Development of 27 New Microsatellite Markers for the Shanny *Lipophrys Pholis*

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Short Report

Keywords: Microsatellite, Genetic diversity, Blenniidae, Lipophrys pholis

Posted Date: January 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1279170/v1

Abstract

The shanny *Lipophrys pholis* is an intertidal fish widely distributed throughout the Northeast Atlantic. Characterized by limited adult mobility and a long pelagic larval duration, the shanny stands as an ideal model to better understand larval dispersal and connectivity dynamics, which are critical parameters implicated in marine conservation and management. To this aim, we developed 27 highly polymorphic microsatellite markers, presenting an average allelic diversity of 20.1 alleles per locus and heterozygosity ranging from 0.619 to 1. This set of newly developed microsatellite markers will be useful in providing critical insight into the processes shaping *L. pholis* gene flow and connectivity patterns and can be used to investigate local parentage lineages.

Introduction

Connectivity among geographically distant populations is a key aspect of marine organisms' ecology, as it shapes metapopulation dynamics, determines genetic diversity, and drives resiliency of populations to environmental disturbances and exploitation [1]. Understanding population connectivity has proven valuable in identifying dispersal-related processes, with important consequences for conservation and management through the design of ecologically relevant protected areas and fisheries management units [2–4]. These connectivity dynamics have been shown to vary widely according to life-history traits and oceanographic characteristics among marine organisms, including intertidal fish [5]. In particular, pelagic larval duration has long been associated with dispersal capabilities, with often greater genetic homogeneity for species with a long planktonic phase [6, 7].

This may be the case of the shanny *Lipophrys pholis* (Linnaeus, 1758, Pisces: Blenniidae), a common resident fish of the intertidal rocky shores of the Northeast Atlantic. *L. pholis* presents a wide distribution, ranging in latitude from Norway to Mauritania and in longitude from the Azores and Madeira in the Atlantic to the Western Mediterranean [8]. Due to its high abundance as well as its restricted home range [9, 10], it has been used as a model species in marine physiology [11–13] and in studies of exposure to genotoxins [14, 15]. Its capacity to act as a sentinel species makes this species especially useful for biomonitoring ecosystem health, namely for detecting organic contaminants [16, 17] as its life-history traits reflect local environmental conditions: in particular, after a long larval dispersal period of 57 to 73 days [18], they remain in the same intertidal pool for the rest of their life cycle, and adult migration is limited to a few metres [19]. These characteristics also make it an ideal model for investigating differentiation in marine fishes with a long pelagic larval duration.

Earlier genetic studies, based on mitochondrial DNA, suggest that *L. pholis* Atlantic opulations can be separated into two groups: a population comprising fish ranging from the European coastline (UK to Morocco) to Madeira, and a genetically distinct Azorean group, with a lack of phylogeographic structure and high genetic diversity throughout its range implying large-scale connectivity between shanny populations along the European coastline [20, 21]. These patterns of high genetic connectivity do not necessarily oppose small-scale demographic processes [22]. In fact, cross-scale studies linking local and

broader-scale patterns of larval exchange are considered important areas of research [23] and have proven valuable in shedding light on scale-related demographic exchange [24], suggesting that *L. pholis* may present yet undetected finer scale genetic differentiation patterns. Most of the previous genetic studies on *L. pholis* are based on mitochondrial (CR, D-Loop, 12S, and 16S) and nuclear (S7) sequence data but do not provide information on fine-scale connectivity [20, 21, 25]. Only one recent publication has developed and used microsatellite markers but did not focus on genetic structure [26].

Microsatellites have been a widely used tool in genetic studies since the 1990s because of their high polymorphism, their abundance, and their dispersion throughout eukaryotic genomes [27]. By using a panel of several microsatellite loci, a unique genotype profile can be produced for each organism tested, leading to their individual identification. To date, only four specific microsatellite markers have been previously published for the shanny, which is insufficient to conduct population structure and parentage analysis [26]. The goal of this study was to develop an additional set of *de novo* microsatellites for *L. pholis* to extend the existing set, in order to investigate the Northeast Atlantic population structure.

Materials And Methods

Biological samples and DNA extraction

After collection, fin clips were preserved in 70% alcohol, and frozen until DNA extraction was performed at the laboratory. Genomic DNA was extracted from all samples using the QIAamp® 96 DNA QIAcube® HT robotic workstation (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA templates were then diluted at 50 ng/µL and stored at -24°C.

