Journal of Heredity
June 2018, Volume 109, Issue 5, Pages 510-519
http://dx.doi.org/10.1093/jhered/esy015
http://archimer.ifremer.fr/doc/00433/54505/

Crossing phenotype heritability and candidate gene expression in grafted black-lipped pearl oyster *Pinctada margaritifera*, an animal chimera

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Abstract :

Grafting mantle tissue of a donor pearl oyster into the gonad of a recipient oyster results in the formation of a chimera, the pearl sac. The phenotypic variations of this chimera are hypothesized to be the result of interactions between the donor and recipient genomes. In this study, the heritability of phenotypic variation and its association with gene expression were investigated for the first time during *P. margaritifera* pearl production. Genetic variance was evaluated at different levels, 1) before the graft operation (expression in graft tissue), 2) after grafting (pearl sac tissue expression in chimera) and 3) on the product of the graft (pearl phenotype traits) based on controlled bi-parental crosses and the F1 generation. Donor related genetic parameter estimates clearly demonstrate heritability for nacre weight and thickness, darkness and colour, surface defects and grade, which signifies a genetic basis in the donor oyster. In graft relative gene expression, the value of heritability was superior to 0.20 in for almost all genes, while in pearl sac, heritability estimates were low ($h^2 < 0.10$) (except for *CALC1* and *Aspein*). Pearl sac expression seems to be more influenced by residual variance than the graft, which can be explained by environmental effects that influence pearls sac gene expression and act as a recipient additive genetic component. The interactions between donor and recipient are very complex and further research is required to understand the role of the recipient oysters on pearl phenotypic and gene expression variances.

Keywords : Heritability, Phenotype, Gene expression, Pearl oyster, Pinctada margaritifera

Introduction

Quantitative genetics is a powerful framework to explore the complex genetic architecture of phenotypic traits (Kruuk, & Hadfield 2007). The fraction of the phenotypic variability that is of transmittable genetic origin is called heritability (Falconer & Mackay 1996; Roff 1997; Lynch & Walsh 1998). Quantitative genetic approaches have been designed to determine to what degree this phenotypic variation is genetically rather than environmentally determined (Falconer, 1989). Broad-sense heritability (H^2) estimates the proportion of phenotypic variation due to all genetic effects, whereas narrow-sense heritability (h^2) estimates the proportion of phenotypic variation due to heritable genetic variation alone (Visscher *et al*. 2008). Recent reports of substantial heritability for gene expression and new estimation methods using marker data highlight the relevance of investigating heritability in the genomics era. At the transcriptome level, gene expression profiling has become a popular technique used to quantify regulatory changes in messenger (m)RNA expression. Indeed, gene expression acts as an intermediate phenotype between genotypes and complex traits (Nica and Dermitzakis 2008; Li *et al.* 2012; Goldinger *et al.* 2013). To investigate heritability, the expression profile of a gene in a segregating population can be treated as a quantitative trait and its additive genetic variance estimated (Visscher *et al.* 2008; Powell *et al.* 2013; Wright *et al.* 2014; McCairns *et al.* 2016). Genetic variation underlying gene expression levels has been well established and reported in the literature, with the transcript levels for the majority of genes being heritable to some degree (Price *et al.* 2011; Grundberg *et al.* 2012; Powell *et al.* 2012b), but inconsistency in heritability principles raises questions about the transmission process.

Heritability is of great relevance for breeding strategy as it measures the potential response to selection (Lynch and Walsh 1998; Falconer 1989; Mousseau 1998). In cultivated populations, the selection procedure chosen needs to be the best adapted to the breeding plan, allowing assessment of genetic parameters in few generations based on a small effective population. In the context of cultured pearl production by the *Pinctada* genus, the complexity of the graft leading to a chimera type complex makes it more complicated to understand the heritability of any phenotypes or

candidate gene expression. In the plant kingdom, the heritability of grafted-induced phenotypic changes suggests that regulatory processes underlying the scion-rootstock communication also involve a genetic component (Tsaballa *et al.* 2013). Some studies have demonstrated the exchange of genetic material between cells in grafted plants (Stegemann & Bock 2009). Recently, increasing effort has been made to determine how macromolecules are transferred between scions and rootstocks in grafted plants to reveal the mechanism that controls graft-induced changes in plant traits (Paultres *et al.* 2016). Grafting is characterized by tight connections between cells with different genomes, providing the possibility of interactions or cell communication between genetically divergent cells, resulting in a profound perturbation of the cellular environment (Cao *et al.* 2016). Chimeras provide one of the most interesting environments in which to investigate the transmission of genetic material and the resulting phenotypic variation. Thus, the phenotypic variations of the chimera are hypothesized to be the result of interactions between the different genomes.

