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Genetic Structure and Effective Population Size through Time: A Tale on Two Coastal Marine Species with Contrasting Life-History Patterns

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Abstract

Background: Species with planktonic larvae are more likely to show temporal genetic variation, due to differences in larval mortality and dispersal ability. The shanny *Lipophrys pholis* is a typical benthic rocky intertidal fish with its dispersion limited to a long larval stage. In contrast, the sand smelt *Atherina presbyter* has a very short planktonic life, small size and weak swimming capabilities, which translates into reduced dispersion potential.

Methods: A total of 226 shanny specimens (collected in 2003, 2013 and 2014) and 281 sand smelts (collected in 2005, 2012, 2013 and 2014) were screened for genetic variation using the mitochondrial control region. Genealogies, genetic diversities, temporal structures and contemporary effective population sizes were assessed.

Results: Haplotype networks showed deep genealogies with multiple levels of diversification and no temporal structure. Genetic diversity indices showed little variation among sampling periods and were generally high. For *L. pholis* significant genetic differentiation was detected between 2013 and 2014, while no significant differences were detected between sampling periods in *A. presbyter*. The shanny showed lower effective population size per generation when compared to the sand smelt (which yielded lack of evidence for genetic drift for the first two periods of the study).

Conclusion: These results highlight the fact that temporal changes in the gene pool composition need to be considered when evaluating population structure, especially for species with long pelagic larval dispersion, more vulnerable to fluctuations in the recruitment.

Keywords: Population genetics; Planktonic larval dispersion; Temporal structure; Effective population size

Introduction

In a context of climate change and anthropogenic influences on aquatic ecosystems, great importance is being given to the genetic biodiversity monitoring [1]. Assessments of changes in genetic diversity and their trajectories over time provide critical information for both fisheries and endangered-species management. Nevertheless, concerning population genetic structure, and from a practical standpoint, it has often been assumed that the observed spatial patterns are stable over time [2,3]. Several studies, however, show that the assessment of population differentiation may be biased by sampling on a single occasion [4,5]. The use of a temporal scale helps to overcome this issue and is being used with increasing frequency [6-8].

Temporally replicated sampling allows both to address the temporal stability of a population and to estimate the effective population size (N_e). N_e is defined as the number of breeding individuals of a population that has the same optimum amount of dispersion allele frequencies subject to random genetic drift [9]. This metric is particularly sensitive to recent events and is related to the absolute size, calculated by census (N). By providing a measure of genetic diversity, contemporary or short-term N_e can be used to predict the adaptive potential of a population, as a function of its vulnerability to stochastic forces, i.e., to genetic drift [10].

The measurement of N_e in natural populations is difficult, hence the considerable interest in the development and implementation of genetic methods to estimate it [11, for a review see 10]. One of the most commonly used approaches for the estimation of contemporary variations of N_e has been the temporal method [12], dependent on allele frequency estimates of a population in two or more points in time. It is assumed that the observed genetic drift between two sampling times will be much sharper in a smaller population. The model also assumes that generations are discrete, although many species present age structure with overlapping generations. To overcome this limitation, Jorde and Ryman [13] derived a method which incorporates the effects of age structure, showing that the magnitude of changes in allele frequencies is determined, not only by the actual size and range of samples, but also by birth rates and specific survival of every age. More recently, a modified approach developed by Jorde and Ryman [14] provided a direct extension of the model, enabling the use of sampling time cohorts born with only a certain number of years of separation. For unexploited species, N_e stability (or lack thereof) may serve as an indicator of the species robustness (faced with anthropogenic and natural effects) allowing for a better conservation management of both the species and its habitat.

The dispersal capabilities of a given organism have often been described as influencing population genetic structure [15-17]. Species with longer pelagic larval duration (PLD) are more vulnerable to fluctuations in oceanographic conditions, and their recruitment is expected to suffer greater variation, when compared with species with reduced PLD. For the latter, population temporal stability is most expected (irrespective of the effective size of the population and its propagule production). In the present work, two species with contrasting early life histories were chosen in order to evaluate the influence of this ecological trait in the temporal stability / structure of a population.