Development of new microsatellite markers

Microsatellite sequences were retrieved from a pool of genomic DNA of nine *L. pholis* individuals. To ensure large microsatellite screening and maximize amplification success of all samples, individuals were chosen from six sample sites spanning more than 50 kilometers of coastline in Galicia, Spain (Corrubedo Cape, Couso Cape, O Grove, Ons Island, Cies Island, Silleiro Cape). After extraction, he DNA was sent to GenoScreen (Lille, France) for high-throughput genomic sequencing and library preparation, using the approach described in Abdelkrim (2009) [28] to provide a database of microsatellites. The paired-end 2x250 sequence run was done on an Illumina MiSeq platform, and the resulting reads were merged by the Usearch software. Final analysis of the raw sequences and primer design was done using QDD v.3. Following this initial sequencing step, 2331 primer pairs were obtained, and 50 pairs were selected among them based on repeat number, motif (di-, tri-, or tetranucleotide), and PCR product size (≥ 100 bp). Primers were then tested individually using Polymerase Chain Reaction (PCR) at temperatures ranging from 53°C and 65°C to determine optimal hybridization temperature. PCR was performed with 11 µl reaction volume, containing 5 µL of Master Mix Type-it 2X (Qiagen) containing all materials required for the PCR amplification (HotStarTaq *Plus* DNA Polymerase, dNTP mix with 200 µM each, and PCR

buffer containing 6mM $MgCl_2$), 6 µL RNase-free water, 1 µL of forward and reverse primers (2 µM diluted in TE pH 8 buffer), and 1 µL containing 50 ng of genomic DNA. PCR conditions were: an initial denaturation step at 95 °C for 5 min, 40 cycles of 95°C for 30 s, chosen annealing temperature (53°C to 65°C) for 90 s, 72 °C for 30 s, and a final 30 min extension step at 60 °C. Electrophoresis was carried out at 100 V on 2% agarose gels for microsatellite size characterization as well as to detect polymorphism preliminarily. Thirty microsatellites were chosen and included in five multiplex panels designed by combining loci of different allele sizes and identical primer annealing temperatures. In order to proceed with genotyping, forward primers were labeled with fluorescent dyes FAM, YAKYE, ATTO550, and ATTO565 (Eurofins, Luxembourg). Amplifications were carried out with PCR conditions identical as previously described. PCR products from all samples were sent to GenoScreen for fragment visualization using an Applied Biosystems 3730 Sequencer, where an internal ladder was added to each sample to size accurately (GeneScan 500 LIZ, Applied Biosystems). Allele sizes were scored in GENEMAPPER software v.5 (Applied Biosystems), with ambiguous peaks assigned as missing data to avoid erroneous genotyping.

Data analysis

For this study, 42 *L. pholis* samples originating from Ons Island (42°23'28.4 N "8°55'24.0 W) within the Islas Atlánticas Marine Park (Pontevedra, Spain) were used. The software MICRO-CHECKER v 2.2.3 [29] was used to detect null alleles, potential scoring errors as well as test for large allele dropout. Allele frequencies, total number of alleles N_a and effective number of alleles N_e were calculated in GenAlEx 6.503 [30]. Observed (H_o) and expected (H_e) heterozygosities were identified through GENETIX v 4.05.2 [31], which was also used to compute $F_{IS,}$ the inbreeding coefficient, and linkage disequilibrium (LD).

Results And Discussion

Out of the 50 primer pairs that were tested in this study, 30 polymorphic loci exhibiting clear amplification profiles were successfully developed. Analysis with MICRO-CHECKER revealed the presence of null alleles in three loci (AG08, AC17, AGG47). These loci were removed from the dataset for further analysis, and no large allele dropout or allele scoring error detected in the rest of the marker set. No significant linkage disequilibrium was detected between locus pairs, which is consistent with the previously used S7 nuclear marker for which a relatively low value of 13.67% of locus pairs was in LD [24].

The new panel of microsatellite markers was characterized by an almost equal distribution of motif abundance percentages, with a majority of di- (37%) followed by tri- (33%) and tetranucleotide repeats (29%), compared to previously developed markers which were exclusively dinucleotides [26]. Although less abundant than dinucleotides in the genome, tri- and tetranucleotide repeats are traditionally sought-after due to the large number of base-pairs differentiating alleles: this makes variations in the number of repeats simpler to identify during the genotyping process, therefore removing possible biases, for

instance stuttering [32, 33]. In fine, 27 loci were successfully developed, with the screening of 42 individuals revealing a total of 568 alleles in the dataset for the Ons Island location, which was deemed robust enough to study the structure and parentage of the *L. pholis* population [34].

