

Pearl Formation: Persistence of the Graft During the Entire Process of Biomineralization

S. Arnaud-Haond,^{1,*} E. Goyard,¹ V. Vonau,¹ C. Herbaut,² J. Prou,^{1,†} D. Saulnier^{1,†}

¹Laboratoire d'Aquaculture tropicale, IFREMER/COP, BP 7004, 98719 Taravao, Tahiti, French Polynesia

²Laboratoire de Génétique Moléculaire Humaine, Université du Pacifique, Tahiti, French Polynesia

Received: 8 March 2006 / Accepted: 24 August 2006 / Published online: 1 December 2006

Abstract

Most bivalves species of the genus *Pinctada* are well known throughout the world for production of white or black pearls of high commercial value. For cultured pearl production, a mantle allograft from a donor is implanted into the gonad of a recipient oyster, together with a small inorganic bead. Because of the dedifferentiation of cells during the first steps of the host oyster's immunological reaction, so far the fate of the graft and its exact role in the process of pearl formation could not be determined via classical histological methods. Here we report the first molecular evidence of the resilience of the graft in the recipient organism by showing that cells containing genome from the donor are still present at the end of pearl formation. These results suggest the existence of a unique biological cooperation leading to the successful biomineralization process of nacreous secretion in pearl formation.

Keywords: Pearl oyster — biomineralization — grafting — pearl sac — pearl — genotype

Introduction

During grafting, a live tissue fragment or graft prepared from the mantle of a donor pearl mollusc is implanted into the gonad of a recipient pearl mollusc together with a small inorganic bead, often referred to as the nucleus, in contact with the graft.

*Present address: CCMAR and Center for Marine Sciences, F.C.M.A., and University Algarve Gambelas, P-8005-139 Faro, Portugal

†Present address: Laboratoire de Génétique et Pathologie, IFREMER, BP 13317390, La Tremblade, France

Correspondence to: S. Arnaud-Haond; E-mail: sarnaud@ualg.pt or Sophie.Arnaud@ifremer.fr

Cultured black pearls from *Pinctada margaritifera* show a wide range of color and luster, which are the most important characteristics determining their commercial value. This variation is likely to be influenced by both environmental and genetic factors. The latter may depend on the recipient oyster, but professional grafters commonly consider that the color and luster of pearl are often related to the phenotypic properties of the donor (rather than of recipient) oyster (Wada and Komaru, 1996). The grafting process induces a great upheaval in the recipient organism, including an immunological reaction and cell differentiations that make it difficult to track the different cells until the development around the bead of a follicle called a "pearl-sac" and the beginning of nacreous secretion. For that reason, none of the histological studies performed during this early stage were able to determine clearly the initiation of the graft cells (Herbaut et al., 2000).

In the absence of evidence, opinions diverge as to the role and fate of the mantle allograft in the recipient. On one hand it may influence the very first steps of pearl sac formation and the secretion of the very first nacreous layers of the pearl before being rejected by the immunological response of the host. On the other hand, if it persists in the host during the whole course of pearl formation, it would imply an exceptional rate of graft success, as well as a unique biological cooperation between cells from distinct individuals with distinct genotypes in the nacreous secretion process. In an attempt to clarify its role and presence throughout the course of biomineralization, we tried to detect its presence using molecular methods, to screen for the occurrence of a foreign genotype in the pearl sac of the recipient oyster. We compared the genomes in the muscles and pearl sacs of 80 black-lipped pearl oysters, *Pinctada margaritifera*, at the day of harvesting (about 18 months after the graft), using three codominant polymorphic molecular markers (Arnaud-Haond et al., 2002, 2003a,b).

Materials and Methods

Sampling and Extraction. Two series of 50 and 30 pearl oysters were collected 18 month after grafting, at the time of pearl collection. The pearl sac was extracted with the pearl inside, and the cells layer around the pearl were carefully split until a layer of one cell width surrounding the pearl was obtained. This layer and a piece of the adductor muscle of the recipient oyster were both labeled identically and preserved in 80% ethanol. The second series of 30 pearl oysters was sampled and processed after the first series of 50 had been entirely analyzed. This was done to make sure that the discrepancies observed between the genotypes of pairs of muscles and pearl sacs was a repeatable observation, and could not be attributed to some incident mixing of the sample during the sampling or genotyping processes.