The shanny *Lipophrys pholis* L. 1758 (Pisces: Blenniidae) is a rocky intertidal resident fish, very common in west European shores. Its distribution area comprises a large coastal region from Norway to Mauritania and from the Azores and Madeira to the entrance of the Mediterranean [18]. The demersal eggs are guarded by the male [19], hatching after 16 days with 5.0mm total length at a temperature of 17°C

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[20]. Hatching larvae are well developed, settling at 13-14mm TL after a pelagic larval duration of ca. 29 days at a temperature of 15.5-17.5ºC [20]. Juveniles and adults show restricted movements due to their weak swimming capabilities, and are thought to stay in the same rocky stretch for life [21].

The sand smelt *Atherina presbyter* Cuvier 1829 (Pisces: Atherinidae) is an inshore marine fish, occasionally entering coastal lagoons and estuaries. Its distribution ranges from the British Isles and southern North Sea to the Canary Islands, Mauritania and Cape Verde [22], and it has also been reported for the Azores archipelago [23]. The sand smelt spawns in very shallow waters, the demersal eggs attach to vegetation and larvae hatch after 15-16 days with 6.7-7.5 mm TL at a temperature of 15ºC [24]. Hatching larvae are well developed and ready to start exogenous feeding. This very short larval stage likely limits passive dispersal. Adults of sand smelt are active swimmers in the water column, although their migratory movements are probably difficult along exposed shores.

Our current understanding of the gene flow in *L. pholis* and *A. presbyter* points to contrasting patterns. Previous studies suggest panmixia for the shanny across large part of its distribution area [25,26], as would be expected given the long duration of their pelagic phase. In contrast, previous studies on the sand smelt, with its very short planktonic life and overall reduced dispersion potential, strongly suggest a structure of isolation by distance that points to reduced gene flow between localities separated by some hundreds of kilometers [27]. Both species revealed deep and complex genealogies, showing evidences of a Pleistocene signature [25,27,28].

In this work we used these two fish species with contrasting PLD to evaluate the variability in the mitochondrial control region at distinct sampling times in the western Iberian coast. Our focus was on the comparison of population structure obtained by sets of temporally independent samples (at the phylogeographic level), and on the contrast of the current state with that of at least one previous generation. Additionally, we aimed to estimate and compare the effective sizes of the populations and their recent evolution.

Materials and Methods

Sampling

Juveniles of the year were collected near Lisbon: *L. pholis* in rocky tide-pools (S. Pedro do Estoril 38° 42' N, 9° 22' W), and *A. presbyter* in channels (Fonte-da-Telha 38° 34' N, 9° 11' W, and S. Pedro do Estoril 38° 42' N, 9° 22' W). For the shanny, samples were collected in 2003 (N = 30), 2013 (N = 97) and 2014 (N = 99). Sand smelt samples were collected in 2005 (N = 34), 2012 (N = 91), 2013 (N = 61) and 2014 (N = 95). Most samples were newly collected for this study, however, the sequences corresponding to the first temporal sample of both species were already included in previous works [25,29,30] (Table S-1 in supplementary materials). Whenever possible only a small piece of dorsal or caudal fin was clipped and the fish was promptly released. The smallest specimens had to be euthanized due to the impossibility of collecting the samples without killing them. All samples were preserved in 96° ethanol and deposited in ISPA-IU/MARE collections.

DNA extraction, amplification and sequencing

Total genomic DNA extraction was performed with the REDExtract-N-mp kit (Sigma-Aldrich). A fragment from the mitochondrial control region (CR) was amplified using the primers Lpro1 (5'-ACTCT CACCC CTAGC TCCCA AAG-3') and HDL1 (5'-CCTGA AGTAG GAACC AGATG CCAG-3') [31]. Polymerase Chain Reactions (PCR) were performed in a 20 µl total reaction volume with 10 µl of REDExtract-N-ampl PCR reaction mix (Sigma - Aldrich), 0.8 µl of each primer (10 µM), 4.4 µl of Sigma-water, and 4 µl of template DNA. An initial denaturation at 94ºC for 3' was followed by 35 cycles (denaturation at 94ºC for 30", annealing at 55ºC for 30" and extension at 72ºC for 1') and a final extension at 72ºC for 10' on a Bio-Rad Mycycler thermal cycler. The same primers were used for the sequencing reaction, and the PCR products were purified and sequenced at STABVIDA (Portugal, www.stabvida.net) and GATC (Germany, www.gatc-biotech.com).

