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Genetic characterization of wild, broodstock and seed samples of *Polititapes rhomboides* (Bivalvia: Veneridae): Implications for hatchery seed production

Ginna M. Chacón [a], Alberto Arias-Pérez [a,1], Ruth Freire [a,2], Luisa Martínez [a], Justa Ojea [b], Ana Insua [a,*]

[a] Departamento de Bioloxía-Facultade de Ciencias and Centro de Investigacións Científicas Avanzadas (CICA), Universidade da Coruña, 15071 A Coruña, Spain
[b] Centro de Cultivos Marinos de Ribadeo-CIMA, Xunta de Galicia, 27700, Ribadeo, Lugo, Spain

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ABSTRACT

The banded carpet shell *Polititapes rhomboides* is a fishery resource and a suitable candidate for the development of native clam aquaculture in Europe. Here, we analyzed nine microsatellite loci in wild samples to provide estimates of genetic diversity and population differentiation for NW Spain, the main production area of this clam. We also analyzed wild-caught broodstock and hatchery-reared seed to investigate the genetic consequences of seed production for stock enhancement purposes by mass spawning. We examined the seed sample as a whole (total seed), but also subdivided it into three size classes to assess the genetic consequences of size grading. While wild samples, broodstock included, displayed minimal or no genetic differences, the total seed showed a reduction in allelic richness (12–24 %) and a significant level of differentiation (F_{ST} = 0.026−0.043) with respect to wild samples. After performing parentage assignment, we detected a drastic reduction in the effective number of breeders (N_e) compared to the census number (85 %), and an inbreeding rate of 0.036 per generation. The low N_e and high inbreeding rate were mainly due to high variance in reproductive success. The seed size classes also showed a decrease in allelic richness and significant genetic differentiation, but we did not find significant differences in parental contribution to each size class. Our results may help the genetic-assisted management of wild populations, give insight into the genetic composition of the seed produced by mass spawning and provide a basis for the development of more effective hatchery practices and sustainable stock enhancement programs in *P. rhomboides*.

1. Introduction

European production of clams, cockles and arkshells is dominated by the Japanese carpet shell *Ruditapes philippinarum,* a clam endemic to Indo-Pacific waters and introduced in Europe in the second half of the 20th century (Flassch and LeBorgne, 1992; Jensen et al., 2004), where it has now naturalized and even hybridizes with native species (Hurtado et al., 2011; Habtemariam et al., 2015). According to FAO statistics (www.fao.org) from 2013 to 2017, the global production (capture and aquaculture) of this non-native species was over 33,000 tonnes per year. The production of native clam species such as the grooved carpet shell *Ruditapes decussatus*, the pullet carpet shell *Venerupis corrugata* and the banded carpet shell *Polititapes rhomboides* amounted to around 6,000, 2, 100 and 500 tonnes, respectively, during that same period. In Spain—-specifically in the northwest, where 90 % of the Spanish clam production is harvested—production of *R. philippinarun* in 2013–2017 was similar to that of the three other clams combined (www.pescadegalicia.gal). The success of *R. philippinarum* production can be attributed to several causes including high fecundity and growth rates, successful cultivation and commercial production of spat (CABI, 2020). This paradoxical situation, in which a non-native species monopolizes the production of clams, warns of the need to rethink the priorities for aquaculture activities on the European coast. Aquaculture development of European native clams would increase the diversification of species in the market, stimulate local economic development and preserve the regional gastronomic identity.

* Corresponding author.
E-mail addresses: ginnamargchaconr@gmail.com (G.M. Chacón), alberto.arias@uca.es (A. Arias-Pérez), ruth.freire.alvarez@gmail.com (R. Freire), m.l.martinez@udc.es (L. Martínez), justa.ojea.martinez@xunta.gal (J. Ojea), ana.insua@udc.es (A. Insua).
[1] Present address: Departamento de Biomedicina, Biotecnología y Salud Pública, Universidad de Cádiz, 11510, Puerto Real, Cádiz, Spain.
[2] Present address: Institute for Quantitative Genetics and Genomics of Plants, Heinrich Heine University Düsseldorf, 40225, Düsseldorf, Germany.

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As in *R. philippinarum* and other aquaculture species, European native clam production could be increased through hatchery-based stock enhancement programs. In such programs, individuals are spawned and reared for a while in a hatchery and then released into a wild area of interest, where they are expected to contribute to the reproductive output of the enhanced population and to the fishery harvest (Bert et al., 2007). Although this practice has become very common for many species, it requires a careful genetic monitoring to minimize negative effects on genetic diversity, population structure, fitness and effective population size of wild populations (Bert et al., 2007; Hedgecock and Coykendall, 2007; Camara and Vadopalas, 2009; Fisch et al., 2015; Grant et al., 2017).

The first aspect to consider in hatchery-based stock enhancement is the management of the broodstock and the offspring produced. The origin, number, sex ratio and mating system of the broodstock determine the genetic suitability of the offspring for stock enhancement purposes. Since hatchery-produced offspring can be released at sites other than where the broodstock was collected, population genetic structure should be considered because of potential genetic differences. In this scenario, releasing hatchery-produced offspring may reduce genetic diversity within wild populations, reduce genetic differences between populations and disturb potential adaptations to local environmental conditions (Johnson, 2000; Bert et al., 2007; Grant et al., 2017). In addition to the use of breeders genetically compatible with the recipient wild population, broodstock number should also be taken into account given that it determines the genetic diversity of progeny. The high fecundity of many aquaculture organisms makes the production of large numbers of offspring from a small number of broodstock possible. However, these offspring might capture a limited proportion of the natural genetic diversity, increasing the risk of inbreeding depression in subsequent generations. Similar effects can be expected when a skewed sex ratio is used or an unequal contributions of broodstock individuals to the progeny occurs (Bert et al., 2007; Grant et al., 2017). To save labour, hatcheries commonly use mass spawning, which consists of maintaining broodstock in a tank where fertilization occurs randomly when males and females release gametes. However, this practice can have detrimental consequences by increasing the variance in reproductive success due to unpredictable contributions of individual breeders (Lind et al., 2009). On the other hand, hatchery offspring management such as size grading to reduce size variation and increase survival, could also potentially impact genetic diversity, given that parental contribution to each size grade may be non-uniform (Frost et al., 2006; Borrell et al., 2011; Loughnan et al., 2013).