In the case of pearl bivalve aquaculture based on a grafting operation, previous genetic studies have primarily focused on determining genetic parameters for shell growth, aiming to detect any significant genetic variation for shell growth in the pearl mussel *H. cumingi* (Jin 2012) and in the pearl oysters *Pinctada fucata martensii* (He *et al.* 2008; Wang *et al.* 2010) and *P. maxima* (Kvingedal *et al.* 2010). For *P. margaritifera*, genetic analyses based on heritability estimations are still lacking for both quantitative pearl traits and expression levels of some biomineralization genes. A study was made on *P. maxima*, with the estimation of the genetic parameters (heritability and genetic correlations) of commercially important pearl traits (Jerry *et al.* 2012). The production of cultured pearls is both unique and biologically complex in comparison to any other aquaculture industry. *P. margaritifera* produces valuable pearls as a result of the biomineralization process of a mantle graft originating from a donor oyster, inserted together with a nucleus, into the gonad of a recipient oyster (Southgate 2011). The grafting process therefore associates two distinct genotypes, each of which maintains its own genetic identity throughout the life of the grafted organism (the recipient), but which survive together as a genetic chimera due to a unique symbiotic relationship (Mudge *et al.*

2009). Exploring the heritability of candidate gene expression in the graft tissue (donor) and pearl sac (chimera), and the heritability of pearl phenotypic traits (product of the chimera) is vital to understanding the phenotypic variations induced by the grafting process and the recipient environment.

This original study aimed to evaluate *P. margaritifera* genetic variance for both pearl traits and biomineralization gene expression levels, based on a multi-cross design that made it possible to consider parental and segregating progeny contributions at three material levels: 1) the mantle graft tissue gene expression, 2) the pearl sac tissue (chimera) gene expression and 3) the final product at harvest, the pearl phenotypes. Most previous studies have estimated the genetic contribution to phenotypic traits and, more recently, examined relative gene expression, but they have rarely crossed the traits and the gene expression in the same analysis. In the present study, heritability will then be estimated from parents to progenies within different bi-parental crosses, making it possible to evaluate character transfer in a two-generation framework. The representative panel of genes encoding proteins involved in the biomineralization process that we screened in the graft and pearl sac were: 1) aragonite: *Pif-177*, *MSI60* and *Perline*; 2) calcite (*Aspein*, *Shematrin* and *Prismalin*); and 3) for proteins implicated in both layers, *Nacrein* (Marie *et al.* 2012; Joubert *et al.* 2010; Xiang *et al.* 2013).

Materials and methods

Experimental design

Nine bi-parental *P. margaritifera* families (named A1, B2, D2, F5, G6, H6, H7, I6 and I7) were produced in the Ifremer hatchery system facilities in Vairao, Tahiti, French Polynesia, using female and male broodstock from Mangareva Island (Gambier Archipelago, French Polynesia). Spawning was induced by thermal shock (Ky et al. 2015a). Nine families were produced in two distinct periods (i.e. two separate controlled breedings, #1 and #2), 5 families (A1, B2, D2 and F5) using 4 females and 3 males (in March 2013), and 4 families (G6, H6, H7, I6 and I7) using 3 females and 2 males (in August 2013). Figure 1 illustrates the breeding design, showing that individuals 2, 6 and 7 (males) and H and I (females) were used in multiple combinations. Artificial breeding, larval rearing and oyster culture procedures were conducted using the protocol developed by Ky *et al*. (2013).

Individuals of the nine families that would be used as donor oysters were randomly selected and transferred by air to Mangareva Island (Gambier Archipelago), allowing the oysters to be cultured in natural environmental conditions. Two months prior to nucleus implantation, oysters from the nine progenies were taken from the rearing station and stored ready for use in the grafting procedure.

Grafting procedure

As the grafting operation itself may influence cultured pearl quality, all grafts were performed under standard production conditions by a single expert at the Regahiga Pearl Farm using a single nucleus size of 1.8 BU (5.45 mm diameter; Imai Seikaku Co. Ltd., Japan). All recipient pearl oysters were obtained by natural spat collection from the wild in the Mangareva lagoon. They were selected based on visible health status (colour of the visceral mass and gills), shell size appearance, and muscle resistance when prising the shells slightly open.

A total of four different experimental grafts were performed; two using the parents of the two breeding designs (one per breeding) and two others using the progenies (one per breeding). For

breeding #1, all the five parents were used as donors, covering a total of 229 grafts, with for A (*n* = 28 grafts), B (*n* = 36), D (*n* = 37), F (*n* = 29), 1 (*n* = 23), 2 (*n* = 36), 5 (*n* = 40). For breeding #2, 200 grafts were produced with a standard 40 grafts per parent. Concerning the progenies, 20 donors per families were used, with donors providing 1260 grafts for breeding #1 and 2000 grafts for the breeding #2. At 45 days post-grafting, recipient oysters were checked to estimate nucleus retention and oyster mortality rates as described in Ky *et al*. 2014. After this check, recipient oysters that had retained their nuclei were drilled and fixed to chaplets for long term culture and each chaplet was labelled according to the corresponding donor oyster for traceability.

