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Short communication

Genetic structure and historical demography of the shanny *Lipophrys pholis* in the Portuguese coast based on mitochondrial DNA analysis

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1. Introduction

The shanny *Lipophrys pholis* (Linnaeus, 1758) (Pisces: Blenniidae) is a rocky intertidal resident fish whose distribution comprises a large coastal region in Northeastern Atlantic, limited by Norway, Mauritania, and the Azores Islands (Zander, 1986). *L. pholis* has demersal eggs guarded by the males (Almada et al., 1992) that hatch after 16 days with 5.0 mm total length at a temperature of 17 °C (Faria et al., 2002). Hatching larvae are well developed, settling at 13–14 mm TL after a pelagic larval duration of ca. 29 days at a temperature of 15.5–17.5 °C. After settlement and metamorphosis, this benthic species is thought to forage within a home range at high water and return to a particular set of rock pools or crevices at low tide (Dodd et al., 2000), with its movements being restricted to a small area.

In the Portuguese coast the stretches of rocky intertidal are widely separated by tens of kilometers of sandy shores. After settlement, the movements of *L. pholis* between the different rocky stretches are virtually impossible, and connectivity among populations must be ensured by larvae.

Fish larvae have long been considered passive particles at the mercy of ocean currents, tides, and weather events (Montgomery et al., 2001). For reef fishes, ecological evidence has demonstrated that passive dispersal alone cannot explain larval distributions (e.g., Leis and Carson-Ewart, 1998). Several studies have focused on early life-history traits (e.g., Doherty et al., 1995) and larval behaviour (e.g., Marliave, 1986; Miller and Shanks, 2004; Neilson and Perry, 1990) and tried to correlate them to dispersal potential. It would be interesting to understand if a coast with the range (ca. 700 km) and fragmentation of the Portuguese one bears just one or several populations of *L. pholis*.

Using mtDNA data, Stefanni et al. (in press) showed that the populations of the shanny can be separated in two distinct groups: one including fish from the Western European shores (from Scotland to Portugal and Madeira) and another comprising fish from the Azores.

The present genetic structure of a species can only be fully interpreted if one considers the influence of historical events and the complex interactions of biology, geography, and climatic shifts (Hewitt, 2000). It is known that during glaciations, the Atlantic shores of Western Europe underwent drastic changes in sea surface temperature. Alveirinho-Dias et al. (1997) showed that at the last glacial maximum (LGM, ca. 18.000 years ago), the waters along the Portuguese coast were many degrees colder than today, and the polar front was located at the latitude of Lisbon (38°42′N, 9°10′W), or even south of that point. Thus, in its present composition, the ictiofauna of this geographical area is very young and must have been established after the end of the last glaciation, in the last 10 thousand years or so.

In the present work, mitochondrial DNA control region sequences were used to study populations of *L. pholis* along the Portuguese west and south coasts, specifically focusing on the following questions: (1) What is the degree of genetic differentiation of *L. pholis* along the Portuguese coast?; (2) Is there evidence of population expansion during Pleistocene for this species?; and (3) If no genetic differentiation is found, can we distinguish the effects of a post-glacial expansion from present day connectivity?

2. Materials and methods

Eighty nine specimens of *L. pholis* were collected between July 2003 and March 2005 from three rocky platforms along the Portuguese coast (Fig. 1): Cabo Mundo (41°11′N, 8°42′W) ($n=27$), Estoril (38°42′N, 9°22′W) ($n=31$), and

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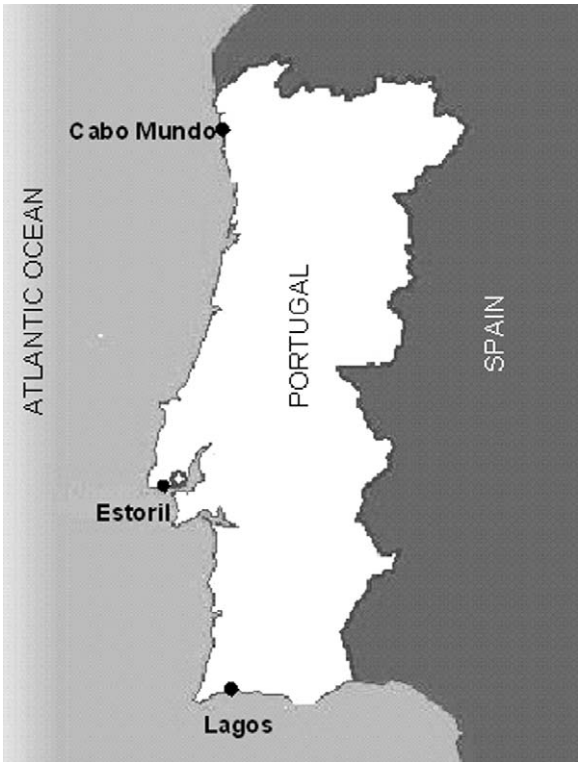

Fig. 1. Sampling locations for *Lipophrys pholis* along the Portuguese coast.