This study focuses on *P. rhomboides*, a species naturally distributed from Norway to the Iberian Peninsula and the Mediterranean and Atlantic coast of Morocco (Tebble, 1966). The main production area of this clam is NW Spain where it constitutes a valuable and high demand resource with a higher market price than *R. philippinarum*. Although hatchery-based stock enhancement strategies could increase production of *P. rhomboides*, genetic data to support sustainable culture and fishery management are very scarce. Only one recent work (Chacón et al., 2020) provides estimates of genetic diversity and population differentiation for the NW Spanish coast based on the cytochrome oxidase c subunit 1 (*cox1*) gene. Nuclear genetic variation was studied in a small sample from this coast to develop microsatellite markers (Chacón et al., 2013). Here, we analyze nine microsatellite loci in wild samples, a wild-origin broodstock and hatchery seed produced by mass spawning in order to make informed decisions regarding the exploitation of wild populations and seed production for stock enhancement purposes. The specific aims of this study are: (1) provide estimates of genetic diversity and population differentiation of NW Spain clams, (2) assess the efficiency of mass spawning in capturing and maintaining wild genetic diversity, (3) identify the main causes of potential genetic implications of mass spawning through individual parental contribution assessment, and (4) evaluate the genetic consequences of seed size grading in the hatchery.

2. Materials and methods

2.1. Sampling, DNA extraction and microsatellite genotyping

We sampled a total of 690 specimens of *P. rhomboides*: 300 commercial-size clams from six localities (50 specimens each) along the NW Spanish coast (Fig. 1); 91 sexually mature individuals from Vigo-Tirán used as broodstock; and 299 seeds produced by the broodstock. The seed was obtained by mass spawning which consisted of placing the broodstock in a sea water tank where fertilization took place randomly when males and females spontaneously released gametes. Following fertilization, we sexed the broodstock by detection of eggs or sperm in gonad tissue. To simulate the size grading practice, we split the seed into three size classes: large (~7 mm), medium (~5 mm) and small (~3 mm). Dissected adults and seed were preserved in 96 % ethanol.

We extracted genomic DNA from muscle or foot tissue of adults according to Fernández-Tajes and Méndez (2007) or following a Chelex-boiling extraction procedure (modified from Estoup et al., 1996), where a small piece of tissue ($1-2$ mm^3) is incubated in 100 μL of a 10 % Chelex® 100 (Sigma-Aldrich) solution at 100 °C for 20 min. We also employed this procedure for DNA extraction from seed using either the whole individual or a small piece of tissue depending on the specimen size.

We genotyped nine microsatellite loci (Vrb14, Vrh124a, Vrh117, Vrb35, Vrh124b, Vrh99a, Vrh257, Vrh243 and Vrb114), previously developed by Chacon et al. (2013). We carried out multiplex reactions in a 12.5 μL volume with 100 ng of DNA, $0.1-0.5$ μM of each primer (see Chacón et al., 2013) and 1x QUIAGEN Multiplex PCR Master Mix. The thermal cycler protocol consisted of 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 90 s at 57 °C and 60 s at 72 °C, followed by 30 min at 60 °C. Amplification products were loaded into an ABI PRISM 3130xl sequencer (Applied Biosystems) and analyzed using GeneMapper v.3.7 (Applied Biosystems).

2.2. Data analysis

We used data from wild samples, broodstock and hatchery-produced seed. But we analyzed the seed sample as a whole (hereafter referred to as total seed) to evaluate the efficacy of mass spawning for the conservation of wild genetic diversity, and also divided it into three size classes (large, medium and small) to assess the genetic consequences of size grading. We calculated the number of alleles, observed heterozygosity (H_o) and unbiased expected heterozygosity (H_e) of Nei (1978) for each microsatellite locus and sample with Genetix v.4.05.2 (Belkhir et al., 2004). We obtained the allelic richness (R_s), a measure of genetic diversity that compensates for uneven population sample size, with Fstat v.2.9.3 (Goudet, 2001) using a rarefaction method. Two estimates of R_s were obtained: one considerings all wild samples plus the total seed (R_s^1) and one considering the wild samples plus the seed size classes (R_s^2). Differences in R_s, H_o and H_e among samples were tested by a Friedman test with the statistical package SPSS 16.0 (SPSS Inc.). When p-values for the Friedman test were significant, we performed a *post hoc* analysis based on the Wilcoxon-Nemenyi-McDonald-Thompson procedure (Hollander and Wolfe, 1999) using an R function (Galili, 2010). We estimated null allele frequencies for each locus and sample with the FreeNA software (Chapuis and Estoup, 2007).

We tested Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between pairs of loci within each locality using Genepop v.4.0 (Rousset, 2008). We also used this program to calculate the inbreeding coefficient (F_{IS}) following Weir and Cockerham (1984). To determine statistical significance, we employed a Markov chain method using 10,000 dememorizations, 5,000 batches, and 5,000 iterations per batch. To check for departures from selective neutrality, we used both the Ewens–Watterson homozygosity test (Watterson, 1978, 1986) and the Ewens–Watterson–Slatkin exact test (Slatkin, 1994, 1996) using Arlequin 3.11 (Excoffier et al., 2005).