Pearl quality variables

After 18 months of culture in Regahiga lagoon, the cultured pearls were harvested and placed separately in compartmented boxes that allowed traceability between the pearls and corresponding donor oyster family. Once harvested, cultured pearls were cleaned and five variables were measured to characterize their quality (Figure 2):

- The size of the cultured pearls was assessed by measuring nacre thickness and weight.
- Cultured pearl shape was characterized in two ways: the presence / absence of circle(s) and the shape category (“b” for baroque and semi baroque, “o” for oval and drop, “r” for round and semi-round pearls).
- The colour of the pearls was evaluated on the basis of the darkness of their colour and their visually perceived colour category, which is conferred by pigments (bodycolor: grey, white and yellow), and secondary colours (overtone: green, aubergine and peacock).
- The cultured pearl grade was determined for each sample according to the official A–D Tahitian classification (Journal Officiel 2001 n° 30, 26 July 2001) from the most to least valuable quality: A, B, C, D and Rejects (*rebuts*).
- The surface defects and lustre (components of cultured pearl grade) were determined separately so that they could be analysed independently.

Quality traits were evaluated as described in Ky *et al.* (2013). To ensure homogeneity in parameter assessment, all evaluations were made visually (without a jeweller's loupe) by 2 operators working together and cross checking.

Gene expression variables

The formation of the molluscan shell nacre is regulated to a large extent by a matrix of extracellular macromolecules that are secreted by the shell-forming tissue and the mantle (Ellis and Haws 1999). Recently, the number of genes identified as coding for molluscan shell matrix components has increased (Susuki *et al.* 2009, Miyamoto *et al.* 2005, 2013, Marie *et al.* 2012, Joubert *et al.* 2010, Montagnani *et al.* 2011, Huang *et al.* 2012, Suzuki and Nagasawa, 2013, Shi *et al.* 2013). In order to identify variability in gene expression in the graft process, we sampled 3 to 5 grafts per donor during the graft operation and pearl sacs during harvest (preserved in RNAlater® and stored at –80°C for subsequent RNA extraction). In order to minimize the mixture of recipient tissues, the pearl sacs were excised from host oysters by removing the outer layers with a surgical blade until a thin (< 0.5 mm) layer tissue surrounding the pearls remained, and immediately transferred and preserved into 2.0 ml tubes with RNAlater®". We then evaluated relative gene expression by screening aragonite-related genes (*Pif-177, MSI60* and *Perline*), calcite-related genes (*Aspein, Shematrin5, Shematrin9* and *Prismalin*) and one gene implicated in both layers (*Nacrein*) (Blay *et al*. 2017) (Table 1). Total cellular RNA was extracted from the initial graft tissues and harvested pearl sacs (final graft stage) using TRIZOL reagent (Life Technologies) according to the manufacturer's recommendations. For each sample, 3 µg of total RNA were treated with DNase using a DNA-free Kit (Ambion). For each sample, 0.5 µg of total RNA were reverse-transcribed using a Transcriptor First Strand cDNA Kit (Roche) and amplified by real-time PCR on a Roche Light Cycler® 480 using a set of forward and reverse primers (Blay et al. 2017). Two other genes were used as "housekeeping genes": *REF1* (Joubert *et al.* 2014) and *GAPDH* (Lemer *et al.* 2015). The amplification reaction details are provided in Blay et al., 2017. All measurements were performed on duplicate samples and all analyses were based on the Ct values of the PCR products. Relative gene expression was calculated using two

reference genes *GAPDH* and *REF1*, by the 2-ΔCt method (Livak and Schimittgen, 2001), as follows: Relative expression $_{(\text{target gene, sample x})} = 2^{\wedge -(\Delta Ct \text{ sample, sample x} - \Delta Ct \text{ calibrator, sample x})} = 2^{-\Delta\Delta Ct}$. Here, the ΔCt calibrator is the mean of the ΔCt values obtained for the tested gene. The delta threshold cycle (ΔCt) is calculated by the difference in Ct for the target and reference genes. The relative stability of the *GAPDH* and *REF1* combination was confirmed using NormFinder (Stability value for best combination) (Anderson *et al.* 2004).

Statistics

The normality of data distribution and homogeneity of variances were tested for pearl size and graft and pearl sac relative gene expression data using the Shapiro–Wilk test and Bartlett's test. When necessary, transformations were used to adjust data to this distribution (logarithm or square root).

