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# SNP-based parentage analyses over two successive generations demonstrates the feasibility of efficient production of inbred lines in the Pacific oyster (*Crassostrea gigas*) by self-fertilization of simultaneous hermaphrodites despite severe inbreeding depression

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**Abstract :**

The Pacific oyster, Crassostrea gigas, is a species in which true male and true female well as hermaphrodite individuals have been reported. Among the latter, most are sequential, with one or more sex changes throughout their live, while a few (<2%) are simultaneous hermaphrodites (SH) which may self-fertilize. Sex determinism and its functional bases remain unclear is this species. In this study, one SH oyster was found among a mature broodstock and used to produce a progeny by self-fertilization. Several thousand offspring were obtained for this family (SF1) but only 73 SF1 oysters survived following an episode of mass mortality at the spat stage. Six of them were conditioned for reproduction and three were found to be SH, and one produced a limited but viable progeny by self-fertilization (SF2). Oysters were sampled and genotyped with 226 SNP markers, confirming that the SF1 and the SF2 oysters were produced by self-fertilization over two successive generations. To our knowledge, it is the second study reporting the feasibility of production on an inbred line of Pacific oysters by self-fertilization using a simultaneous hermaphrodite as primary genitor, and the first study to generate two successive generations of selfing. Observed mean inbreeding coefficient of SF2 oysters produced by selfing for two generations was 0.61, ranging from 0.46 to 0.80. This coefficient was lower than the expected theoretical value (0.8125), suggesting that the most homozygous offspring died at early stages due to inbreeding depression. Our study demonstrates that inbred lines of Pacific oysters can be produced by self-fertilization but may exhibit higher heterozygosity than theoretically expected due to better survival of the most heterozygous offspring.

**Highlights**

► Second evidence of oyster produced by self-fertilization beyond the larval stage. ► Oysters produced by self-fertilization can produced a viable progeny. ► Inbreeding rate was lower than expected.

**Keywords** : self-fertilization, inbreeding, Pacific oysters, hermaphroditism, Crassostrea gigas

## 1. Introduction

Crossing between related individuals, including selfing, gynogenesis and androgenesis, is used in numerous plant and animal species to produce isogenic or highly inbred lines. Such lines are key resources that are widely used to study the effect of the environment on phenotypes, mapping quantitative trait loci or for genetic improvement through heterosis. Gynogenesis and androgenesis can successfully produce isogenic lines in only one or two generations (e.g. in the rainbow trout: (Quillet et al., 2007)), but those approaches remain unsuccessful in many species. For oyster species, gynogenesis was successful in *Crassostrea gigas* (Guo and Gaffney, 1993; Li and Kijima, 2006), while Li et al. (2004) managed to induce haploid androgenesis embryos that died before reaching the D-shaped stage. Alternatively, self-fertilization of hermaphroditic species results to faster inbreeding than crossing between siblings (Falconer and Mackay, 1996). Self-fertilization has been reported in mollusk species especially in functional simultaneous hermaphrodites, such as giant clams *Tridacna derasa, T. squamosa, T. crocea* (Zhang et al., 2020), scallops *Argopecten irradians* (Zheng et al., 2012), *Argopecten purpuratus* (Martinez et al., 2007), *Argopecten circularis* (Ibarra et al., 1995), *Aequipecten tehuelchus* (Narvarte and Pascual, 2003) or *Pecten maximus* (Beaumont, 1986; Beaumont and Budd, 1983). For successive hermaphrodite species, such as cupped oysters, several articles also reported attempts to produce inbred progenies using cryopreserved sperm to fertilize sex-reversed females. Lannan (1971) first reported successful fertilization of ova in *C. gigas* using this approach, but no information was given about the faith of the obtained embryos. A similar approach was also successful in *C. virginica* and self-fertilization was confirmed using microsatellite genotyping of the resulting eyed-larvae, but none managed to survive beyond the larval stage (Yang et al., 2015).Survival beyond settlement and metamorphosis of larvae resulting from selfing using

cryopreserved sperm remained to be demonstrated due to high mortality of inbred progenies. This likely to be related to the strong inbreeding depression observed at early life stages resulting from the high genetic load that has been well demonstrated in *C. gigas* through the study of segregation distortion (Launey and Hedgecock, 2001). Meanwhile, inbred lines have been successfully developed by brother-sister mating for genetic improvement through crossbreeding (Hedgecock and Davis, 2007; Yin and Hedgecock, 2019).