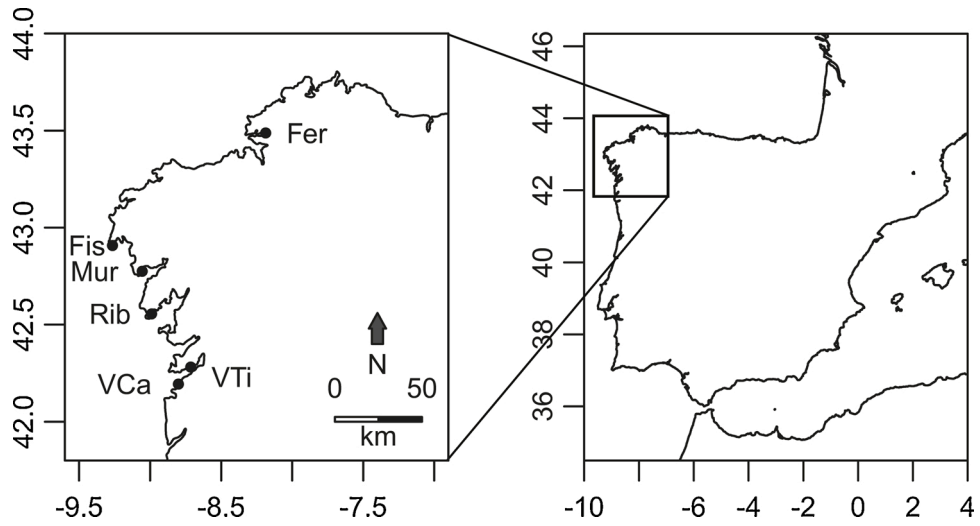

Fig. 1. Location of sampling sites of *P. rhomboides* along NW Spain. Fer: Ferrol. Fis: Fisterra. Mur: Muros. Rib: Ribeira. VTi: Vigo-Tirán. VCa: Vigo-Canido.

To assess genetic differentiation among samples, we computed the pairwise F_{ST} statistic of Weir and Cockerham (1984) in Genetix, and determined statistical significance by a nonparametric permutation approach (10,000 permutations). We also calculated F_{ST} values using FreeNA software, which implements the Excluding Null Allele (ENA) correction method to avoid positive bias induced by the presence of null alleles (Chapuis and Estoup, 2007). In addition, we performed a hierarchical analysis of molecular variance (AMOVA) locus by locus (10,000 permutations) in ARLEQUIN v.3.11 (Excoffier et al., 2005). Finally, to perform a Bayesian clustering analysis we used the program STRUCTURE v.2.3.1 (Pritchard et al., 2000). The number of potential clusters (K) was set from 1 to the number of predefined populations (8 and 10) and 10 runs were computed for each value. To obtain the average permutated individual Q-matrices, we used a burn-in and Markov chain Monte Carlo repetitions of 100,000 and 250,000 in CLUMPP v.1.1.2 (Jakobsson and Rosenberg, 2007) and we plotted results in DISTRUCT (Rosenberg, 2004).

We used the sequential Bonferroni correction (Rice, 1989) to adjust significance values when multiple tests were performed.

We carried out parentage assignment using the maximum likelihood approach implemented in CERVUS 3.0.7 (Kalinowski et al., 2007). CERVUS calculates the broodstock allele frequencies and provides several statistics, such as number of alleles, polymorphic information content (PIC), non-exclusion probabilities and null allele frequency to determine the suitability of loci. The frequency of each allele for each locus is then used in a simulation analysis to examine the feasibility of parentage analysis and to calculate critical values of likelihood ratios (Delta values). Based on these critical values of likelihood ratios, the confidence of parentage assignments is determined when parentage analysis with real data is finally carried out. For simulation analysis, we selected the option “parent pair (sexes known)” and the following parameters: 10,000 offspring, 100 % of loci typed, 1 % of loci mistyped, a minimum number of five loci typed and Delta confidence levels of 95 %.

We used chi-square goodness of fit tests to explore the differential contribution within females and males. To test differential contribution of female and male broodstock to the different size classes, we applied a re-sampling procedure described in Borrell et al. (2011). One hundred thousand contingency tables were pseudo-randomly generated while keeping the observed marginal totals. The frequency of tables where a parent showed a number of offspring equal or higher than the observed was computed. Low frequencies (less than 5 %) suggested departures from the null hypothesis of equal contribution to the three size groups. We conducted the procedure in the R statistical environment (R Development Core Team, 2019) and generated contingency tables with the "r2dtable" function of the "stats" package.

We calculated the effective number of breeders (N_e) with the formula $N_e = 4N_dN_s/(N_d + N_s)$ (Falconer, 1989), where N_d and N_s are the census number of female and male broodstock, respectively, to account for the effect of unequal sex ratio and the number of parents used as boodstock. The same calculation was performed using the number of dams and sires assigned in parentage analysis to take into account that not all broodstock individuals may contribute to the offspring. We also calculated N_e according to $N_e = 4N_{ed}N_{es}/(N_{ed} + N_{es})$, where N_{ed} and N_{es} are the effective number of dams and sires, respectively, to take also into account the differences in reproductive success. We calculated N_{ed} following Lande and Barrowclough (1987) as $N_{ed} = (N_dK_d - 1)/[K_d + (V_d/K_d) - 1]$, where N_d is the number of dams, K_d the mean number of offspring per dam and V_d the variance of K_d. This same method was used to calculate N_{es}. We determined the rate of inbreeding (ΔF) as $\Delta F = 1/(2N_e)$ according to Falconer (1989).

3. Results

3.1. Genetic diversity and differentiation in wild samples and hatchery-reared seed

We genotyped nine microsatellite loci (Table S1) in a total of 690 individuals: 391 wild individuals from six localities, including 91 used as broodstock, and 299 offspring seed (analyzed as a whole and also classified in three size classes). All loci were polymorphic with a number of alleles per locus between six and 34. Fifty-eight out of the 99 locus-sample combinations conformed to HWE after sequential Bonferroni correction. Aside from six with negative F_{IS} values, all departures from HWE showed positive F_{IS} values. Linkage disequilibrium tests performed by sample revealed 52 significant comparisons after sequential Bonferroni correction, all but one in the seed samples. None of the two neutrality tests showed deviations from neutrality for any locus-locality combination after sequential Bonferroni correction (data not shown).

