Correction Method for Null Alleles in Species with Variable Microsatellite Flanking Regions, A Case Study of the Black-Lipped Pearl Oyster *Pinctada margaritifera*

SARAH LEMER, ELISABETH ROCHEL, AND SERGE PLANES

From the USR 3278 CNRS-EPHE; the Centre de Recherche Insulaire et Observatoire de l’Environnement (CRIOBE), BP 1013, Papetoai, Moorea, French Polynesia; and the Centre de Biologie et d’Ecologie Tropicale et Méditerranéenne, Université de Perpignan, 66860 Perpignan, France.

Address correspondence to Sarah Lemer at the address above, or e-mail: sarah.lemer@univ-perp.fr.

In bivalves, heterozygote deficiencies and departures from Hardy–Weinberg equilibrium (HWE) in microsatellite analysis are common and mainly attributed to inbreeding, genetic patchiness (Walhund effect), or null alleles. We checked for the occurrence of null alleles at 3 microsatellite loci in 3 populations of black-lipped pearl oyster, *Pinctada margaritifera*, using a step-by-step method to reamplify homozygotes and null individuals with redesigned primer pair combinations. After amplification with original primer pairs, the 3 populations exhibited null alleles, absence of structure, and significant departure from HWE for all 3 loci due to heterozygote deficiencies. After 3 reamplification steps, with modified primer sets, all loci were corrected for null alleles. Once corrected, all populations appeared at HWE, demonstrating that null alleles were responsible for the initial disequilibrium of the populations. Furthermore, analysis from corrected genotypes demonstrates significant genetic differentiation for one population from the other 2.

**Key words:** bivalve, genetic structure, Hardy–Weinberg deviation, heterozygote deficiency

Microsatellite markers are characterized by high mutation rates ranging between $10^{-2}$ and $10^{-6}$ per locus per generation (Dallas 1992; Goldstein et al. 1995). Such high mutation rate leads to important genetic variation and allelic diversity making microsatellites useful genetic markers for population genetic studies (Kalnokwski 2002), relatedness analyses (Wagner et al. 2006), and parentage screening (Planes et al. 2009). Even though the development of microsatellite libraries has become a relatively common and rapid technique (Zane et al. 2002), technical problems such as genotyping errors (stuttering or large allele dropout) and occurrence of null alleles remain difficult to resolve and rarely considered.

Null alleles can be caused by suboptimal polymerase chain reaction (PCR) conditions or mutations on one or both of the primer-binding sites (Dakin and Avise 2004). Their occurrence is manifested as weak or null amplification of one or both alleles leading to artificial homozygote excess in samples compared with Hardy–Weinberg equilibrium (HWE) (Foltz 1986). Microsatellite null alleles are widespread, but some groups, in particular marine invertebrates, demonstrate particularly high frequencies of null alleles (Hare et al. 1996; Hedgecock et al. 2004). Shifting primer regions along the flanking sequence has been previously explored in rare cases, as a solution for loci exhibiting null alleles (Shearer and Coffroth 2004; Brownlow et al. 2008). However, so far this approach only ameliorated the null allele problem without eliminating it (i.e., fewer null alleles but loci still exhibiting heterozygote deficiencies and populations still deviate from HWE).

Like most marine bivalves, the black-lipped pearl oyster, *Pinctada margaritifera*, exhibits a high proportion of null alleles in microsatellites that were tailored to this species (Herbinger et al. 2006). Considering the important bias induced by null alleles on population genetic analysis, we developed a method to decrease their occurrence in order to obtain correct genotypic data. In this study, we demonstrate that null alleles alone were responsible for heterozygote deficiencies observed in the 3 populations of *P. margaritifera* for the 3 loci (Pmarg37, Pmarg45, and Pmarg68) previously developed by Herbinger et al. (2006).
Materials and Methods

As a model, a total of 166 individuals of *P. margaritifera* were collected from 3 different atolls in the central Tuamotu archipelago, French Polynesia (Motutunga *n* = 60, Tepoto *n* = 56, and Tuanake *n* = 50). Total DNA was extracted from ethanol-preserved mantle and abductor muscle tissue using DNeasy Blood and Tissue kit (Qiagen), following the manufacturer’s instructions. Multiplex PCRs with the 3 loci were performed in 10 μl using Qiagen Multiplex PCR (Qiagen) components and concentrations. PCR products were processed on a Beckman Coulter sequencer CEQ 8000 genetic analysis system, and microsatellites were scored visually from electropherograms.