We first evaluated the "family effect" on culture pearl trait and gene expression among the progenies of the controlled breeding (Table 2). An ANOVA was performed to test for "family effect" on cultured pearl weight, thickness and gene expression in the graft among the progenies. To test for "family effect" on pearl sac gene expression, ANOVA was performed on progenies of breeding #1 only (pearl sacs from breeding #2 were not harvested). If the overall test was significant, a Dunn procedure, including Bonferroni correction for multiple tests, was performed among all pairs of families. Qualitative classes based on cultured pearl surface defects, lustre, grade, darkness and circles were re-encoded to give quantitative scores that would enable the mean value of progenies to be obtained for each criterion, thus allowing them to be ranked. For each criterion, Kruskall–Wallis tests were then applied to compare the progenies. For the cultured pearl colour and shape categories, differences and family effects were evaluated using χ^2 tests (Ky *et al*. 2013).

Quantitative genetic parameters and variance components were estimated using an animal model (Kruuk 2004) based on the donor oyster family relationships. The analyses were implemented in R© software using the Markov Chain Monte Carlo for generalized linear mixed models

(MCMCglmm) package (Hadfield 2010). We considered the phenotype y_i of the individual *i* as a variation around the average population phenotype μ as a function of the pedigree of the individual and other factors. The model was:

$$y_i = \mu + a_i + e_i$$

In this equation, μ stands for the average population phenotype, a_i is called the breeding value and accounts for the influence of the additive effect on the phenotype, and e_i is a residual accounting for the variation not captured by the phenotype. Host genetic variation was considered as a common environmental effect. "Animal" was included as a random effect. The heritability (h^2) was estimated as the ratio of the additive genetic variance to total phenotypic variance.

$$h^2 = \sigma_a/\sigma_p = \sigma_a/(\sigma_a + \sigma_f + \sigma_r)$$

Where σ_a is an estimate of the additive variance, σ_f is an estimate of random variance and σ_r is an estimate of the residual variance. When summed, these three components add up to the total phenotypic variance σ_p.

In all cases, the differences were considered statistically significant when *p* values were lower than 0.05. Statistical analyses were performed using R© software (version 3.2.1).

Results

Cultured pearl quality traits were recorded and analysed on a total of 2241 samples, resulting from grafts made using tissue from the parental and progeny generations. Variations in pearl quality traits and both graft and pearl sac relative gene expressions are given in Tables S1, S2, S3 and S4, supporting information. Results are presented in three sections below corresponding to: 1) family and cohort effects on pearl quality and gene expression, 2) heritability of cultured pearls traits, and 3) heritability of gene expression levels.

Family effect on pearl quality traits and gene expression

The average nacre thickness among the 2241 harvested pearls was 0.11 cm, with minimum and maximum values of 0.01 and 0.37 cm, respectively. The average nacre weight was 0.62 ± 0.29 g, with minimum and maximum values of 0.05 and 3.35 g, respectively. The nacre weight and thickness for each family and parent are given in Table S1, supporting information. Very highly significant "family effects" were demonstrated for the nacre thickness and weight ($p < 0.0001$) (Table 2). The cultured pearl quality traits are described in Table S2, supporting information. Overall, analyses comparing differences in pearl quality indicators among the nine families showed a significant "family effect" for all traits except lustre (Table 2).

Analyses comparing differences in relative gene expression of the graft from the nine families showed a highly significant "family effect", with the main differences between the nine families (Table 2). This effect was the least significant for relative expression of the *Pif-177* gene ($p = 0.01$). Relative gene expressions in the graft among the eight genes are given in Table S3, supporting information. All differences between the nine families for each gene are shown in Fig S1, supporting information.

The comparison of the relative expression of the eight genes in the pearl sac showed a significant "family effect" for four genes *Aspein* ($p = 0.01$), *MSI60* ($p = 0.01$), *Shematrin9* ($p = 0.01$) and *Nacrein* ($p = 0.04$) (Table 2). Pearl sac relative gene expressions are given in Table S4, supporting

information. All differences between the families for each gene in pearl sac are shown in Fig S2, supporting information.

The high variability across samples for the gene expression data could be explained by the correlation between the gene expressions with the pearl's surface quality (Blay *et al*., 2017). Moreover, in *P. margaritifera,* we have a lot of variability in pearl culture quality at the time of harvest, with surface defects, surface deposits and grade classification, which could justify the range of standard deviation observed.

Heritability of cultured pearl traits

Heritability estimates for donor-derived pearl quality traits are given in Table 3. A moderate to low heritability was found for darkness (h^2 = 0.37; 95% CI [0.30, 0.44]), nacre weight (h^2 = 0.34; 95% CI [0.27, 0.41]), nacre thickness (h^2 = 0.29; 95% CI [0.22, 0.36]), surface defects (h^2 = 0.21; 95% CI [0.15, 0.28]), grade (h^2 = 0.19; 95% CI [0.11, 0.25]), colour (h^2 = 0.16 ; 95% CI [0.11, 0.23]) and lustre (h^2 = 0.12; 95% CI [0.06, 0.18]). For these heritable expression traits, the genetic variance component explained between 12 and 37% of the total variance. However pearl shape and presence / absence of circle(s) showed low heritability values (h^2 = 0.02; 95% CI [0.00, 0.06] and h^2 = 0.05; 95% CI [0.01, 0.10], respectively) attributable to the donor.