Genetic variation statistics per sample are shown in Table 1. Bro displayed the largest number of alleles (117) and LSe the lowest (58). Except for VTi and LSe, all samples displayed private alleles at low frequencies (≤ 0.02). A loss of 13 alleles was detected when comparing the total seed to the broodstock. This loss was more pronounced when size classes were taken into account (LSe: 59; MSe: 21; SSe: 27). Bro displayed the highest R_s ($R_S^1 = 11.028$; $R_S^2 = 8.618$) and See ($R_S^1 = 8.368$) and LSe ($R_S^2 = 6.444$) the lowest. Although we detected a reduction in the R_s of the seed compared to that of the broodstock and the other wild samples (12–24 % in R_s^1), especially in the case of LSe

Table 1
Genetic diversity of *P. rhomboides* samples.

Samples (code)	N	N_A(average)	P_A	R_s^1	R_s^2	H_e	H_o
Ferrol (Fer)	50	92 (10.222)	3	10.120	7.940	0.684	0.574
Fisterra (Fis)	50	86 (9.556)	1	9.482	7.648	0.669	0.535
Muros (Mur)	50	94 (10.444)	3	10.320	7.939	0.660	0.557
Ribeira (Rib)	50	93 (10.333)	3	10.203	7.972	0.690	0.560
Vigo-Tirán (VTi)	50	94 (10.444)	0	10.350	7.972	0.680	0.563
Vigo-Canido (Vca)	50	92 (10.222)	3	10.132	7.876	0.687	0.606
Boodstock (Bro)	91	117 (13.000)	2	11.028	8.618	0.687	0.592
Total Seed (See)	299	104 (11.556)	2	8.368		0.662	0.592
Large seed (LSe)	23	58 (6.444)	0		6.444	0.598	0.473
Medium seed (MSe)	174	96 (10.667)	1*		6.793	0.657	0.592
Smal seed (SSe)	102	90 (10.000)	1*		6.930	0.679	0.618

N: Sample size. N_A: Total number of alleles (average number of alleles per locus). P_A: Private alleles. * Private alleles included within the total seed. R_s^1: Average allelic richness based on a minimum sample size of 48 individuals. R_s^2: Average allelic richness based on a minimum sample size of 23 individuals. H_e: Average expected heterozygosity. H_o: Average observed heterozygosity.

Table 2
Pairwise F_{ST} values between samples of *P. rhomboides* without the ENA correction.

	Fis	Mur	Rib	VTi	VCa	Bro	See[a]	LSe[b]	MSe[b]	SSe[b]
Fer	0.000	0.008*	0.000	0.002	0.000	0.000	0.028**	0.056**	0.028**	0.025**
Fis		0.009*	0.002	0.003	0.003	0.001	0.026**	0.043**	0.028**	0.023**
Mur			0.002	0.002	0.002	0.010**	0.050**	0.064**	0.052**	0.047**
Rib				0.000	0.000	0.003	0.035**	0.058**	0.036**	0.031**
VTi					0.005	0.003	0.030**	0.041**	0.030**	0.030**
VCa						0.005*	0.043**	0.063**	0.044**	0.040**
Bro							0.029**	0.049**	0.030**	0.026**
See								–	–	–
LSe									0.014*	0.021*
MSe										0.001

[a] Sequential Bonferroni correction carried out using the data from wild samples and total seed.
[b] Sequential Bonferroni correction carried out using the data from wild samples and seed size classes.
* Significant at 5% level.
** Significant after sequential Bonferroni correction.

(16–25 % in R_s^2), we only found significant differences between Bro and LSe ($P = 0.006$) and MSe ($P = 0.017$). H_e ranged from 0.598 (LSe) to 0.690 (Rib) and H_o from 0.473 (LSe) to 0.618 (SSe), with no significant differences detected between samples.

Pairwise multilocus F_{ST} analysis without (Table 2) and with ENA correction (Table S2) provided similar levels of genetic differentiation. Wild samples, including Bro, showed very low F_{ST} values (≤ 0.01). After sequential Bonferroni correction, only Mur and Bro showed significant genetic differentiation. On the contrary, all comparisons between wild and seed samples (both total and size class seed) showed significant differences ($F_{ST} = 0.023-0.064$). We found the largest genetic differentiation between LSe and wild samples. The seed size classes did not show significant differences. According to mean F_{ST} values, the genetic differentiation level between seed and wild samples was more than one order of magnitude larger than that detected among wild samples.

AMOVA results (Table 3) indicated that most of the variation is due to differences among individuals (96.444 %, $P < 0.0001$), but the percentage of variation explained by differences between wild and seed samples (3.063 %, $P = 0.0002$) is higher than that explained by differences among samples within the wild and the seed group (0.493 %, $P < 0.0001$). STRUCTURE analyses, with total seed (Fig. 2a) and seed size classes (Fig. 2b), both displayed K 2 as the most probable value according to the DeltaK statistic (Fig. S1), revealing two genetically differentiated clusters: one formed of wild samples and another one formed of the seed.