World oyster production jumped from 3.6 million tons in 2000 to 5.7 million tons 2017 (FAO, 2019), and the Pacific oyster is the one of the main oyster species cultivated worldwide. Its sex determinism and associated molecular mechanisms have been investigated but remains unclear (Dheilly et al., 2012; Han et al., 2021; Yue et al., 2018; Zhang et al., 2014), notably because this species shows dioecy, sequential/successive hermaphrodites as well as a low frequency (<2%) of simultaneous hermaphrodites (Broquard et al., 2020; Guo et al., 1998). To our knowledge, only one previous study reported the production of oysters by using a simultaneous hermaphrodite. Thus, Hedgecock et al. (1995) used Pacific oysters produced by selfing, confirmed using allozyme markers, to generate inbred lines indicating that selfing oysters can survived beyong the larval stage, and that they are capable to produce a new generation by crossing males and females . In our study, we report the production of viable adult Pacific oysters over two successive generations of selfing of simultaneous hermaphrodites. The main objective was to demonstrate the feasibility to produce inbred oysters by self-fertilization over successive generations, confirm parentage using molecular DNA markers and estimate the level of inbreeding of the resulting progeny.

## 2. Materials and methods

Unless specified, all crosses and rearing of the biological material was conducted at Ifremer's shellfish hatchery in La Tremblade, Charente Maritime, France.

*2.1. Broodstock*

The pedigree of the oysters is given in supplementary Table 1 and illustrated in Fig.1. Bi-parental crosses were performed in March 2013 by crossing wild oysters samples in the Marennes-Oléron Bay in 2012 as described by Azéma et al. (2017). Among the resulting full-sib families, some of them (including family "G0-H17") proved to be highly resistant to OsHV-1 and were therefore selected for the next generation. Among those, G0-H17 oysters were conditioned in a 240 L tank in January 2015: seawater temperature was gradually increased to 21°C over one week. Seawater flow was 400 L/h, and a cultured phytoplankton diet (*Isochrysis galbana*, *Tetraselmis suecica*, and *Skeletonema costatum*) was provided *ad libitum* (i.e. 50,000 cells/mL). Mass spawning was obtained following a thermal shock on March 2015 using 14 full-sib G0-H17 oysters, producing the G1-H17 progeny (Fig.1). Individual sex of each G0-H17 oyster used in this cross was not recorded but we assumed that each offspring resulted from two parents, i.e., one male and one female as reported by the pedigree provided in supplementary table 1.

*2.2. First selfing-cross using a simultaneous hermaphrodite to produce family SF1*

In June 2016, oysters of the G1-H17 progeny were ready to reproduce (i.e. presenting mature gonads), and a new generation of the H17 progeny was planned to be produced by stripping the gonad of the progenitors. This step required the sex determination with a microscope. One G1-H17 oyster was found to be a simultaneous hermaphrodite (SH1), *i.e.* presenting both male and female gametes with a higher proportion of sperm than ova. Gametes were collected by stripping the gonad, and then diluted in seawater in a 1 L beaker for 5 min to

allow self-fertilization (Fig.1). The ratio eggs/sperm was much higher (rough estimation of 1/10,000) than the one basically used in hatchery practices (1/100). Then, gametes and early embryos were sieved on 100-µm and then 20-µm mesh screens to separate large and small tissue debris, respectively, including the sperm, from eggs. Unfertilized and fertilized eggs, retained on the 20-µm mesh screen, were transferred in a 30 L tank filled with 25°C using UV-treated (40 mj/cm$^2$) and filtered (5 µm) seawater. Gill tissue of the SH1 was sampled and stored in ethanol 100% at ambient temperature for further DNA analyses. Following larval development, eyed-larvae were settled on cultch from day 15 to day 18 post-fertilization. Around 5000 larvae successfully settled but a mortality outbreak due to *Vibrio europaeus* (formerly described as *V. tubiashii* for mortality reported in France (Dégremont et al., 2021; Travers et al., 2014)) led to high mortality at 1 mm size. In December 2016, 73 surviving SF1 oysters were counted.