Heritability of relative gene expression levels

Heritability estimates for donor-derived relative gene expression in the graft are given in Table 3. With the exception of *Pif-177* transcript levels, which showed only a low heritability, *MSI60* (h^2 = 0.42; 95% CI [0.24, 0.63]), *Perline* (h^2 = 0.47; 95% CI [0.22, 0.72], *Nacrein* (h^2 = 0.37; 95% CI [0.22, 0.54]), *Aspein* (h^2 = 0.33; 95% CI [0.14, 0.51]), *Prismalin* (h^2 = 0.44; 95% CI [0.27, 0.6]), *Shematrin5* (h^2 = 0.35; 95% CI [0.21, 0.52]) and *Shematrin9* (h^2 = 0.25; 95% CI [0.11, 0.41] showed moderate to high heritability.

Regarding relative gene expression in the pearl sac, heritabilities are given in Table 3. Except *Aspein*, which showed a high heritability (h^2 = 0.41; 95% CI [5E-5, 0.77]), and *Nacrein*, which showed a moderate heritability (h^2 = 0.25; 95% CI [5E-5, 0,67]), expression levels of all other genes had low

heritabilities ($h^2 < 0.10$).

Discussion

The present approach in a complex animal graft model evaluates the genetic parameters of relative gene expression of the graft tissue (at grafting time), the pearl sac tissue (at harvest), together with the pearl quality trait phenotypes (i.e. the product of the grafting procedure). We report for the first time in *P. margaritifera* the variation together in the phenotype and in the transcription response (i.e., gene expression).

Gene expression heritability

It is well known that traits under genetic control are likely to demonstrate higher heritability values than those whose variability is highly influenced by the environment (Fisher 1930; Falconer and Mackay 1996; Lynch and Walsh 1997). The data of the present study indicate that the parental effects on gene expression level are much stronger in the mantle graft, than in the pearl sac. From our study, the value of heritability was moderate to high ($h^2 > 0.20$) in graft relative gene expression for almost all genes (only *Pif-177* showed a low heritability; $h^2 = 0.11$), whereas heritability values were low for expression in the pearl sac ($h^2 < 0.10$) (except for *Nacrein* ($h^2 = 0.25$) and *Aspein* ($h^2 = 0.41$)). The pearl sac therefore seems to be more influenced by residual variance than the mantle graft provided by the donor oyster. The residual variance can mainly be explained by environmental effects that influence pearl sac gene expression and act as a recipient additive genetic component. Unfortunately, we were unable to estimate recipient oyster-derived genetic parameters for relative gene expression because recipient oysters were obtained from natural spat collection in which we could not control the natural variance. Previous studies observed a significant correlation between recipient shell size and harvested pearl size in *P. fucata martensii* (Wada and Komaru, 1996), and in *P margaritifera* (Ky *et al.* 2017; Blay *et al.* 2017). So pearl size is likely to have a recipient additive genetic component. The low heritability levels of gene expression in pearl sac corroborate a recipient additive genetic component and suggest that the deposition of nacre on the pearl may be dependent on the capacity of the recipient oyster to gather energy and allocate it to cellular growth and nacre deposition processes (Wada and Jerry 2008; Le Pabic et al. 2016). Moreover, this low heritability

could be induced by grafting, particularly by the recipient cellular environment. A recent histological examination and chronological description of pearl sac development in *P. margaritifera* (Kishore and Southgate 2016) showed that complete attachment of the mantle graft tissue to the host tissues had taken place by 14 days after grafting. The newly developed pearl sac could barely be distinguished as foreign tissue present in host oysters at this stage. In fact, the pearl sac had attained the visible characteristics of the host tissue and could not be differentiated as foreign tissue by 18 days after grafting. In the plant kingdom, grafting is characterized by a tight connection between cells, providing the possibility of interactions or cell communication between different cell lineages and resulting in a profound perturbation of the cellular environment (Cao *et al.* 2016). Grafting involves contact between heterologous cells at the stock and scion junction. It has been previously shown that that cells of the scion and stock individuals become linked to each other, and that genetic material (macromolecules including DNAs, RNAs and protein) can be transported between them (Jackson *et al.* 2001; Li *et al.* 2013; Wang *et al.* 2016; Cao *et al.* 2016). Recently, several studies have reported that endogenous small RNAs can be transmitted in chimeras during grafting and induce epigenetic modifications such as DNA methylation and RNA silencing, without changing the DNA sequence (Haque *et al.* 2007; Molnar *et al.* 2010; Wu *et al.* 2010; Li *et al.* 2013; Wang *et al.* 2016). Interactions between scions and rootstocks are complex but they mutually affect the graft zone and research has increasingly attempted to uncover the processes involved in these interactions (Wang *et al.* 2016). In the present animal model, detailed mechanisms enabling intercellular molecular transport need further research in order to confirm or refute their likeness with plant kingdom chimera and propose mechanisms that could help us to understand how this environment could moderate heritability in pearl sac gene expression.