3.2. Parentage assignment, parental contribution, effective number of breeders and rate of inbreeding

Genetic diversity statistics and exclusion probabilities for the broodstock are shown in Table S3. The combined non-exclusion probability for parent pair was 0.00001. Simulation of parental analysis using a strict confidence level (95 %) revealed an assignment ratio of 100 % for parent pairs. Parental assignment was carried out on 299 offspring resulting from a mass spawning of the 91 potential progenitors (48 females and 43 males). Of the 299 individuals, 269 (90 %) were assigned to a parent pair (Table 4). For the three seed size classes, the percentage of assignment ranged from 83 to 91 %. Eleven females and five males failed to contribute to the offspring, and significantly unequal contribution was detected for dams ($\chi^2 = 1860$, $P < 0.001$) and sires ($\chi^2 = 489$, $P < 0.001$) in the total seed. The number of offspring assigned to each dam (Fig. 3a) ranged from one to 116 (mean = 7.3; variance = 375.6). Two dams (D29 and D54) contributed to 51 % of the offspring. The number of offspring assigned to each sire (Fig. 3b) ranged from one to 54 (mean = 7.1; variance = 93.6). In this case, six sires (S03, S20, S33, S32, S46 and S48) produced near half (47 %) of the

Table 3
Results of AMOVA performed between wild samples (Fer, Fis, Mur, Rib, VTi, VCa and Bro) and seed size classes (LSe, MSe and SSe) of *P. rhomboides*.

Source of variation	Sum of squares	Variance components	% variation	Fixation indices
Among groups	70.726	0.09598	3.06282	$F_{CT} = 0.03063$*
Among samples within groups	39.313	0.01544	0.49273	$F_{SC} = 0.00508$*
Within samples	4112.735	3.02244	96.44445	

* Significant values ($P < 0.00001$).

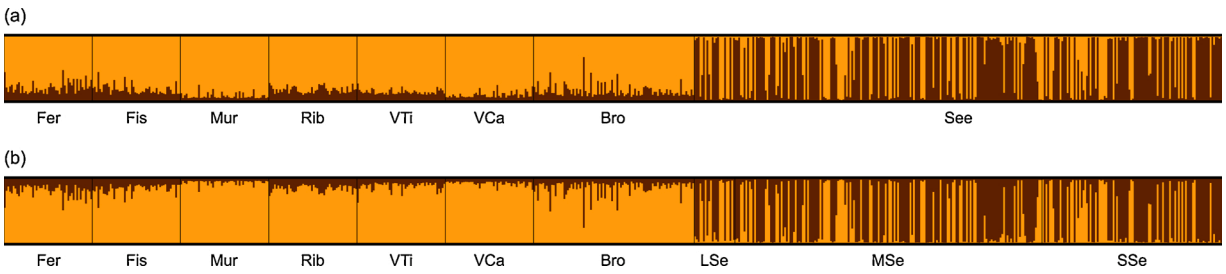

Fig. 2. Genetic structure of *P. rhomboides* samples obtained with STRUCTURE using two clusters, the most likely number of clusters as suggested by the estimated likelihood of K and the DeltaK statistic. Analysis performed using wild plus total seed samples (a), and wild plus seed size class samples (b). Individuals are represented as vertical bars divided into segments of different color that represent their estimated membership coefficients in the two clusters.

Table 4
Parentage assignment in *P. rhomboides* and estimations of effective number of breeders and rate of inbreeding.

	Offspring seed			
	Total	Large	Medium	Small
N	299	23	174	102
Broodstock	91			
Females (N_d)	48			
Males (N_s)	43			
Parent pair assignement	269 (90 %)	19 (83 %)	159 (91 %)	91 (89 %)
Dams (N_d')	37	10	28	26
Sires (N_s')	38	11	33	32
K_d	7.270	1.900	5.679	3.500
V_d	375.647	2.322	191.485	51.860
K_s	7.079	1.727	4.818	2.844
V_s	93.642	0.618	38.903	11.555
N_{ed}	4.626	8.482	4.115	5.197
N_{es}	13.881	16.587	13.286	15.236
N_e^1	90.725			
ΔF^1	0.006			
N_e^1/N_d+N_s	0.997			
N_e^2	74.987	20.952	60.590	57.379
ΔF^2	0.007	0.024	0.008	0.009
N_e^2/N_d+N_s	0.824			
N_e^3	13.878	22.448	12.567	15.501
ΔF^3	0.036	0.022	0.040	0.032
N_e^3/N_d+N_s	0.153			

N: Number of offspring. N_d and N_s: Census number of females and males of the broodstock. N_d'and N_s': Number of dams and sires with offspring in parentage analysis. N_{ed} and N_{es}: Effective number of dams and sires. K_d and K_s: Mean number of offspring per dam and sire. V_d and V_s: Variance of K_d and K_s. N_e: Effective number of breeders. [1]: N_e estimation using N_d and N_s. [2]: N_e estimation using N_d' and N_s'. [3]: N_e estimation using N_{ed} and N_{es}. ΔF: rate of inbreeding.

offspring. Except for the sires' contribution to LSe, a significantly unequal contribution was also evident when the seed size classes were considered (dams: $\chi^2 = 92$ (LSe), 1254 (MSe) and 566 (SSe), $P < 0.001$; sires: $\chi^2 = 322$ (MSe) and 167 (SSe), $P < 0.001$). We did not detect significant differences in the level of contribution to each of the seed size classes for either dams or sires.

A total of 123 parent pairs were assigned (Table S4), and the number of offspring ranged from one to 23 (mean = 2.2; variance = 9.1). Dams produced progeny with a number of sires ranging from one to 23; and sires produced progeny with a number of dams ranging from one to 10.

We obtained different estimates of the effective number of breeders and rate of inbreeding (Table 4). For the total seed, N_e, calculated taking into account the 48 females and 43 males used for mass spawning, was very close (90.7) to the census number (91). When considering the number of dams (37) and sires (38) that contributed to the total seed, N_e dropped to 75 (18 % reduction compared to the census number of broodstock). When both sex ratio and the variance in reproductive success were taken into account, N_e fell to 13.9 (85 % reduction). For the seed size classes, N_e (LSe: 21; MSe: 60.6; SSe: 57.4) was similar to the number of parents contributing to the offspring when the effect of sex ratio was accounted for, but when the variance in reproductive success was also considered, N_e was larger (LSe: 22.4) or smaller (MSe: 12.6; SSe: 15.5) than the number of dams and sires assigned. According to the N_e estimates, the highest rate of inbreeding was detected when the variance in reproductive success was taken into account: 3.6 % for the total seed and 2.2–4 % for the seed size classes.