Lagos (37°05′N, 8°41′W) ($n = 31$). A very small piece of dorsal fin was clipped and preserved in 96°C ethanol.

Total genomic DNA extraction was performed using the protocol of Sambrook et al. (1989) with some modifications. A fragment from the mtDNA control region was amplified using the primers described in Ostellari et al. (1996): L-pro1 and H-DL1. Details of DNA extraction, PCR, and sequencing may be requested from the authors. The obtained sequences were deposited in the GenBank Data Base (Accession Nos. DQ154169-4257; see Appendix in supplementary material).

Relationships among haplotypes were analyzed with a parsimony network estimated with TCS version 1.18 (Clement et al., 2000). The software PAUP* 4.0b (Swofford, 2000) was used to estimate the uncorrected p distances between all sequences.

The ARLEQUIN software package version 2.000 (Schneider et al., 2000) was used to estimate the genetic diversity, to access population differentiation and to search for signs of past growth. The following diversity indices were computed: number of polymorphic sites, gene and nucleotide diversity (Nei, 1987), and average number of pairwise differences (Tajima, 1983). Analysis of molecular variance (AMOVA; Excoffier et al., 1992), pairwise F_{ST}, and exact test for population differentiation (Raymond and Rousset, 1995) were performed. Mismatch analysis (Rogers, 1995; Rogers and Harpending, 1992) and Fu's F_s (Fu, 1997) test were performed to test for a possible bottleneck and population expansion.

The program LAMARC (Kuhner et al., 2005), based on the coalescent theory of Kingman (1982a,b), was used to estimate the effective population size and g (the exponential growth parameter in units of μ^{-1}). Estimations of θ and g were accomplished with 10 replicate runs using the default settings.

3. Results

The 89 sequences obtained define 67 haplotypes, 55 of which are represented in a single specimen. A total of 83 polymorphic sites and 93 mutations (80 transitions, 12 transversions, and 1 indel) were found out of a 380 bp fragment of the mtDNA control region. All populations showed high values of gene and nucleotide diversity (Table 1).

The statistical parsimony procedure yielded one network (Fig. 2), with the exception of three haplotypes, which could not be connected together under the confidence limit of 95% (Templeton et al., 1992). This network revealed a lack of geographical structure. Within it there are several ambiguous connections, suggesting parallel substitutions, and haplotype 8 was inferred to be the ancestral one, as it yielded the highest outgroup weight (0.092) (Castelloe and Templeton, 1994). The uncorrected p distances were 0.0300 (SE = 0.0111), 0.0279 (SE = 0.0132), and 0.0269 (SE = 0.0152) within Cabo Mundo, Estoril, and Lagos groups of samples, respectively, and 0.0311 (SE = 0.0125) between Cabo Mundo and Lagos, 0.0312 (SE = 0.0099) between Cabo Mundo and Estoril, and 0.0329 (SE = 0.0137) between Estoril and Lagos. The average p distance between the two more distant localities (Cabo Mundo and Lagos) is not greater than those between Cabo Mundo and Estoril and between Estoril and Lagos, suggesting that, at the scale of the present study, no effects of isolation by distance can be detected.

AMOVA of the D-loop sequence data revealed that the overall F_{ST} value was not significant (significance based on 1023 permutations), indicating no genetic structure within the studied area ($F_{ST} = -0.0017$, $P = 0.659$). All pairwise F_{ST} values were smaller than 0.001, and none of them were significant ($P > 0.05$). Similarly, exact tests of population differentiation indicated no significant difference ($P > 0.05$) for any pair of populations. Given these results, in the subsequent analyses the samples were pooled together, and treated as one single population.

Table 1
Diversity measures for the populations of *Lipophrys pholis*

Population	n	nh	S	H	π
Cabo Mundo	27	24	51	0.991	0.033
Estoril	31	27	58	0.991	0.031
Lagos	31	26	52	0.990	0.032
Total	89	67	83	0.990	0.032

n, number of individuals; nh, number of haplotypes; S, number of polymorphic sites; H, gene diversity; and π, nucleotide diversity.