Moreover the present heritability values based on pedigree assignment explained only a minority of the expected heritable fraction. Although the majority of transcripts appear to have genetic variation for expression in the graft, less than 50% of the total phenotypic variation is typically explained by additive effect. Other non-additive genetic effects contribute significantly to

transcriptional variation. This variance is known as “missing heritability” and its underlying factors and mechanisms are not precisely established (Trerotola *et al*. 2015). Gene expression heterogeneity can be influenced by cell cycle position, stochastic expression or epigenetic effects (Parts *et al.* 2014). In recent decades, some studies clarify that non-genetic sources of heritable phenotypes play a role in phenotypic variations (Jablonka & Lamb 2008; Danchin *et al.* 2011; Laland *et al*. 2014). In particular, epigenetic modifications (defined as heritable changes in chromatin structure and DNA methylation) impact gene expression (Migicovsky & Kovalchuk 2011) without affecting the underlying genomic sequences (Gibney *et al.* 2010; Trerotola *et al.* 2015). Epistatic components need to be integrated by estimating the contribution of non-genetic factors (Koch, 2014). In the present study, further work needs to be done on epistatic variance. Furthermore, genetic regulation does not only happen at the transcription level, further investigation on the expression of matrix protein in the pearl sac at the protein level should be made.

Relationship between pearl phenotype and gene expression

Gene expression levels constitute an intermediate step toward final phenotype expression (Hubner *et al*. 2005; Emilsson *et al.* 2008; Chakravarti *et al*. 2013; Parts *et al.* 2014). Some studies have combined genetic data and genome-wide gene expression analysis to try to understand the genetic basis of gene expression (Brem *et al.* 2002; Schadt *et al.* 2003; Cheung *et al.* 2003). In such studies, mRNA levels are considered as a phenotypic value, which is subjected to variation (as every phenotypes) due to experimental, environmental and/ or genetic sources. These variation and associated heritability could thus be estimated. It was first demonstrated that, within populations, statistically significant estimates of heritability were found for gene expression in a much larger proportion of genes than would be expected by chance (Schadt *et al.* 2003; Cheung *et al.* 2003). Such evidence of heritability for gene expression is important because statistical power to detect gene variants that affect gene expression depends on heritability (Visscher *et al.* 2008). In the present study, it was not possible to combine genetic data and genome-wide gene expression analysis, but it

was possible to combine data on gene expression in the pearl sac with phenotype traits to show the relationship between the final pearl phenotype and gene expression level.

Nacrein and *Aspein* were the only transcripts in the pearl sac for which the heritability estimates are rather high or moderate. Shell matrix proteins play a key role in the shell bio-mineralization process. Some genes encoding the proteins of the calcified matrix have been identified and are known to be specifically involved in the formation of the nacreous layer and/or prismatic layer, playing a role in crystal nucleation, orientation, polymorph and morphology during deposition of the two shell layers (Joubert *et al.* 2010; Montagnani *et al.* 2011; Marie *et al.* 2012). *Aspein* is thought to play a key role in calcite precipitation (Takeuchi *et al.* 2008; Isowa *et al.* 2012). In contrast, *Nacrein*, which is found in both the nacreous and prismatic layers of the shell, exhibits carbonic anhydrase activity and probably functions as a supplier of bicarbonate ions and a regulator of calcium carbonate crystal formation (Miyamoto *et al.* 2013; Liu *et al*. 2012; Suzuki & Nagasawa 2013). In previous studies, we found a significant correlation between relative gene expression of *Aspein* in the pearl sac with the component of quality traits (surface defects, lustre and grade), and with colour. Furthermore a significant correlation was found between *Nacrein* relative gene expression with colour of the pearl (and no significant correlation in graft tissue) (Blay *et al.* 2017). These two candidates seem to be involved in pearl quality and colour phenotypes. However, further work is still required to refine our understanding of the exact role of *Aspein* and *Nacrein* in the pearl phenotype, because our studies revealed high levels of additive expression in pearl sac, thus providing evidence for a genetic basis for this variation, which could be used in breeding programs.