4. Discussion

Both fisheries management and aquaculture development for *P. rhomboides* require responsible approaches based on existing levels of genetic variation within and between populations to minimize genetic impacts on wild beds. In this work, we provide estimates of genetic diversity and differentiation of wild samples from NW Spain, the main production area of this species, to help define management units necessary for fishery management and stock enhancement programs. Moreover, we characterize wild-derived broodstock and the seed produced in hatchery conditions to assess the genetic consequences of mass spawing and size grading. Genetic assessment of these two common hatchery practices are necessary to determine if production and management of hatchery-produced seed of *P. rhomboides* for stock enhancement programs should be improved and which measures, if any, should be adopted in order to capture the wild genetic variability. As in many other studies in the field (e.g. Lind et al., 2009; Hold et al., 2013; Straus et al., 2015; Xu et al., 2019), we examined genetic variation at microsatellite loci, which are presumed to be selectively neutral. If there are differences in neutral variation, it is most prudent to assume that there may also be differences in adaptive variation (Camara and Vadopalas, 2009; Lind et al., 2009).

4.1. Deviations from HWE, linkage disequilibrium and null alleles

Several samples and loci examined in *P. rhomboides* displayed significant departures from HWE. In most cases, this was associated with a deficit of heterozygotes (positive values of F_{IS}), as has been frequently observed in marine bivalves (e.g., Giantsis et al., 2014; Rico et al., 2017; Papetti et al., 2018). Heterozygote deficiencies are usually attributed to inbreeding, selection, Wahlund effect or null alleles. The high fecundity, external fertilization, and high larval dispersal capacity of *P. rhomboides*, as well as an unequal distribution of heterozygote deficit across loci rule out inbreeding as the main cause. Results of the neutrality tests do not support the hypothesis that the microsatellite loci are under selection. Population admixture seems unlikely due to the absence or low level of genetic differentiation detected in wild samples and the maintenance of the seed in individualized tanks. However, in most cases, we detected null alleles at relatively high frequencies, which suggests that they are the main contributing factor to the observed deficit of heterozygotes. Nevertheless, in a few instances, we detected heterozygote excesses, particularly in the seed, which may be related to a small effective number of breeders (see below). When N_e is small, allele frequencies in males and females can be different due to sampling error, which would

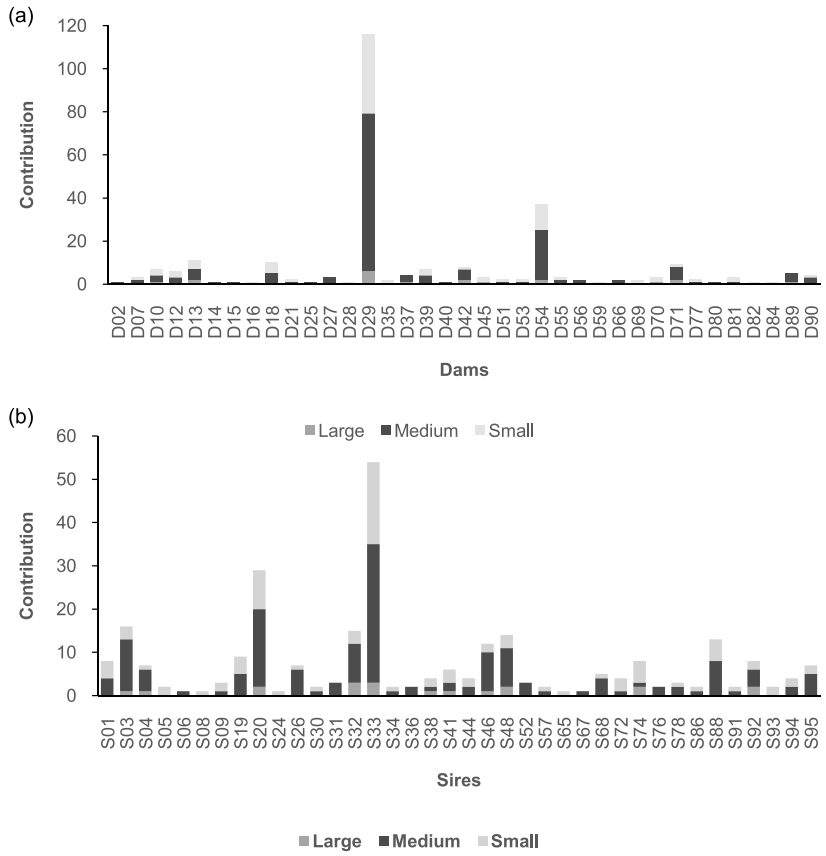

Fig. 3. Contribution to offspring of dams (a) and sires (b) of *P. rhomboides*. Each color represents the number of individuals from each size class.

cause an excess of heterozygotes in the progeny with respect to HWE expectations (Luikart and Cornuet, 1999; Balloux, 2004). Seed samples also exhibited a high incidence of locus combinations with significant linkage disequilibrium. This may be attributable to kinship among the seed (Hedgecock et al., 2007), since we detected a high proportion of half-sibs.

Null alleles may inflate levels of genetic differentiation and may also be problematic for parentage analyses (Chapuis and Estoup, 2007; Carlsson, 2008). To avoid a positive bias of F_{ST} values, we applied the ENA correction (Chapuis and Estoup, 2007), but estimates were very similar to those obtained when we omitted such correction. Therefore, it seems that the frequency of null alleles detected in *P. rhomboides* does not have a remarkable effect on our estimates of genetic differentiation. The use of microsatellite loci affected by null alleles in parentage analysis may cause incompatible genotypes (mismatches) between parents and offspring and thus result in false exclusions of true parentage (Wang, 2010). Since, according to Cervus analysis, seven out of the nine microsatellite loci displayed null alleles in the broodstock at frequencies near 0.05 or higher, it cannot be discarded that the 10 % unassigned seed of *P. rhomboides* results mainly from the existence of such alleles. Other causes why all progeny could not be assigned include other scoring errors (e.g., incorrect identification of alleles) or contamination (Miller et al., 2014).