The procedures of DNA extraction, precipitation, and storage were similar to those described in Sambrook et al. (1989), using approximately 0.5 g of chopped and subsequently air-dried tissue. The nucleic acid pellet obtained after precipitation in 100% ethanol was washed with 70% ethanol, air-dried, resuspended in 100 to 200 μ l of deionized water, and preserved at -20°C . Concentration of DNA extraction was estimated via fluorometry, and all extractions were rediluted to obtain a standardized concentration of DNA of 50 ng/ μ l for each extract.

Polymerase Chain Reaction (PCR) and Genotyping. Three markers developed (Arnaud-Haond et al., 2002) via the direct amplification of length polymorphism (DALP; Desmarais et al., 1998) and the exon primers intron crossing (EPIC; Palumbi, 1995) methods were used: pinucl2, pinucl3, and pinaldo (Table 1).

PCR was performed in a 20- μ l reaction volumes with final concentrations of 300 μ M each dNTPs, 1.8 mM MgCl_2 , 0.4 μ M of each primer, about 100 ng of template DNA, $1\times$ Taq buffer, and 0.75 U of Taq

polymerase. For all PCR reactions, a negative control was used, replacing DNA with nanopure water, to ascertain the absence of contamination of the PCR reaction. This negative control would always lead to the absence of PCR product. PCR products were separated through 6% denaturing polyacrylamide gels (acrylamide-bisacrylamide, 29:1, 7 M urea) using $1\times$ Tris-borate-EDTA buffer, running each pair of samples (the DNA extracted from pearl sac and from the corresponding adductor muscle) in neighboring lanes to facilitate comparison of the genotypes revealed by PCR in each tissue. The gels were then silver stained according to Bassam et al. (1991).

Results and Discussion

For the three loci pinucl2, pinucl3, and pinaldo, among the respectively five, five, and six alleles observed over all the samples analyzed in Polynesia (Arnaud-Haond et al., 2002, 2003b, 2004), respectively three, three, and six were observed in the two series of grafted oysters analyzed. In the first series of 50 oysters analyzed at the time of pearl collection, differences in the genotype of the muscle and pearl sac cells were revealed in respectively 43%, 44%, and 7% of the sample pairs successfully genotyped. Owing to the high sensitivity of PCR, it is very probable that an admixture of host and donor genotypes were amplified from the isolated pearl sac, but in any case the discrepancies between the genomes from muscle and pearl sac demonstrate the presence of donor cells as well as their corresponding genome in the pearl sac at the moment of pearl collection, 18 months after grafting. Indeed in some cases, three bands appeared in the lane corresponding to the pearl sac, resulting from the simultaneous amplification of distinct alleles from donor and recipient oysters that were both present in the pearl sac. Similar results were obtained on the second series of 30 oyster analyzed after. Joint analyses of both series distinguished about 65% pairs of distinct genotypes in

Table 1. Detail of the Loci Used, with the Sequence of Each Primer, Range of Size of the Fragments on the Basis of Sequences, and Names of Alleles Observed Polynesian Archipelagoes

Locus	Primers	Range size	alleles
Pinucl2	TAGGGTACAGTCCGAGGTGTTCCG TCATTAATTGTCGAAAACCTTGTC	270–320	(210) (220) (230) (240) (250)
Pinucl3	CCCAAAATTGTTCCGGGGAGC CCCAAAATTGTTCCGGGGAGC	170–190	(090) (100) (110) (120) (130)
Pinaldo	CTTTGCAACGGCTGTATAGC TAGGCCACCCAATCAGAGTTC	80–100	(070) (080) (085) (090) (095) (100) (110)

Alleles observed in the two series of grafted pearls analyzed are indicated in bold.