Sequences obtained were deposited in GenBank (Accession numbers KR028543-KR028984). Additional sequences from previous works [25,29,30] were retrieved from GenBank (Table S1 in supplementary materials). Sequences were edited with Codon Code Aligner (http://www.codoncode.com/index.htm) and aligned with Clustal X2 [32].

DNA statistical analyses

The appropriate model of sequence evolution for the CR of each species was determined using jModeltest program v.2.1.7 [33,34], under the Akaike Information Criterion (AIC) [35]. Parsimony networks estimated with TCS version 1.21 [36] were used to analyze relationships among haplotypes and to compute out group weights.

ARLEQUIN software package v.3.5 [37] was used to estimate the genetic diversity [k - mean number of pairwise differences [38]; π- nucleotide diversity and h - haplotype diversity [39]] within each sampling period, to perform analyses of molecular variance (AMOVA) [40] and to compute pairwise F_{ST}s. The χ^2-test developed by Salicru et al. [41] was used to access the significance of the differences in haplotype diversity values among temporal samples. The software Contrib v1.02 [42] was used to estimate haplotype richness R, using rarefaction to correct for sample-size bias. In order to test for possible bottlenecks and population expansion, mismatch analysis [43,44], Fu's [45] *Fs* and Tajima's [38] *D* tests were performed. In the absence of a specific calibration for the CR of these species, estimates of effective population size, their changes with time and the age of populations were computed using the mutation rate of 5% for the CR [46].

LAMARC 2.1.10 [47] was used to estimate female effective population size (*Nef*) and the exponential growth parameter (*g*), using a MCMC approach with 10 runs of 12 short chains of 1,000 steps and three long chains of 20,000 steps, with a burn-in of 10,000.

Past population demography of *L. pholis* and *A. presbyter* were inferred using the linear Bayesian skyline plot (BSP) [48] model as implemented in BEAST v.1.8.2 [49], employing the Bayesian MCMC coalescent method and a strict clock. The Bayesian distribution was generated using results from five independent runs of 50 million MCMC steps, obtaining effective samples sizes (ESS) of parameter estimates of over 200, with a burn-in of 10%. The time to the most recent common ancestor (t_{MRCA}) and the median and corresponding credibility intervals of the BSP were depicted using Tracer v.1.6 [50].

TempoFs [14] was used to estimate genetic drift (F_s) and contemporary effective population size (*Ne*) under the temporal method of allele frequency shifts. The program reports F_s' (genetic drift corrected for sampling plan) and N_e per generation (in this case, adapted to mtDNA). For this approach we used a sampling plan II (individuals sampled before reproduction and not returned to the population; [51]) and a generation time of 2yr for *L. pholis* [21,52] and 1yr for *A. presbyter* [53].

Results

A 380bp fragment of the CR of *L. pholis* was analyzed; 226 sequences were obtained corresponding to 171 haplotypes with a total of 108 polymorphic sites (Table S1). Differences among haplotypes corresponded to 105 transitions, 26 transversions and 8 indels. For the shanny only 12 (out of 171 haplotypes) were shared between sampling periods. For *A. presbyter* a total of 281 sequences for the CR was obtained with a final alignment of 368bp, comprising 155 haplotypes; 70 polymorphic positions were found (63 transitions, 19 transversions and 6 indels) (Table S1). For the sand smelt 25 haplotypes (out of 155) were shared between sampling periods. For both fishes, the best fit evolutionary model was the generalized time-reversible (GTR) + invariable sites (I) [54].

The haplotype networks showed deep genealogies with multiple levels of diversification and no apparent temporal structure for both species (Figure 1 and 2). For *L. pholis* the ancestral haplotype (outgroup weight 0.053) includes specimens collected in the three sampling periods (Figure 1). Several haplotypes, including the estimated ancestral one, were in the centre of star-like patterns and were temporally wide spread. The inferred genealogy was very deep, with some haplotypes reaching 17 mutational steps from the ancestral one. In the case of *A. presbyter*, although less diverse, the network also showed several star-like patterns around several haplotypes (Figure 2). Some of the branches reached a maximum of 16 mutational steps from the ancestor (outgroup weight 0.051), which comprises individuals from all sampling periods.

Genetic diversity indices showed little variation among sampling periods and were generally high (Table 1). For both species, levels of haplotype diversity were not significantly different among sampling periods (*L. pholis*: $\chi^2 = 3.022$, $p = 0.388$; *A. presbyter*: $\chi^2 = 4.342$, $p = 0.362$).