Table I Characterization of the 27 microsatellites developed for this study

Locus	Primer sequence	Repeat motif	T_a (°C)	Size range (bp)	N	N_a	N_e	H_e	H_o	F_{IS}	Genbank accession number
AAAC45	F:GGTTAAAGTCCAGTCTTGATGCC R:ATCACCTGTGAGCCACATGTT	$(TGTT)_{14}$	53	122 - 242	41	18	9.8	0.9091	0.9268	-0.01979	OL690375
AAC46	F:ACCAGATTAAACTGTGACCAGCA R:CCTGAGCTTCAGTCTCCAGC	$(TTG)_{14}$	53	117 - 165	41	13	3.9	0.7498	0.7317	0.02439	OL690376
AAG27	F:TCCTTGAGAAGTGGGAATGGT R:TGGACAAGAACCAAGTGATGAG	$(AAG)_{21}$	53	197 -320	42	14	5.9	0.8399	0.881	-0.04946	OL690377
AC23	F:CTTGACACTCTGGTCGCTGG R:CGGATTCTATATCAATGCGATGGC	$(TG)_{24}$	53	264 - 302	41	8	4.3	0.7772	0.7805	-0.00432	OL690378
AGC06	F:TACCTTTCCTGCTCCCTGTCT R:AATCAGCTTCCATCTGAGAACT	$(TGC)_{32}$	53	181 -295	41	29	18.7	0.9581	1	-0.04425	OL690379
ATCC29	F:AGTCATCACACCAGTGCAGAA R:GCCTGGAACTTAGGGACACA	$(CCAT)_{20}$	53	206 - 290	42	15	10.1	0.9116	0.8571	0.06047	OL690380
ACAT28	F:GCATGAAGGCCCACTGGT R:TAGTTGAGAGTCACCTGGGA	$(ACAT)_{21}$	55	118 -210	42	17	9.1	0.9005	0.881	0.02192	OL690381
AC15	F:ACACTCTGTCCTCAGCTTGG R:ACAGAAACTCAAGTTGCCGC	$(GT)_{27}$	55	265 - 367	42	27	11.5	0.924	0.8571	0.07316	OL690382
ATC31	F:GAGGAAGAAGATCGAGCCGG R:TGAGACAAAGCTGCTGGAGG	$(TGA)_{19}$	55	242 - 366	42	16	7.9	0.8835	0.9048	-0.02433	OL690383
AT43	F:TTCTTGCTCTCGGAATCGGG R:CAAATGCACTCACCAGAGGT	$(TA)_{14}$	55	253 - 291	41	17	4.9	0.8043	0.8049	-0.00076	OL690384
AC41	F:ACGACATGTGTACTTCCTGCA R:GGTTTCATTCACAGCCGCAG	$(AC)_{16}$	57	103 - 181	42	20	6.3	0.8514	0.881	-0.03514	OL690385
AGAT02	F:TGATCCATATTGCATGCACATG R:AGAAATTGCTGATGTCCGGT	$(ATCT)_{37}$	57	218 - 354	42	24	17.0	0.9527	0.9286	0.02559	OL690386
AGC25	F:TGACACATGTGCTCCAGTGG R:GGACACGGAGACATGCTCAT	$(AGC)_{24}$	57	157 - 253	42	21	13.5	0.9372	0.9286	0.00929	OL690387
AG11	F:CGCAGCGCTCTGGATTAAAC R:GCTTCAGTAACAGGTCGCCT	$(TC)_{29}$	57	143 - 213	41	24	17.5	0.9545	1	-0.04826	OL690388
AG16	F:GTCGGCATTAGCACACGTTG R:AACTGAAAGCCTGCTGTGGT	$(AG)_{27}$	57	260 - 338	40	21	15.3	0.9465	0.975	-0.03049	OL690389

Table I (continued)