Because amplification failed in many individuals and there was obvious dominance of homozygotes, second primer pairs were designed, for each locus, from the initial cloned sequences (Table 1) using online software Primer 3 v 0.4.0 (Rozen and Skaletsky 2000). Modified primers were either internal, external, or extensions to the original primer sequence. The original and modified primers were combined to obtain 4 different primer couples for each locus. After the first amplification with the original primer pairs (Primer set I, Table 1), all remaining homozygotes and null genotypes (Table 1) were re-amplified and rescreened with a second combination of primer pairs (Primer set II). Again, all the remaining homozygotes and null genotypes from this second amplification were amplified and screened with a third combination of primer pairs (Primer set III). A fourth amplification was not necessary in this study. Only 8 of the 166 individuals simultaneously failed to amplify or were homozygote at the 3 loci after the first amplification. Thus, the second (Primer set II) and the third (Primer set III) amplification steps were not performed in multiplex PCRs. In addition, a selection of 8 heterozygotes, already identified from primer set I, were amplified and screened with the 3 primer pairs for all loci to ensure that all alleles showed constant and expected differences in size related to primers shifts. After each new amplification, presence of null alleles in the updated genotype data sets was estimated with Microchecker (Van Oosterhout et al. 2004). Fstat (Goudet 1995) was used to calculate expected (*H*<sub>e</sub>) and observed (*H*<sub>o</sub>) heterozygosities and to test genotypic distributions for conformity to HWE (10 000 permutations). Allele number per locus, fixation index (*F*<sub>IS</sub>), and pairwise *F*<sub>ST</sub> values among the 3 sites were computed with Arlequin 3.1 (Excoffier et al. 2005). *P* values for pairwise *F*<sub>ST</sub> were obtained after 10 000 permutations of the data set and were corrected by sequential Bonferroni correction (Rice 1989) for multiple comparisons. In order to compare with the updated genotype data sets, presence of null alleles, *F*<sub>IS</sub> within populations, and conformity to HWE were also estimated after all amplification steps for each new data set taken independently (i.e., data sets obtained with primer set II and primer set III independently).

![Table 1](https://academic.oup.com/jhered/article-abstract/102/2/243/789249)
Results

After amplification with Primer set I, Microchecker detected presence of null alleles and departure from HWE for all 3 loci in all locations. In addition, there were scoring errors due to stuttering for the locus Pmarg68 in samples from Motutunga and Tepoto. $F_{IS}$ estimates were all significantly positive for each locus, indicating heterozygote deficiencies within all populations (Figure 1). None of the 3 pairwise tests of differentiation ($F_{ST}$) performed between locations were significant at this stage (Table 2).

After the second amplification step (Primer set II), all 3 populations reached HWE at 2 loci: Pmarg45 and Pmarg68 (Table 1). There was still evidence of null alleles and significant $F_{IS}$ ($0.079$) at the third locus Pmarg37. $F_{ST}$ values appeared to be significant between Tepoto and Motutunga ($F_{ST} = 0.012$) and between Tepoto and Tuanake. ($F_{ST} = 0.008$) (Table 2).

After the third amplification step (Primer set III), $F_{IS}$ estimates did not significantly differ from zero at any locus and therefore all frequencies conformed to HWE (Figure 1 and Table 1). $F_{ST}$ values increased between Tepoto and the 2 other populations (Table 2).

Following the 3-step PCR, the number of alleles increased by 1 for locus Pmarg45, remained stable for locus Pmarg37, and decreased by 2 for locus Pmarg68 (Table 1). Such a decrease in the number of alleles for locus Pmarg68 is counterintuitive and resulted from the correction of scoring errors due to stuttering encountered in the initial genotypic data.

When analyzing the data sets obtained with primer set II and primer set III independently, we detected evidence of null alleles, departure from HWE, and significant $F_{IS}$ estimates (ranging from 0.18 to 0.69) for all 3 loci indicating heterozygote deficiencies within all populations (Supplementary Table 1). In addition, none of the pairwise tests of differentiation ($F_{ST}$) performed with the independent data set were significant (Supplementary Table 2).

Discussion

Contrary to traditional PCR approach, the 3 step-PCR method revealed genotypes that conformed to HWE for all loci and in all populations, demonstrating that the heterozygote deficiencies in $P. margaritifera$ only result from the presence of null alleles due to mutation on the primer-binding sites.

Development of a step-by-step process was necessary because no primer set alone was sufficient to eliminate null alleles. This observation reveals that primer-binding sites of both DNA strands contain mutations and emphasizes the high variability in the microsatellite flanking regions, commonly encountered in several taxa (i.e., lepidopterans, Meglecz et al. 2007; nematodes, Johnson et al. 2006; and marine molluscs, Weetman et al. 2005). Therefore using only one modified or extended reverse primer, as described in Shearer and Coffroth (2004) is not necessarily sufficient to correct for null alleles. Brownlow et al. (2008) recommended testing primer reliability for a locus by redesigning another primer pair. According to the authors, if the 2 primer sets show evident discrepancies, such as changes from homozygotes to heterozygotes, both primer pairs should be considered unreliable. Our work reveals that one unique reliable primer pair may not exists for a locus and that a combination of primers, giving complementary information, is required to score accurate genotypic data. In the present case, 3 amplification steps, each with different primer sets, were necessary to reach conformance to HWE.

The corrected data set displayed only one additional allele (for locus Pmarg45), indicating that null alleles should not be consistently considered as original alleles. This low number of new allele encountered can be explained by the mutational model of the microsatellite repeat units that allows allele size homoplasy (Estoup et al. 2002) as well as by our small sample size (166 individuals and 3 loci). Interestingly, and contrary to previous findings (Chapuis and Estoup 2007), the presence of null alleles in the initial

![Figure 1](https://example.com/figure1.png)
data did not lead to detection of genetic differentiation between populations. Instead, obtaining correct genotypes revealed that the Tepoto population significantly diverges from the other 2, probably because the overall genetic information available was increased.

We therefore suggest that this approach should be systematically undertaken when encountering heterozygote deficits in order to separate evolutionary process inducing HWE deviation from technical PCR artifacts that can lead to false outcome. This approach certainly requires increasing laboratory work efforts but is essential to correct for null allele before proceeding with population genetic analysis or drawing conclusions about Hardy–Weinberg disequilibrium and its relation to species history life traits.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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References


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