When testing temporal differentiation, AMOVA showed non-significant genetic differentiation for both the shanny ($F_{ST} = 0.009$, $p = 0.074$) and the sand smelt ($F_{ST} = 0.002$, $p = 0.312$). However, for *L. pholis* significant genetic differentiation was detected between 2013 and 2014 ($F_{ST} = 0.013$, $p = 0.028$), while no significant differences were detected between sampling periods in *A. presbyter*. For both species, and given the absence of temporal structuring, the sequences from all sampling periods were pooled together for further analyses.

The results of the neutrality tests indicated negative and highly significant values for Fu's *Fs*, suggesting population expansion for both species; Tajima's *D* were non-significant, although negative (Figure 3). Analyses of mismatch distributions did not differ significantly from the model of sudden expansion for both the shanny (SSD = 0.007, $p = 0.134$) and the sand smelt (SSD = 0.006, $p = 0.342$) populations. Two peaks were detected in the graph of *L. pholis*: a stronger peak at 14 differences and another one at 4 differences. The graph of *A. presbyter* revealed a younger genealogy with a stronger peak detected at 10 differences and another one at 2 differences (Figure 3). The estimated time for demographic expansion yielded 378 thousand years (ky) for the shanny and a younger date was estimated for the sand smelt (283ky) (Figure 3).

The demographic parameters estimated with Lamarc were consistent with the ones obtained with the mismatch analyses, with the confidence intervals for the age of populations globally overlapping for both species (Figure 4). For *L. pholis* the BSP revealed a rapid population growth in the last 50 ky, reaching a female effective of about 20,000 individuals in present-day (Figure 4A). The estimated value for t_{MRCA} was ~522ky. The skyline plot for *A. presbyter* shows that this species experienced a faster population growth around 30ky before present, with a maximum female effective size of about 15,000 individuals (Figure 4B). t_{MRCA} for the sand smelt was ~386ky.

The *Ne* estimates per generation for the shanny ranged from 94 (first interval) to 612 (second interval). Conversely, allele frequency changes - genetic drift (*Fs'*) were higher for the second period (0.010 vs 0.008). In the case of *A. presbyter* estimates of *Fs'* were negative for the first and second periods. For the third period (2013-2014), *Ne* per generation was estimated as 1282, with a corresponding *Fs'* of 0.001.

Discussion

The results presented above simultaneously reveal similarities and divergences between these populations of *L. pholis* and *A. presbyter*. For both species, the phylogeographic patterns obtained here are consistent

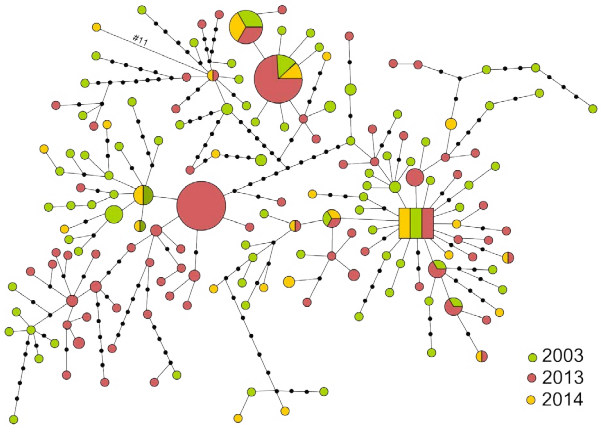

Figure1: Haplotype network for the CR of *Lipophrys pholis*. The haplotype with the highest out group probability is displayed as a square, other haplotypes as circles. The area of the circles is proportional to each haplotype frequency. Colors refer to the year of sampling. In the case where haplotypes are shared among sampling periods, shading is proportional to the frequency of the haplotype in each period.