4.2. Genetic characterization of wild samples

Wild samples of *P. rhomboides* showed similar genetic diversity: no significant differences were detected in any of the parameters (R_s, H_e and H_o). Previous estimates from nuclear markers in this species come from the analysis of 22 microsatellite loci in a small sample (20 individuals) from Fer (Chacón et al., 2013). The mean values of H_e (0.668) and H_o (0.501) estimated from Chacón et al. (2013) are similar to those calculated in this study ($H_e = 0.684$; $H_o = 0.574$) for the same locality using a lower number of loci and a higher number of individuals. Other genetic diversity estimates for *P. rhomboides* come from the analysis of mitochondrial *cox1* gene in the same localities examined here (Chacón et al., 2020) and, with a few minor exceptions, no significant differences in haplotype and nucleotide diversity among localities were reported. The availability of estimates of genetic diversity at both the nuclear and mitochondrial levels will allow us to track through time this important population parameter and consequently detect potential changes due to fishery and aquaculture activities in NW Spain. It should be noted that *P. rhomboides* exploitation was until now dependent on natural recruitment; therefore, it can be supposed that genetic diversity of *P. rhomboides* has been less eroded than that of other clam species whose seed is produced at industrial scale.

Overall, we found no significant genetic differentiation between the wild samples. F_{ST} values were always lower than 0.01 and significant after Bonferroni correction only between Bro and Mur. Moreover, both AMOVA and Bayesian clustering analysis support a high degree of homogeneity among the wild samples. This agrees with the analysis of the *cox1* gene that detected no genetic differentiation after sequential Bonferroni correction among the same localities (Chacón et al., 2020). However, minimal or subtle genetic differentiation cannot be refuted, and this hypothesis should be tested by a temporal survey. Knutsen et al. (2011) demonstrated in the coastal Atlantic cod that weak genetic differentiation (average $F_{ST} = 0.0037$) can be biologically meaningful, corresponding to separate, temporally persistent, local populations. A panmictic or very weak genetic differentiation scenario is compatible with results obtained in other bivalves from the same region, including the wedge clams *Donax trunculus* (Nantón et al., 2017; Fernández-Pérez et al., 2018) and *D. vittatus* (Fernández-Pérez et al., 2017, 2019), the cockle *Cerastoderma edule* (Martínez et al., 2013, 2015) and the queen scallop *Aequipecten opercularis* (Arias et al., 2010). Other invertebrate

species such as the sea urchin *Paracentrotus lividus* (Tourón et al., 2018) or the shore crab *Carcinus maenas* (Domingues et al., 2010) also displayed genetic homogeneity in NW Spain. This suggests that the larval dispersal potential of *P. rhomboides* coupled with a favorable water circulation pattern could explain the absence of genetic structure.

4.3. Genetic characterization of the total seed

Genetic characteristics of the seed produced under hatchery conditions differed from those of the broodstock and other wild samples. Although we did not find significant differences in R_s^1, H_e and H_o, a reduction of genetic diversity in the total seed was evident. It should be noted that 13 alleles found in the broodstock were not detected in the seed, and that R_s, a parameter that does not depend on sample size, showed a reduction of up to 24 % and a minimum of 12 % compared to the broodstock and other wild samples, respectively. A decrease in allelic richness combined with no remarkable decline in heterozygosity is not unexpected given that allelic richness is sensitive to the number of alleles while heterozygosity is sensitive to their frequency (Greenbaum et al., 2014). Although heterozygosity reductions have been previously described in some mollusk species such as *Crassostrea gigas* (Li et al., 2009), allele loss is more common (Lind et al., 2009; Hold et al., 2013; Borrell et al., 2014; Straus et al., 2015). The allelic richness reduction detected between seed and broodstock of *P. rhomboides* was greater than that reported for *R. decussatus* reared in the same hatchery facility (~13 %, Borrell et al., 2014), but compared to wild samples, both clams showed similar levels of reduction (14 % in *R. decussatus*). Other cultured bivalve showed more pronounced reductions (Lind et al., 2009; Straus et al., 2015). Beside allele loss, seed also showed two private alleles (i.e., absent from the broodstock and other wild samples). The presence of these alleles could be attributed to scoring errors, contamination or spontaneous mutations (Miller et al., 2014).

In contrast with the results obtained for wild samples, all analyses related to population structure (F_{ST}, AMOVA and Bayesian clustering analysis) detected significant genetic differentiation between the total seed and wild samples (broodstock included). Mean F_{ST} values showed that the differentiation between seed and wild samples was an order of magnitude higher than the differentiation detected among wild samples. According to the AMOVA analysis, 3.06 % of variance is due to differences between wild and seed samples, and the Bayesian clustering analysis revealed two clusters: one composed of wild samples and one of seed. This finding is in line with other studies that also demonstrate that a single generation of hatchery rearing is enough to produce an offspring genetically different from its broodstock (Lallias et al., 2010a; Borrell et al., 2014; Segovia-Viadero et al., 2016). Consequently, using wild individuals as broodstock, proves to be insufficient to avoid the production of seed with a distinct genetic composition and additional measures are necessary. Although we did not find evident genetic structure in the region studied, the data show that the level of genetic differentiation between seed and wild samples from different sites varies (e.g., $F_{ST} = 0.026$ for Fis-See and $F_{ST} = 0.050$ for Mur-See). This degree of differentiation highlights the importance of genetic assessment of hatchery produced seed to support decisions about choosing restocking areas (i.e., sites of seed release).