### *2.3. Second selfing-cross using a simultaneous hermaphrodite from family SF1 to produce family SF2*

In January 2018, as described for the production of the G1-H17, six SF1 oysters were conditioned in a 240 L tank so that ripe oysters were obtained in March 2018. All were opened, sampled for DNA analyses and their sex was determined using a microscope. Three SF1 oysters (SH2, SH3, and SH4) were found to be simultaneous hermaphrodites. For each one, self-fertilization was done as described for the production of SF1. Two crosses failed to generate larvae, whereas a few D-larvae (<2000) were obtained from SH2, producing family SF2 (Fig. 1). Around 150 eyed pediveliger larvae were observed and settled from day 16 to day 23 post-fertilization. Seed (n~70) were transferred at Ifremer's nursery in Bouin (Vendée, France) in May 2018. Fifty individuals (weighing 15g) were sacrificed in June 2019. For each individual, a piece of gills was sampled and stored in ethanol 100% for further DNA analysis.

*2.4. Genotyping*

A SNP-genotyping array (Illumina infinium), comprising 226 markers and developed by Lapègue et al. (2014) was used to genotype oysters in order to perform parentage assignment and to estimate inbreeding coefficient of SF2 oysters. The list of those markers is provided in supplementary Table 2. DNA extraction and genotyping were performed by Labogena (Jouy-en-Josas, France). For the parent of the SF1 family, and the parent of the SF2 family, DNA extraction and SNP-genotyping were done in duplicates. In total SNP-genotyping was done for 50 SF2, six SF1 and one G1-H17 oysters (supplementary Table 1).

Parentage assignment and genetic parameters for each parent (SH1 and SH2) and their progenies (i.e. number of alleles per locus, observed and expected heterozygosity, Polymorphic Information Content…) were determined using Cervus 3.0.7 (Kalinowski et al., 2007). The theoretical inbreeding coefficient F at each generation t was calculated to be 0.25 for SH1 (assuming a first generation of crossing between full brother and sister), and 0.625 and 0.8125 for SF1 and SF2 oysters from Falconer and Mackay (1996):

$$Ft = \Delta F\ (1-F_{t-1}) + F_{t-1}$$

Where $\Delta F$ is the rate of inbreeding, expected to be 0.5 for self-fertilization.

Ft was also estimated from the observed genotypes for each SF1 and SF2 oysters, as $\Delta F$ measures the fraction of heterozygosity that disappears each generation, and so, could be higher or lower than 0.5.

The consensus sequences of each 226 SNP from the SNP-genotyping arrays were compared against the National Center for Biotechnology Information (NCBI) by searching for similarities at the nucleotide level using BLASTN. The E-value cutoff was 1e-10. Only

markers without doubt on their position were kept (206 SNPs), and then, we get the physical position and the name of the linkage group on which each marker is located.

Finally, PLINK v1.9 software (Purcell et al., 2007) was used to compute observed and expected homozygous genotype counts for each individual of SF1 and SF2 families, as well as SH1 oyster of the G1-H17 family, and reports method-of-moments F coefficient estimates using the 206 mapped SNPs.

## 3. Results and discussion

In our study, the four simultaneous hermaphrodite oysters were identified using microscope by observing a few eggs bathing in sperm involving a low fecundity and resulting into limited number of offspring (Table 1). However, it might be possible to observe simultaneous hermaphrodites with many eggs and a few sperm, which will still be enough to fertilize eggs. These oysters could show a higher fecundity, and a higher likelihood to obtain larger progenies by selfing, but such oysters would need to observe large numbers of mature oysters and it may therefore be practically difficult to identify by microscopic observation of gonad samples. An alternative approach would be to maintain the gametes in seawater for one or several hours, and check the presence of swimming sperm or/and the polar body extrusions and the first cleavages of fertilized eggs.

Out of four SH oysters observed in our study, two successfully produced viable D larvae (approximately 20,000 and 2000 larvae for the SF1 and SF2 families, respectively), and subsequently spat (Table 1). The duration of their larval development was similar to those observed for bi-parental crosses or mass spawns that were raised at the same time of the SF1 and SF2 families (data no shown). Unfortunately, abnormal mortality caused by pathogenic bacteria of *C. gigas* was observed for the SF1 oysters at 1 mm size as well as for other oyster

families produced by mating at least two parents, reducing drastically the number of SF1 oysters at the spat stage from 5000 to less than 100 (Table 1). For the SF2 oysters, although the number of spat was low, they show relatively good survival from the spat stage suggesting that oysters produced by self-fertilization may not exhibit higher mortality than outbred oysters in our controlled facilities from the spat stage. This is in agreement with results reported by Plough (2018), who observed no appreciable genetic mortality of inbred spat 40 days after settlement.