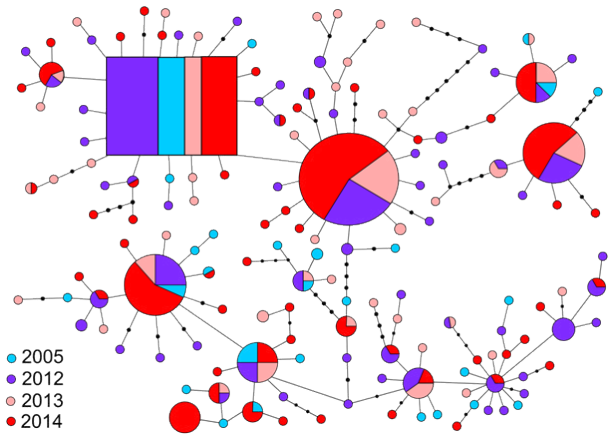

Figure 2: Haplotype network for the CR of *Atherina presbyter*. The haplotype with the highest out group probability is displayed as a square, other haplotypes as circles. The area of the circles is proportional to each haplotype frequency. Colors refer to the year of sampling. In the case where haplotypes are shared among sampling periods, shading is proportional to the frequency of the haplotype in each period.

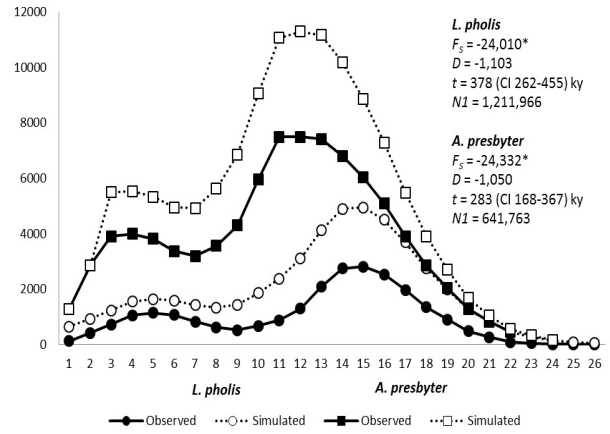

Figure 3: Mismatch distributions for the CR of *Lipophrys pholis* and *Atherina presbyter*. The lines represent the observed frequency of the pairwise differences among haplotypes; the dashed lines indicate the expected distributions based on the model of sudden population expansion. Circles – *L. pholis*. Squares – *A. presbyter*. Neutrality tests: *Fs* (Fu's), *D* (Tajima's). Mismatch distributions: *t* time in years (upper and lower bounds of 95 % CI in parenthesis), *N1* female effective population size after the expansion.

with the range-wide population studies previously published [25,27-29]. On the other hand, *L. pholis* presents higher genetic diversity levels and higher percentage of private haplotypes between sampling periods when compared with *A. presbyter* (Table 1).

The genealogies of both species confirmed signatures of events clearly older than the Last Glacial Maximum (LGM, ~18kya) [55], with lineages coalescing in early Pleistocene. However, when one takes a closer look, it is possible to notice that the shanny population is older (Figure 3 and 4). It is a known fact that the North Atlantic surface water cooled between 4ºC and 5ºC during the LGM [56], therefore, waters around the Iberian Peninsula were suited for bearing populations of the shanny [25,28], but not for the sand smelt, who must have been pushed southwards during glacial times [27,29].

This work highlights the fact that temporal changes in the gene pool composition need to be taken into account when evaluating population structure. Although no global temporal structure was found for both species, *L. pholis* presented significant differences among the most recent sampling periods. Possible explanations for this difference between these species are related to their early life-history traits. Having a relatively long PLD allows for potentially long dispersal in the shanny, meaning that this species is more prone to interannual oceanographic fluctuations. As expected, this affects its recruitment