Final phenotype

Heritability allows a comparison of the relative importance of genetics and environment in the variation of traits within and across populations, and is a proxy parameter for predicting the response to selection (Visscher *et al.* 2008). Whatever the mechanism implied in pearl formation, the most important is the final pearl phenotype and its heritability. Our results clearly demonstrate heritability for nacre weight and thickness, darkness and colour, surface defects and grade, signifying

an important donor oyster effects with a genetic basis, while shape and presence / absence of circle(s) with low heritability were not strongly heritable or attributable to the donor. In fact, pearl shape is known to be mostly influenced by environmental factors (Ky *et al.* 2015b). This study confirms the significant genetic role that the implanted mantle graft plays in the biomineralization process of cultured pearls (Arnaud-Haond 2007; McGinty *et al.* 2010; Blay *et al*. 2017). When heritability is high, the corresponding trait could be improved by selecting donor oysters with high genetic merit. Sufficient additive genetic variance in a selected trait is a prerequisite for selective breeding and good breeding efficiency is possible when levels are high (Gjedrem and Baranski 2010). From an applied point of view, this has major implications for any genetic selection strategies and for the black pearl industry in French Polynesia. Variation in additive and non additive genetic factors and environmental variance are population specific, meaning that heritability depends on the population. Therefore, the heritability in one population cannot be used to predict the heritability in another population for the same trait, although in practice heritabilities of similar traits are often similar across populations in the same or different species (Visscher *et al.* 2008). Therefore, selection programs aimed at improving traits such as pearl size, colour, darkness, surface defects and grade should be achievable through targeted donor oyster selection, whilst further work is required to understand the role of the recipient oysters on pearl phenotypic variance and gene expression variance.

Conclusion

The current study showed, for the first time, an additive genetic component attributable to donor oysters for gene expression in graft tissue and, to a lesser extent, in the pearl sac and for harvested pearl phenotype (excluding pearl shape and circle). The interaction between donor and recipient are very complex and research has increasingly attempted to uncover the processes involved in these interactions and the resulting graft-induced phenotypic changes, for example by studying molecular mechanisms and endogenous factors. Moreover, establishing a direct link between pearl phenotype and candidate gene expression remains an important next step if we are to understand its role in *P.*

margaritifera selection potential in a breeding program. This study provided a good understanding of heritability estimates for pearl phenotypes and gene expression in this chimera model.

Acknowledgements

This study was supported by grants from the "*Direction des Ressources Marines et Minières*", through the TripaGEN project (2016–2019). The authors would especially like to thank: 1) the technical support for production and rearing of the families, managed by M. Sham-koua; and 2) the SCA Regahiga Pearl farm (Mangareva island, Gambier archipelago, French Polynesia), managed by D. Devaux and S. Nakasai, for their generous help with the grafting experiment and associated technical support throughout the culture period. The authors are indebted to S. Parrad, for her helpful assistance with the molecular work in the laboratory (RT-PCR). C. Blay was jointed funded by an Ifremer PhD grant, the "*Direction des Ressources Marines et Minières*", and CRIOBE EPHE.

Data Availability

In accordance with the Journal of Heredity data archiving policy, we have deposited the primary data underlying these analyses as follows:

- Cultured pearl quality traits for each individuals: Dryad
- Gene expression levels for each individuals: Dryad

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Table and Figure

Table 1. Set of forward and reverse primers used for the biomineralization gene expression analysis in *Pinctada margaritifera*.

Gene name	Function	NCBI Accession Numbers	Forward primer (5'-3')	Reverse primer (5'-3')
Pif-177	Aragonite formation	HE610401	AGATTGAGGGCATAGCATGG	TGAGGCCGACTTTCTTGG
MSI60	Aragonite formation	No accession number but described by B. Marie *et al*.2012	TCAAGAGCAATGGTGCTAGG	GCAGAGCCCTTCAATAGACC
Perline	Aragonite formation	DQ665305	TACCGGCTGTGTTGCTACTG	CACAGGGTGTAATATCTGGAACC
Aspein	Calcite formation	No accession number but described by B. Marie *et al*.2012	TGGAGGTGGAGGTATCGTTC	ACACCTGATACCCTGCTTGG
Prismalin	Calcite formation	HE610393	CCGATACTTCCCTATCTACAATCG	CCTCCATAACCGAAAATTGG
Shematrin5	Calcite formation	HE610376	GTCCGAAACCAAATCGTCTG	CTGTGGTGATGGTGACTTCG
Nacrein	Aragonite and calcite formation	HQ896199	CTCCATGCACAGACATGACC	GCCAGTAATACGGACCTTGG
Shematrin9	Calcite formation	No accession number but described by B. Marie *et al*.2012	TGGTGGCGTAAGTACAGGTG	GGAAACTAAGGCACGTCCAC

Table 2. Significance of the fixed family effect on pearl quality traits and gene expression in *P. margaritifera* progenies; 1918 harvested pearls were examined for nine pearl quality traits, and eight genes coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177, MSI60* and *Perline*), the prismatic layer (*Aspein, Shematrin9*, *Prismalin* and *Shematrin5*), and both the prismatic and the nacreous layers (*Nacrein*) were studied in 164 graft tissue pieces and 73 pearl sacs.