Since the *P. rhomboides* broodstock showed similar genetic diversity to the other wild samples, with no or low levels of genetic differentiation, the changes in the make-up of the total seed do not seem to be due to an under-representation of the wild gene pool, but rather due hatchery-induced genetic bottlenecks in the broodstock. As a general rule, practical breeding schemes use an effective number of breeders of at least 100 (Sonesson et al., 2005), and hence a rate of inbreeding of 0.5 % ($1/2N_e$), but N_e should be at least 150 to have a 95 % guarantee of saving alleles at frequencies of ≤ 0.01 (Tave, 1999). If the contribution to seed of males and females were equivalent, the broodstock used in this work (91: 48 ♀ and 43 ♂), even considering the 82 % (75: 37 ♀ and 38 ♂) that participated in the spawning event, should be enough to obtain a nearly acceptable inbreeding level (<1%). This means that the sex ratio of the broodstock was balanced, with little effect in reducing N_e below the actual parental number. However it was evident an unequal parental contribution and family size variation, with high variance in the mean number of offspring. This resulted in a very low effective number of breeders ($N_e = 13.9$) and a rate of inbreeding (3.6 %) that exceeds recommendations. Therefore, just increasing broodstock number may not be enough to avoid genetic diversity loss.

Large variance in parental contribution has been reported in many other cultured shellfish species including the Pacific oyster *C.gigas* (Boudry et al., 2002; Li et al., 2009; Miller et al., 2014; Xu et al., 2019), the flat oyster *Ostrea edulis* (Lallias et al., 2010a, 2010b), the silver-lipped pearl oyster *Pinctada maxima* (Lind et al., 2009), the geoduck *Panopea generosa* (Straus et al., 2015) and the Pacific lion-paw scallop *Nodipecten subnodosus* (Petersen et al., 2008) and it constitutes the main cause of reduction of N_e in hatcheries that use the practice of mass spawning. Gamete quality, sperm-egg interaction, sperm competition and differential survival among families are the main factors involved (Gaffney et al., 1992; Boudry et al., 2002; Lallias et al., 2010a). To minimize variance in reproductive success several actions have been proposed such as, collecting the oocytes from each female and the sperm from each male separately using an equal proportion of gametes from each parent to provide equal mating opportunities (Xu et al., 2019), conducting simple pair or factorial crosses (Camara and Vadopalas, 2009) and equalizing family size to reduce adaptation to hatchery environment (Fiumera et al., 2004). Making full factorial crosses is labour intensive and can lead to inbreeding if large numbers of individuals are not used (Fisch et al., 2015**).** Effectively equalizing family size requires keeping them in separate tanks for long periods, which can be impracticable for most commercial hatcheries, and can also greatly reduce offspring production (Fiumera et al., 2004). There is no doubt that the fertilization method of mass spawning should be avoided in *P. rhomboides* due to its unpredictable outcome and that controlled crosses must be implemented to maximize seed production and minimize variance in parental contribution. A manageable option for *P. rhomboides,* with little extra effort with respect to mass spawning, may be to carry out partial factorial crosses or multiple mass spawnings with a reduced number of parents, and subsequent pooling of equal quantities of fertilized eggs/embryos/larvae, in line with previous proposals successfully tested for other aquaculture species (Gaffney et al., 1992; Busack and Knudsen, 2007; Straus et al., 2015; Xu et al., 2019).

4.4. Genetic consequences of grading seed by size

The practice of grading seed by size is routinely used in hatcheries to reduce size variance and to increase survival. If genetic differences exist between size classes, restocking an area with only one size class risks increasing the genetic impact of the enhancement program. To simulate this practice in *P. rhomboides*, the total seed was graded into three size classes that were separately analyzed.

The three seed size classes also showed a loss of diversity with significant differences in allelic richness in LSe and MSe compared to the broodstock. All three size classes also showed significant genetic differentiation from wild samples including the broodstock, with LSe displaying a more pronounced differentiation according to the F_{ST} value (0.041−0.064). Although the sample size of LSe ($N = 23$) was lower than that of the other samples analyzed, it seems improbable that the higher F_{ST} values are an overestimation, given that the F_{ST} estimator of Weir and Cockerham (1984) is asymptotically unbiased with respect to sample size and provides a nearly unbiased estimate at moderate population sizes (n = 15–25) and a small number of loci such as 10 (Willing et al., 2012). We did not find significant genetic differentiation among the three size classes, which suggests that these classes are genetically similar. This is in line with the absence of significant differences in dam and sire contribution to each size class. Therefore, we did not find evidence of genetic or parental effects on growth rate in *P. rhomboides* in

contrast with some other aquaculture species in which graded size progenies were examined (Frost et al., 2006; Borrell et al., 2011; Loughnan et al., 2013). This may be due to the lack of association between the microsatellite loci examined and genetic factors involved in growth or to the size classes examined not being a good representation of genotypic variants. Likewise Miller et al. (2014) did not report differences in broodstock contribution to the small and large size classes of six-month old progeny over six crosses of tetraploid *C. gigas*.

5. Conclusion

This work provides the first microsatellite-based estimates of genetic diversity and population differentiation in *P. rhomboides* throughout the coast of NW Spain, reporting minimal or no genetic differences among wild samples, including the broodstock used for hatchery-reared seed, which supports the consideration of NW Spain as a management unit. The genetic changes detected in the seed—lower allelic richness and a significant level of differentiation with respect to all the wild samples—as well as a reduction in the effective number of breeders with respect to the census number, reflect the consequences of the practice of mass spawning in *P. rhomboides* and the need to improve the process of hatchery seed production for stock enhancement programs. Increasing broodstock number and equalizing parental contribution are the main measures that should be implemented. Instead of mass spawning, we suggest partial factorial crosses or several mini mass spawnings followed by the pooling of equal quantities of the corresponding offspring. We found no genetic effects attributable to the size grading carried out, but this aspect should be further investigated using progeny groups with more differentiated growth.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2021.100658.

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