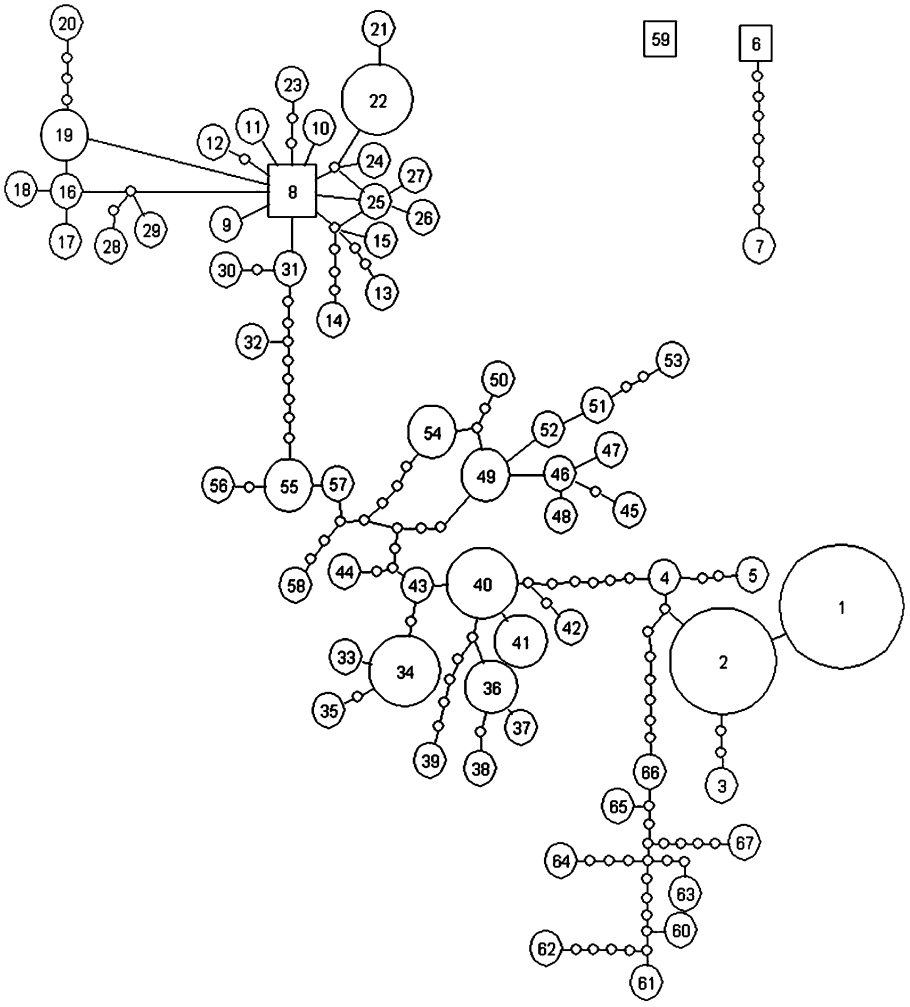

Fig. 2. Statistical parsimony network for the 67 D-loop mtDNA haplotypes of *Lipophrys pholis* in the Portuguese coast. Each haplotype is defined by its corresponding number (see Appendix). The haplotype with the highest outgroup probability is displayed as a square, other haplotypes as circles. The size of the squares or circles is proportional to the haplotype frequency.

The pairwise mismatch distributions were not significantly different from the sudden expansion model for the whole area (Table 2), outcome supported by Fu's test. There are very large discrepancies in estimations of divergence rates for the D-loop of teleosts. In the absence of a specific calibration for the D-loop of blennids we assumed two very distinct rates: 3.6 and 18.6% divergence per site per million years (Domingues et al., 2005; Donaldson and Wilson, 1999, respectively). For the whole Portuguese coast, expansion was estimated to have taken place approximately between 103,000 (95% C.I. 72,566–125,771) and 531,000 years ago (95% C.I. 374,927–649,817), depending on the mutation rate assumed and considering that *L. pholis* attains full maturity in 2 years (Milton, 1983).

Assuming a constant population size for *L. pholis* through time, the average θ value estimated with LAMARC was 0.1901 (SE = 0.0118). Allowing the program to estimate both parameters jointly produced larger estimates of θ (0.4588; SE = 0.0612), and the respective average growth rate was 197.2296 (SE = 18.4142). According to this model, the size of the female population 18 Kya in the LGM was approximately 71.88–93.80% relative to its current size. The approximate age of shanny population, assessed as the age at which N_f drops below 1%, yielded a value of 251,000–1,297,000 years.