The genotypes from the SNP array are given in supplementary Table 3. All SF1 and SF2 oysters were unambiguously assigned to their respective presumed parents, confirming that viable inbred adult oysters can be obtained by selfing and for the first time, over two successive generations. To date, only Hedgecock et al. (1995) used oysters generated by self-fertilization of simultaneous hermaphrodites collected in the wild, to produce a new generation of bi-parental inbred families. Their study has confirmed the pedigree using 24 allozyme markers. One of the two studied lines presented a mean fixation index significantly greater than expected. Otherwise, previous studies focusing on oysters were limited to larval stage and based on cryopreservation of male gametes to fertilize reverted females (Lannan, 1971; Yang et al., 2015)

Summary genetic statistics obtained by CERVUS for the SH1 and SH2 oysters and their progenies are provided for each locus in supplementary Table 4. The SH1 oyster, assuming that it was produced from a cross using full brother and sister, showed 53 and 161 heterozygous and homozygous SNPs, respectively (Table 2). For the two next generations produced by selfing, the mean numbers of heterozygous SNPs decreased from 53 to 41 for the SF1 oysters and 28 for the SF2 oysters (Table 2) while it should theoretically decrease by half at each generation with selfing. Thus, mean observed inbreeding coefficient reached 0.42 and 0.61 for the SF1 and SF2 oysters, respectively, when using the fraction of heterozygosity

that disappears at each generation, and 0.62 and 0.74 when calculated from PLINK (Table 2) (Fig.2). The higher value of the latter may be explained by the low number of SNP used. While the expected theoretical inbreeding coefficient would have been 0.8125 for SF2 oysters, the lower observed inbreeding coefficient may result from lower survival of individuals showing high homozygosity due to inbreeding depression resulting from the high genetic load in *C. gigas* as demonstrated by Launey and Hedgecock (2001). Indeed, without considering the SF1 oysters which had only 6 oysters genotyped, segregation distortion were observed for almost all heterozygous SNPs in SF2 oysters (Supplementary Table 4-sheet SF2) suggesting recessive lethal or nearly lethal alleles closely linked to the SNP (Launey and Hedgecock, 2001). For example, seven SNPs (AM858323_344, AM859785_261, CU684109_266, CU997918_398, EW777868_135, EW779181_638 and EW779181_749), shown in red bold in Supplementary Figure 1 and mapped on LG1 and LG3, had observed heterozygosity >0.90 (Supplementary Table 4-sheet SF2) whereas the frequency in progenies should be closed to 0.5 for each polymorphic SNP. Beside the abnormal mortality caused by pathogenic bacteria of *C. gigas* observed for the SF1 oysters, most of the mortality for the SF1 and SF2 families occurred before and during metamorphosis which is first reported by Launey and Hedgecock (2001) showing that most deleterious genes mostly affect the larval stage or around the time of metamorphosis. However, our study suggests that selfing may be an efficient and rapid method of purging in *C. gigas* (Abu Awad and Roze, 2020). Proceeding successive self-fertilization should purge the lethal alleles, and when a highly inbreed line or nearly isogenic line is obtained, although inbreeding depression reduce their fitness, cross between brothers and sisters would increase hatching and survival at the larval stage in subsequent generations as observed in *Argopecten irradians* (Zheng et al., 2012). Similarly, Brouwer and Osborn (1997) compared the expected and observed heterozygocity over four generations of selfing in diploid alfalfa (*Medicago sativa*) using 40 RFLP loci. Authors

concluded that "inbreeding (…) proceeds more slowly than expected (…) and that inbreeding depression may limit the level of homozygosity obtainable" and that "tests of genotype and allele ratios indicated that the deviations may be due to selection favoring heterozygotes", as theoretically described by Hayman and Mather (1953) and observed when inbreeding numerous species (e.g. *Drosophila melanogaster*: Rumball et al. (1994); *Gallus gallus domesticus*: Mina et al. (1991)).