Table 2. Expected Numbers of Undistinguishable Genotypes Estimated on the Basis of Allele Frequencies and Consequent Pairs of Possible Multilocus Genotypes*

	pinucl2	pinucl3	pinaldo	Overall
Expected	38.86	33.68	5.71	17.52
Observed	43	35	8	25

*Compared to the observed number of pairs of distinct genotypes (when comparing pearl sac and corresponding muscle) after amplification of the loci pinucl2, pinucl3, and pinaldo on a total of 80 pairs of samples analyzed. The χ^2 test applied on the values from the three loci ($\chi^2 = 0.58$; $P > 0.25$) did not allow rejection of the hypothesis that the pearl sac would be systematically constituted of cells from the graft, bearing in some cases a genotype that could not be distinguished from the host genotype.

pearl sacs versus muscle tissue (Table 2). In most cases where differences were highlighted, two bands were observed in the lane corresponding to the amplification product from pearl sac extract (Figure 1), one band being absent in the corresponding muscle PCR product. This implies most recipient oysters were homozygotes, and donors may have been homozygote for the distinct allele appearing in the pearl sac, or heterozy-

gote with only one allele in common with the host oyster.

Once we established the presence of donor cells in the pearl sac 18 months after grafting by the recognition of a foreign genotype present together with the host genotype in the pearl sac, we wanted to screen for the extent of this process. The next question we aimed to answer was therefore whether the grafted cells from the donor systematically persisted in the pearl sac during the entire process of pearl formation, or only occasionally. The probability of occurrence of each possible monolocus genotype was estimated according to allelic frequencies at each locus, assuming Hardy-Weinberg equilibrium and random mating. The expected probabilities that the donor and recipient oyster genotypes were distinguishable were computed on the basis of the multilocus genotype probabilities. We did expect to observe distinct genotypes while comparing PCR products issued from pearl sac and muscle extract in all the cases in which the donor oyster bore at least one allele that was not present in the recipient oyster genome. This probability made it possible to estimate the number of cases in which we expected to observe distinguishable genotypes for at least one locus, under the assumption the donor cells would systematically contribute to pearl sac formation, and the genome of the donor would therefore always be present in the pearl sac. This value was compared to the observed number of cases in which distinguishable genotypes were indeed amplified from extracts of pearl sac and its corresponding adductor muscle (Table 2). A χ^2 test was then performed and showed that V_{obs} and V_{exp} were not significantly different ($\chi^2=0.58$, with a $\text{ddl}=2$; $P > 0.25$), supporting the hypothesis that the pearl sac was systematically bearing cells and genome from the donor oyster.

The pearl sac, the formation of which is induced by the graft process, is composed of a single layer of epithelial cells, the origin of which remained unclear so far because of the process of cell dedifferentiation and proliferation following the grafting (Machii, 1968; Herbaut et al., 2000) that did not allow tracing the fate of the graft cells via classical

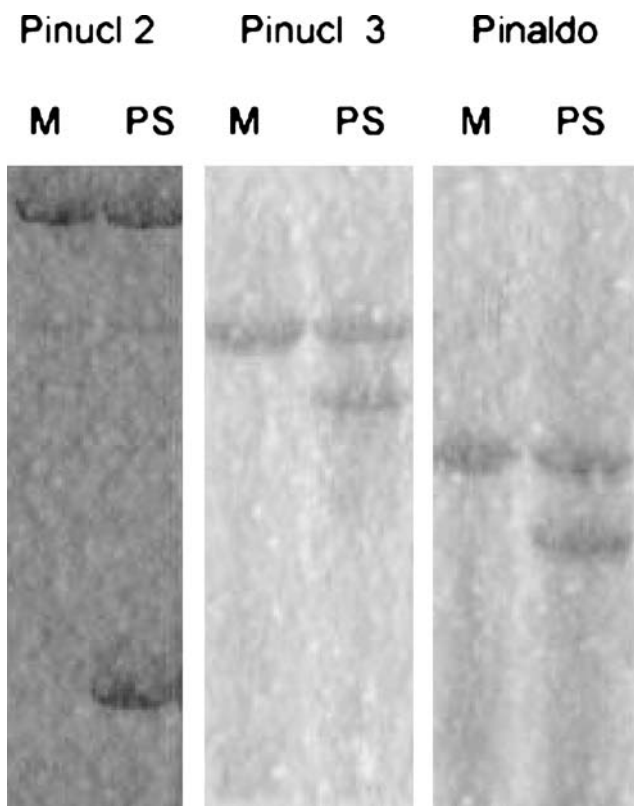


Fig. 1. Electrophoresis gel showing differences between PCR products for the locus pinucl2 amplified from genomic DNA extracted from muscle (M) and corresponding pearl sac (PS) tissues from five oysters. From left to right, each pair of samples shows a different genotype with pinucl2 (M homozygote for the allele 250 and PS heterozygote with the alleles 210 and 250), pinucl3 (M homozygote for the allele 110 and PS heterozygote with the alleles 100 and 110), and pinaldo (M homozygote for the allele 100 and p heterozygote with the alleles 090 and 100).

