, Isle of Lewis—Hebrides; $n=28$, Pembrokeshire; $n=5$, Bude—Cornwall; $n=15$, Eastborne—Sussex; $n=1$, Mullet Peninsula; $n=2$, Pembrokeshire; $n=3$, Orkney; $n=4$, Isle of Mann; $n=3$, Brighton; $n=13$, Rosscarbery Bay; $n=13$, Lannacombe Bay; $n=5$, Guernsey; BMNH 47020, $n=11$, Cornwall.
2. France, 15 specimens: BMNH 47020; $n=10$, Roscoff—Brittany; DOP LIP PHO Fr 1–3, $n=3$, Beg-Meil—Brittany; RMNH 23844, $n=1$, Roscoff—Brittany; RMNH 23864, $n=1$, Roscoff—Brittany.
3. Morocco, 70 specimens: SMNS 13541 $n=24$, Tan Tan; SMNS 13532, $n=1$, Agdu; SMNS 13540, $n=8$, 15 km south of Tan Tan; SMNS 13506, $n=9$, Cap Rhin; SMNS 13538, $n=10$, Tarfaya; SMNS 13536, $n=5$, Sidi Ifni; 13544, $n=13$, Flayounne.
4. Portugal mainland, 14 specimens: DOP LIP PHO Parede 1–14; $n=14$, Parede.
5. Madeira Island, five specimens: BMNH 1863.9.10.12 $n=3$, Lowe 1863; MMF 22421 $n=2$.
6. Porto Santo Island, 1 specimen: RMNH 29592, south west coast.

7. Savages Iles, two specimens DOP LIP PHO Selvagem 1, $n = 1$, Selvagem Grande; MMF 201 $n = 1$, Selvagem Islands.
8. Azores islands (Central group: Fayal and Pico islands), 29 specimens: BMNH 19870, $n = 1$, Porto de Salão, Fayal; DOP LIP PHO Az 1-2, Porto de Salão, Fayal, Clipe-project; DOP LIP PHO Az 3–5, Porto de Salão, Fayal; DOP LIP PHO Az 6–25, north coast Fayal; DOP 161–395-LIP PHO-(6;1) $n = 3$, São Roque, Pico, Expedition Azores 89; (Oriental group: São Miguel and Santa Maria islands), 27 specimens: DOP LIP PHO Santa Maria 1–25, $n = 25$ (lost during transfer of collection); RMNH 35033, $n = 1$, Baía São Lourenço, Santa Maria; RMNH 35032, $n = 1$, south coast São Miguel.
9. Canary Islands, one specimen: DOP 132–395-LIP PHO-(6;1), Tenerife Norte.

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