Interestingly, three of six SF1 oysters, produced by selfing, were also simultaneous hermaphrodites suggesting inheritance for this reproductive characteristic. Simultaneous hermaphrodites are scarcely observed in diploid *C. gigas* (Broquard et al., 2020; Guo et al., 1998) but are more frequent in triploid progenies (Normand et al., 2009). Synchronous mass spawning behaviour, in particular for females (Antonio and Camacho, 2019; Enriquez-Diaz et al., 2009), and strong inbreeding depression due to high genetic load (Launey and Hedgecock, 2001; Plough et al., 2016) are likely to explain why simultaneous hermaphrodites remain rare in *C. gigas* even if this reproductive characteristic might be heritable. If the occurrence of simultaneous hermaphrodites increased for the progenies of one parent showing this characteristic, this should ease the obtention of highly inbred oysters which takes, in theory, 7 generations of successive selfing to reach an inbreeding coefficient of 0.99 against more than 20 generations of crosses between full-sibs (Falconer and Mackay, 1996). Further research is needed on this topic to confirm the inheritance of simultaneous hermaphroditism in *C. gigas*.

Our study demonstrates that the development of highly inbreed lines of *C. gigas* is possible by using self-fertilization of simultaneous hermaphrodites using successive generations of selfing. This method is faster and technically easier to implement than using cryopreserved

sperm from a successive hermaphrodite that change sex to females. The development of highly inbred lines in *C. gigas* will depend on the number of generations needed to purge the genetic load that favour survival of the most heterozygous individuals. Such lines would be of great academic and applied interest to study genetics and epigenetics of traits in *C. gigas*. In any case, parentage assignment using molecular markers will be needed to avoid confirm pedigrees and to ensure absence of uncontrolled fertilization or mixing of progeny during the rearing of inbred lines.

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**Declaration of interests**

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:


Figure 1 Pedigree of the SF-1 and SF-2 oyster families produced by self-fertilization in June 2016 and March 2018, respectively (* no data on sex ratio recorded)

Table 1 Summary of the four simulatenous hermaphrodite oysters (SH) used for self-fertilization

| Oysters | Gametes* | Self-Fertilization | Family name | D larvae number | Larval survival | Days post fertilization for settlement | Spat number |
|---|---|---|---|---|---|---|---|
| SH1 | ♀/♂♂♂♂ | success | SF1 | 20,000 | moderate | D16-D18 | 5000 |
| SH2 | ♀/♂♂♂♂ | success | SF2 | 2,000 | moderate | D16-D23 | 70 |
| SH3 | ♀/♂♂♂♂ | fail | | | | | |
| SH3 | ♀/♂♂♂♂ | fail | | | | | |

*The proportion given are highly approximative, but the number of sperms was much higher than the number of eggs, resulting a « low » fecundity for the four SH.

Table 2 Number of heterozygote SNPs (min, max and mean) and inbreeding coefficients for the two families of *C. gigas* produced by self-fertilization (SF1 and SF2), and their parents

| Family | id | | Number of SNP | | Inbreeding coefficient Ft | | |
|---|---|---|---|---|---|---|---|
| | | | Heterozygote | Homozygote | expected | From ΔF* | Plink |
| G1-H17 | SH1 (parent of SF1) | | 53 | 161 | 0.25 | | 0.51 |
| SF1 | SH3 | min | 36 | 181 | 0.63 | 0.49 | 0.67 |
| | F2 | max | 48 | 169 | 0.63 | 0.32 | 0.54 |
| | | mean | 41 | 176 | 0.63 | 0.42 | 0.62 |
| SF1 | SH2 (parent of SF2) | | 39 | 178 | 0.63 | 0.45 | 0.64 |
| SF2 | OVA19_68 | min | 14 | 203 | 0.81 | 0.80 | 0.87 |
| | OVA19_41 | max | 38 | 179 | 0.81 | 0.46 | 0.65 |
| | SH2 mean | mean | 28 | 189 | 0.81 | 0.61 | 0.74 |

* The inbreeding coefficient Ft was calculated from the SNP array with ΔF obtained from the fraction of heterozygosity that disappears at each generation

- Second evidence of oyster produced by self-fertilization beyond the larval stage,
- Oysters produced by self-fertilization can produced a viable progeny,
- Inbreeding rate was lower than expected,