histological methods. We found a high number (about 65%) of differences between the muscle and corresponding pearl sac genotypes (Figure 1). The proportion of indistinct muscle-pearl sac genotypes is not significantly different from the expected proportion of nondistinguishable genotypes between distinct individuals of the population that contained the host oyster. These results support the systematic survival of grafted mantle cells in the recipient oyster during the whole process of pearl formation, and their participation in the constitution of the pearl sac that secretes nacreous layers. In agreement with empirical observations of professional grafters, this study supports the idea that the part of the pearl properties determined by genetic factors can be influenced by the donor genome, thus making pearl formation a unique example of cooperation among distinct genomes in a biomineralization process. In future genetic selection programs, efforts may therefore be focused on selection not only in the recipient, but also in the donor oyster.

Acknowledgments

We thank Gaby from Takapoto and the Ministry for Pearl Culture in Papeete for providing samples, and two anonymous referees for useful comments on a preliminary version of the manuscript.

References

- Arnaud-Haond S, Boudry P, Saulnier D, Seaman T, Vonau V, Bonhomme F, Goyard E (2002) New anonymous nuclear DNA markers for the pearl oyster *Pinctada margaritifera* and other *Pinctada* species. *Mol Ecol Notes* 2, 220–222
- Arnaud-Haond S, Bonhomme F, Blanc F (2003a) Large discrepancies in differentiation of allozymes, nuclear and mitochondrial DNA loci in recently founded Pacific populations of the pearl oyster *Pinctada margaritifera*. *J Evol Biol* 16, 388–398
- Arnaud-Haond S, Vonau V, Bonhomme F, Boudry P, Prou J, Seaman T, Veyret M, Goyard E (2003b) Spat collection of the pearl oyster (*Pinctada margaritifera cumingii*) in French Polynesia: an evaluation of the potential impact on genetic variability of wild and farmed populations after 20 years of commercial exploitation. *Aquaculture* 219, 181–192
- Arnaud-Haond S, Vonau V, Bonhomme F, Boudry P, Prou J, Seaman T, Goyard E (2004) On the impact of cultural practices on genetic resources: evolution of the genetic composition of wild stocks of pearl oyster (*Pinctada margaritifera cumingii*) in French Polynesia after ten years of spat translocation. *Mol Ecol* 13, 2001–2007
- Bassam BJ, Caetano-Anolles G, Greshof PM (1991) Fast and sensitive silver-staining of DNA in polyacrylamide gels. *Anal Biochem* 196, 80–83
- Desmarais E, Lanneluc I, Lagnel J (1998) Direct amplification of length polymorphism (DALP), or how to get and characterise new genetic markers in many species. *Nucleic Acid Res* 26, 1458–1465
- Herbaut C, Hui B, Herbaut J, Remoissenet G, Boucaud E (2000) The pearl: isolation of outside bodies by molluscs: evolution of the graft and the pearl-sac in *Pinctada margaritifera* (Mollusca, Lamellibranchia). *Bull Soc Zool France* 125, 63–73
- Machii A (1968) Histological studies on the pearl sac formation. *Bulletin of the Pearl Res Lab* 13, 1489–1539
- Palumbi SR (1995) "Nucleic acids II: the polymerase chain reaction". In: *Molecular Systematics*, Hillis D, Moritz C, eds. (Sunderland, MA: Sinauer) pp 205–247
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning*, 2nd ed.. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press)
- Wada KT, Komaru A (1996) Color and weight of pearls produced by grafting the mantle tissue from a selected population for white shell color of the Japanese pearl oyster *Pinctada fucata martensii* (Dunker). *Aquaculture* 142, 25–32