ATCC40	F:ATGTTCAGAGGCTCCATCGC R:AAGTACGAGCCAGTGAGTGT	$(GGAT)_{16}$	57	225 - 325	41	20	11.4	0.9238	0.9512	-0.03004	OL690390
AGAT09	F:GACGCACCCTAACAGCTCTG R:GGAAGGAGACCAAGGACAGC	$(GATA)_{29}$	60	208 - 421	42	35	24.5	0.9707	0.9524	0.01914	OL690391
AGG49	F:TCAGACGAACTCGGAGGTCC R:TTGCCCTGACATCCATCTGG	$(GAG)_{12}$	60	227 - 278	42	10	4.8	0.8032	0.6905	0.14183*	OL690392
ATC30	F:CTGACGCACCCTCACTATGT R:AGCTGCTATACCCTAGTATTGAGA	$(ATC)_{19}$	60	276 - 384	42	19	6.5	0.8571	0.9286	-0.08444	OL690393
ATC44	F:AGAAACCTGCCTTGCTTCAT R:CCACCAACCCAAACTCCCAT	$(CAT)_{14}$	60	202 - 277	42	17	10.4	0.9145	0.9286	-0.01556	OL690394
AT50	F:ACAAGGCATGAAATTGAGTTCCC R:TGACTGTATGGGAGAATATTGGCA	$(AT)_{12}$	60	180 - 206	42	8	2.8	0.6566	0.619	0.05789	OL690395
AGAT04	F:TCATTGCATTTATCATCTTTGGAATTT R:GACCGCCTGACCGATAACAA	$(TAGA)_{34}$	63	208 - 380	42	25	14.8	0.9438	0.9286	0.0163	OL690396
AGC33	F:GTTCCTCGGCTCAGAGCTTT R:AGGAGGGACAATTTGGACGC	$(CTG)_{18}$	63	215 - 356	41	27	14.4	0.9419	0.9512	-0.01004	OL690397
AG20	F:GAAGAGACGCCGGAGTGAAG R:ACGCTCCTCCTGGAAGTCTT	$(CT)_{25}$	63	261 - 311	42	13	6.6	0.858	0.8333	0.02909	OL690398
ATCC38	F:GTCCATGTCCATCCAGCCAT R:GCGACATGTCCTGGGTGTAT	$(CATC)_{17}$	63	119 - 179	41	11	3.9	0.7528	0.6585	0.12657	OL690399
AG22	F:TCCTTAACTGAATCCATATGACTGT R:CACTGAAGGCGGTACTCAGG	$(CT)_{25}$	63	179 - 377	42	31	17.0	0.9527	0.9286	0.02559	OL690400
AG10	F:ACCTCAAATACACCGTGCTTCA R:CTCCTGCGTGCACTCATTGA	$(CT)_{29}$	63	212 - 364	42	42	25.4	0.9722	0.9286	0.04537	OL690401
AG08	F:CACGGTGAGTCAGGAGTTGT R:TCCACGCTGTAAAGGCCATG	$(GA)_{30}$	-	-	-	-	-	-	-	-	OL690402
AC17	F:CGCTGAGATAAGCTGCACCA R:GACGTCACCCATCAGTTGGT	$(TG)_{27}$	-	-	-	-	-	-	-	-	OL690403
AGG47	F:GGAGCACAAGTCAGGACCAT R:GTCTTGACAGGGCAGCATGA	$(GAG)_{13}$	-	-	-	-	-	-	-	-	OL690404

Ta, annealing temperature in degrees; N, number of samples; Na, number of alleles; H_o, observed heterozygosities; H_e, expected heterozygosities; F_{IS}, fxation index following Weir and Cockerham. *indicates signifcant departures from Hardy–Weinberg Equilibrium ($p < 0.05$). AG08, AC17 and AGG47 presented null alleles.

The lowest number of alleles found per locus was 8 (AT50), with the highest 42 (AG10). On average, there were 20.074 alleles per locus (Table I). Expected heterozygosity (H_e) ranged from 0.6566 to 0.9722 with an average of 0.8832, while observed heterozygosity (H_o) ranged from 0.619 to 1 with an average of 0.8754. The difference between H_e and H_o was less than 1% with slightly fewer heterozygotes observed than expected. The new panel of loci showed a high level of genetic diversity and high heterozygosity levels, which is in line with the previous study on *L. pholis* using microsatellites [26], although recent findings seem to suggest this may not be the case throughout its range and may also vary according to sampling years [35, 36]. Similarly to other marine species, such high diversity is likely to be correlated with a combination of several life-history traits: pelagic mode of larval dispersal coupled with long larval duration [37–39], demersal eggs [24, 39], and large population size [40, 41]. Moreover, *L. pholis* also displayed high diversity when other genetic markers were used at a larger scale [20, 21, 25].

Only one marker (AGG49) presented a significant F_{IS} value (p-value < 0.05), suggesting heterozygote deficiency which could be attributed to inbreeding, the presence of null alleles, the Wahlund effect, or selection [42–44]. Loci with possible null alleles were discarded from the set of markers following analysis by MICRO-CHECKER, limiting their interference. Likewise, there is little chance that the Wahlund effect influenced the results since sampling was performed in sample sites with interconnected tidepools, and therefore unlikely to present population substructure.

The successful amplification of a large number of markers (27 *de novo* markers) and the high levels of polymorphism found in the sample suggests that these newly developed microsatellite markers can be

appropriately used in *L. pholis* to make inferences about genetic diversity, family structure and to assess potential dispersal patterns. Having a large panel of microsatellite markers is fundamental in studying patterns of connectivity among populations and these newly developed markers will help shine a light on gene flow patterns in future genetic studies.

Declarations

Acknowledgments

We thank C. Tardy for her help with genotyping and data analysis.

Funding

This work is framed within the project ORGANISE in which A. B. was supported by the Axencia Galega de Innovación (GAIN), Xunta de Galicia (grant ED481B 2018/68).

Compliance with ethical standards

The authors declare that they have no conflict of interest.

Ethical approval

Sampling was conducted by A. B. under a permit delivered by the Atlantic Islands of Galicia Maritime-Terrestrial National Park. A. B. has been trained, is aware of and fully complies with all current ethical guidelines of the European Commission (Article 9 of the Directive 2010/63/EU) and national legislation regarding animal research.

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