Table 2
Parameters of the mismatch distribution, Fu's neutrality test, and their significance for the whole population of *Lipophrys pholis*

Population	τ	θ_0	θ_1	*SSD*	*P*	*Hri*	*P*	F_s	*P*
Portuguese coast	14.525 (10.258–17.779)	0.002	65.596	0.006	0.150	0.005	0.420	−24.344	0.000

τ, time in generations, upper and lower bounds of 95% CI in parenthesis; θ_0 and θ_1, compound parameter representing the mutation rate and the female effective population size before and after expansion, respectively; *SSD*, sum of square deviations and its probability *P*; *Hri*, Harpending's Raggedness index and its probability *P*; *F*s, Fu's neutrality test and its probability *P*.

4. Discussion

The data indicated no significant population genetic structure in *L. pholis* along the Portuguese coast, which may be explained by three not mutually exclusive hypotheses. It may reflect ongoing gene flow. It can result from historical contacts between present-day populations. Finally, it may be the consequence of a colonization event so recent that there was not enough time for mutations to accumulate and population differentiation to occur.

Since the adults of *L. pholis* inhabit restricted rocky intertidal areas, gene flow must be maintained through larval dispersal. Like in other coastal species with demersal eggs, immediately after hatching the newly hatched larvae move to the surface, swim actively and one day after the onset of exogenous feeding occurs (Faria et al., 2002), traits that can be an indication of high dispersal potential. According to Faria et al. (1996), the breeding season in Portugal occurs from October/November to May, and during this period the coast is characterized by a permanent southward surface circulation (30 cm s^{-1} maximum speed), and a countercurrent associated with southerly winds that are formed during autumn–winter (43 cm s^{-1} maximum speed) (Fiúza, 1983; Martins et al., 2002). Thus, the net flow of the surface waters along the shore has a variable direction during winter and spring, depending on the relative proportion of winds that blow from the south which varies greatly along the winter and from year to year. The oceanographic conditions of the studied area may allow shanny larvae to migrate in both directions along the coast, and within the 30 days of pelagic larval duration they can potentially be transported up to 110 km. These distances can be dramatically increased when storm conditions prevail, reaching about 200 km in 6 days (V.C. Almada, unpublished results). These findings are in apparent contradiction with the observations of Marliave (1986), who reported rocky intertidal fish larvae as being capable of resisting offshore, and possibly longshore, dispersal. Nevertheless, it is important to remember that larval dispersal may vary markedly with species, it may differ between sheltered and exposed sites, and, most importantly, even if substantial larval retention occurs, it is sufficient that a fraction of the larvae is transported away from the place of origin to ensure connectivity among populations. Accordingly, present day current regime and biological and behavioural characteristics of the larvae, potentially allow the exchange of individuals in sufficient numbers to prevent population divergence in *L. pholis*.

The results suggest that this species has undergone a population expansion in the Portuguese coast that lasted for several hundred thousands of years. According to the mismatch analysis this expansion may have began after a bottleneck that occurred between 73 and 650 Kya. It is also supported by significantly negative Fu's F_s and large difference between θ_0 and θ_1. Additionally, the approximate age of the population is 251,000–1,300,000 years, clearly long before the LGM.

No signature of the LGM or other similarly recent bottleneck was detected in the mismatch distribution. In addition, the relative female effective population size 18 Kya was only slightly smaller than today's (72–94%).

In the LGM, and probably in other glacial maxima, polar water masses were at the Golf of Biscay, while at the western coast of the Iberian Peninsula the gradient of surface water temperature was extremely steep (from 0.7 °C in the extreme NW to 7.2 °C in the SW in February 18 Kya) (Climap, 1981). At the south coast of Portugal the surface water temperature was even higher (9.0–10.3 °C), equalling several points of the high latitudinal European coastal area in the present time. In such scenario, during glacial maxima, *L. pholis* populations may have persisted along the Iberian Peninsula, just by withdrawing a few hundred kilometers to the south.

All the lines of evidence presented above support the conclusion that the genetic homogeneity of shanny found along the Portuguese coast results from larval dispersal, and was not caused by a colonization event too recent to allow accumulation of mutations and population differentiation.

Stefanni et al. (in press) showed a large homogeneity in D-loop sequences of *L. pholis* populations from Great Britain to Madeira. One of the haplotypes described in Stefanni et al. (one individual from Oban, Scotland AY966030) is also shared by two of our individuals from Estoril—haplotype 8 (the one considered to be ancestral in the parsimony network). This finding suggests that the pattern of high connectivity found in the present work, may occur at a much wider geographical scale along the western European shores.

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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.2005.12.009.

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