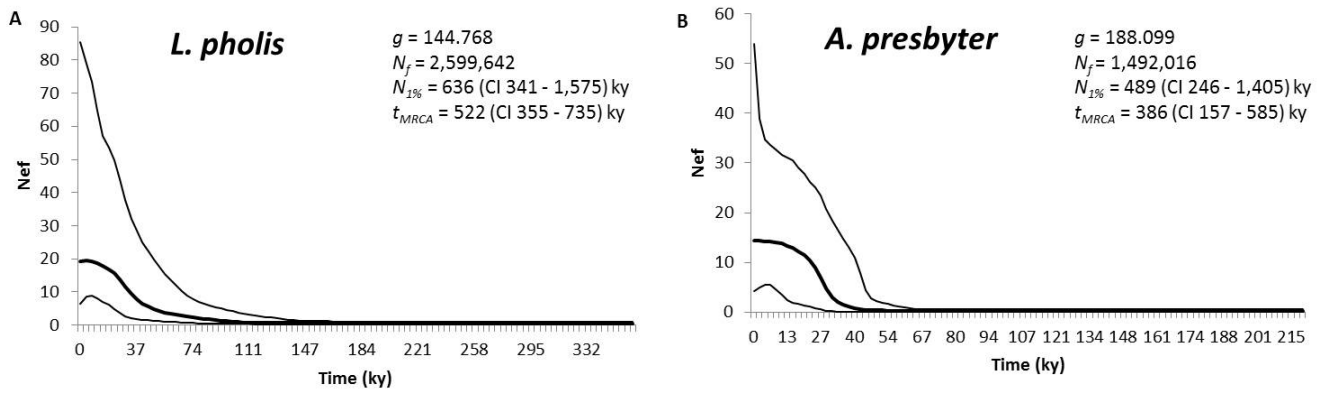

Figure 4: Bayesian skyline plot for the CR of *Lipophrys pholis* and *Atherina presbyter*. The solid line indicates the median estimate, whereas the thinner lines indicate the 95% credibility intervals. A) *Lipophrys pholis*. B) *Atherina presbyter*. Nef - female effective population size. Estimates of population parameters with LAMARC: *g* growth rate, N_f female effective population size, and $N_{1\%}$ age of population, accessed as the age at which N_f drops below 1%. Estimates of t_{MRCA} time to most recent common ancestor with BEAST.

Species	Sampling period	*N*	N_h	*%Ph*	*R*	h	π	*k*
Lipophrys pholis	2003	30	26	69.23	24.267	0.991	0.031	11.614
	2013	97	73	87.61	24.604	0.988	0.028	10.844
	2014	99	88	93.18	27.019	0.997	0.031	11.977
	All	226	171	-	-	0.995	0.030	11.522
Atherina presbyter	2005	34	29	68.96	27.235	0.986	0.022	8.012
	2012	91	63	69.84	26.531	0.984	0.021	7.780
	2013	61	48	68.08	27.975	0.992	0.021	7.866
	2014	95	56	62.5	23.643	0.975	0.022	8.054
	All	281	155	-	-	0.984	0.021	7.931

Table-1: Diversity measures for sampling periods of *Lipophrys pholis* and *Atherina presbyter* based on CR: number of sequences (*N*), number of haplotypes (*Nh*), percentage of private haplotypes (%*Ph*), haplotype richness (*R*), haplotype diversity (*h*), nucleotide diversity (π) and mean number of pairwise differences (*k*).

and, consequently, its genetic temporal structure. Similar results have been found for other species with high dispersal capabilities like the white sea-bream *Diplodus sargus* [57] and the orange-spotted grouper *Epinephelus coiodes* [58]. On the other hand, the sand smelt presents a constant and stable temporal structure, which is in accordance with the expectations, due to its demersal eggs, well developed larvae and adults' swimming capabilities. These features cause *A. presbyter* to present population structure through its distributional area [27] and can also explain its temporal stability. Temporal stability was also found in other marine organisms such as the silver sea bream *Pagrus auratus* [59], the sole *Solea solea* [6] and the northern krill *Meganyctiphanes norvegica* [60]. The interspecific differences found in the present study strengthen the view that life-history features may significantly influence the distribution of genetic diversity and population structure of marine organisms.

Nevertheless, caution should be taken when interpreting these results as the species connectivity might be driven by other early life-history traits, such as larval retention and behaviour. Recruitment is largely affected by larval survival which, in turn, is potentially affected by any biotic, oceanographic or meteorological event [61].

In this work, the estimated effective population size per generation was much higher for the sand smelt, for all periods considered. A high Ne value was also found for other fish species (*Gadus morhua* [62]; *Solea solea* [6]). Genetic stochasticity may play a smaller role in populations with such a large Ne. On the other hand, the shanny presented lower estimates of Ne per generation, which is in accordance with the temporal structure found for some of the periods and its higher vulnerability to stochastic events. Similar results were found for the Ne estimates of *Pagrus auratus* [63], *Raja clavata* [64] and *Clupea harengus* [65], to give only a few examples. Also important is to consider possible migration movements in metapopulations, as gene flow will override the effects of random genetic drift, thus biasing temporal estimates of Ne [66].

In the future, more sampling locations, larger temporal intervals, and additional markers are needed in order to improve the reliability, relevance and generalization of the present results. Further studies with other organisms with varied ecology; life-history and commercial value should also be conducted using this framework in order to detect global patterns, allowing us to predict their ability to adapt to future changes.

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