Pearl Traits	nacre weight	nacre thickness	circle	shape	surface defect(s)	lustre	grade	darkness	colour
(n = 1918)	***	***	**	*	***	ns	***	*	**

Graft	*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*
(n = 164)	**	***	***	***	***	***	***	***

Pearl sac	*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*
(n = 73)	ns	*	ns	*	*	*	ns	ns

p<0.0001 =***, p<0.01 = **, p<0.05 = *, ns = not significant

Table 3. Heritability (and CI 95%) for: a. pearl quality traits in *P. margaritifera* (N = 2241); b. mantle graft tissue relative gene expression levels (N = 176); and c. pearl sac tissue relative gene expression level (N = 80). Eight genes were studied coding for proteins potentially involved in the construction of the nacreous layer (*Pif-177, MSI60* and *Perline*), prismatic layer (*Aspein, Shematrin9*, *Prismalin* and *Shematrin5*), or both (*Nacrein*).

a. Pearl quality traits		nacre weight	nacre thickness	circle	shape	surface defect(s)	lustre	grade	colour	darkness
	h^2	0.34	0.29	0.05	0.06	0.21	0.12	0.19	0.16	0.37
	CI 95%	(0.27, 0.41)	(0.22, 0.36)	(0.01, 0.10)	(1E-3, 0.06)	(0.15, 0.28)	(0.06, 0.18)	(0.11, 0.25)	(0.11, 0.23)	(0.30, 0.44)

b. Graft tissue relative gene expression		*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*
	h^2	0.11	0.42	0.47	0.37	0.33	0.25	0.44	0.35
	CI 95%	(4E-4, 0.29)	(0.24, 0.63)	(0.22, 0.72)	(0.22, 0.54)	(0.14, 0.51)	(0.11, 0.41)	(0.27, 0.60)	(0.21, 0.52)

c. Pearl sac relative gene expression		*Pif-177*	*MSI60*	*Perline*	*Nacrein*	*Aspein*	*Shematrin9*	*Prismalin*	*Shematrin5*
	h^2	0.03	0.09	0.07	0.25	0.41	0.06	0.03	0.05
	CI 95%	(2E-4, 0.11)	(2E-4, 0.27)	(3E-4, 0.23)	(5E-5, 0.67)	(5E-5, 0.77)	(4E-5, 0.21)	(4E-5, 0.15)	(4E-5, 0.23)

Fig 1. *Pinctada margaritifera* crossbreeding design for the production of the nine half-sib families used as graft donors. Females and males were named with letters and numbers respectively.

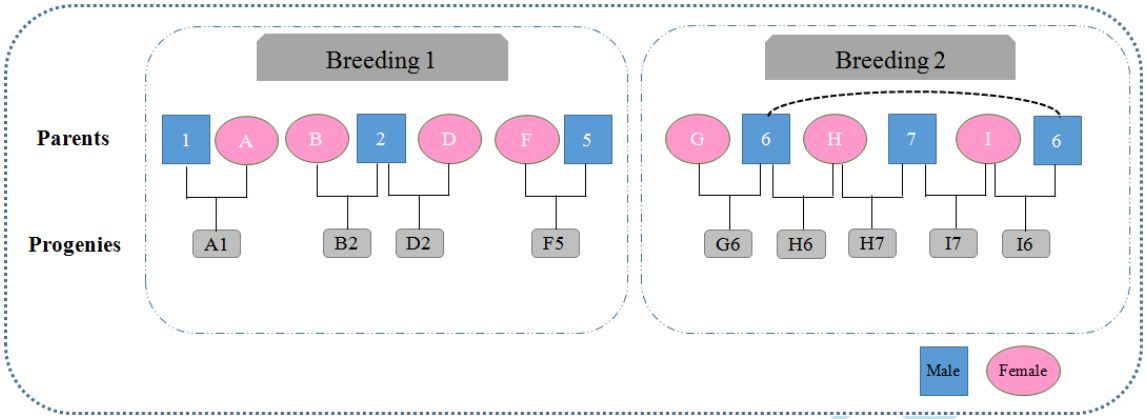
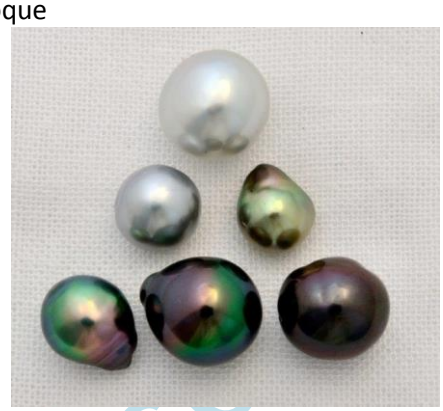


Fig 2. *Pinctada margaritifera* cultured pearls from different colours, showing the main quality traits variables. Shapes and A grade pearls were illustrated in a), b), c), and d), with respectively circle, baroque, drop and round samples. Pictures e), f) and g) represented respectively pearls with numerous surface defects, pearls without lustre and rebut pearls.

a) circle

b) baroque



c) drop

d) round

e) surface defects

f) non lustre

g) rebut