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Development of in situ hybridisation using 16S rRNA gene to monitor black-lip pearl oyster, *Pinctada margaritifera*, larvae in plankton samples

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Abstract – In French Polynesia, the black-lip pearl oyster *Pinctada margaritifera* has been farmed to produce pearls since the 1980s, forming the basis of a major industry. The sustainability of this activity relies on spat collection in the lagoons. However, pearl oyster spat can be difficult to identify for the evaluation of stock variations. It is especially hard to distinguish *Pinctada* spp. larvae at a very early stage of development. In the present study, a whole-mount in situ hybridisation (ISH) technique was developed to allow the discrimination of larvae of closely-related pearl oyster species found in the French Polynesian atolls. Using specific ribosomal 16S-DNA sequence data, we were able to successfully differentiate between *Pinctada margaritifera* and *Pinctada maculata* larvae from 5 to 13 days old. This is the first description of a non-destructive method allowing bivalve larvae discrimination between species within this genus. The method allowed us to successfully identify *P. margaritifera* larvae in natural plankton samples. This result is a key step needed to develop monitoring of *P. margaritifera* larval distribution in French Polynesian lagoons, a procedure which will increase spat collection efficiency and ensure sustainable development of pearl oyster farming.

Key words: Whole larvae in situ hybridisation / 16S rRNA / Plankton / Species identification / Pearl oyster / *Pinctada margaritifera*

1 Introduction

In French Polynesian atoll lagoons, black-lip pearl oyster *Pinctada margaritifera* (L.) farming plays a major socio-economic role. The entire farming activity is sustained by the collection of black-lip pearl oyster juveniles on artificial collectors placed in these lagoons. However, the yield of juveniles on collectors is spatio-temporally unpredictable and the needs of pearl farms cannot always be fulfilled. Efficient management of *P. margaritifera* production requires the ability to predict which sites are most likely to be colonized by larvae, which would allow spat collection areas to be targeted. This goal can be achieved by monitoring the presence of larvae in plankton samples. Thus, species-specific information on larval distributions of the targeted species is needed in order to understand recruitment variations. Like most bivalve species, pearl oysters have a planktonic larval stage with a dispersal potential influenced by biological and physical forces. The success of this larval phase depends on a wide variety of parameters and processes, like temperature, salinity, food availability and predation (Eckman 1996; Troost et al. 2009). To study the effect of these processes on selected species in the field, extensive sampling is required due to the dilute and patchy distribution of larvae over both space and time (Garland et al. 2002). A major obstacle to the quantification of planktonic larval distributions is the identification of sampled larvae. Lagoon ecosystems possess a significant diversity of bivalve species (Pante et al. 2006) and their identification can be very difficult or even impossible, especially during the early stages of development, due to uniform larval morphology (Abalde et al. 2003; Hendriks et al. 2005).

In French Polynesian atoll lagoons, more than 30 bivalve species (Salvat et al. 1985) occur and larvae have never been described for most of these species. In this environment, the main difficulty for the specific identification of *P. margaritifera* larvae is to discriminate them from *Pinctada maculata*, another major species of the *Pinctada* genus. The two species have overlapping reproductive periods and their larvae are of similar size, colour and length of development time before settlement. Until recently, the only technique available for species recognition was direct microscopic observation (Garland and Zimmer 2002; Hendriks 2005). Although this appears efficient with morphologically distinguishable species, it is inaccurate for close species within a genus and impractical when processing large samples, such as in field investigations. A recent study by Paugam et al. (2006) on the identification

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of *P. margaritifera* larvae demonstrated the limit of morphometric approaches. While these authors described the analysis of hinge characteristics as the only method available at that time for identification of the *P. margaritifera* larvae, they stressed that it was expensive and time consuming and proposed the use of molecular identification techniques. For many years researchers have sought new means for discriminating very closely related but genetically distinct species and novel techniques to increase the rate at which specific organisms are quantified, particularly in the context of field surveys. Recent studies highlight the considerable interest shown in molecular identification of bivalve species by immunological (Abalde et al. 2003; Paugam et al. 2003) or quantitative polymerase chain reaction (PCR) techniques (Bendezu et al. 2005; Vadopalas et al. 2006). It appears unlikely that immunological techniques could properly distinguish these two *Pinctada* species, since antibodies have limited taxonomic discrimination resolution below the genus level as proteins of conspecific organisms may be highly conserved (Garen et al., personal communication). As a result, current detection methods rely on molecular techniques, namely polymerase chain reaction (PCR) and in situ hybridisation (ISH). A method for identifying the larval stage of temperate bivalve species by in situ hybridisation of whole larvae has been recently described (Le Goff-Vitry et al. 2007; Pradillon et al. 2007). This technique has the advantage of preserving larval morphology, allowing post-treatments such as biometric measurements, and appears to be more specific than immunological techniques applied to planktonic samples, due to the high level of conservation of the proteins in conspecific organisms, as previously evoked. The previously published ISH protocols were developed based on 18S rRNA genes, which often appear highly conserved between close species and have frequently been used to resolve deep branching orders (Moon-van der Staay et al. 2001; Goffredi et al. 2006). Previous authors have been able to develop species-specific assays using 18S rRNA as probes in an annelid and an echinoderm (Mountfort et al. 2007; Pradillon 2007), but no studies have yet shown the capacity to discriminate between close mollusc species using ISH. In the present study, we further developed the whole-larvae in situ hybridisation method and produced a simple molecular technique for identification of the *P. margaritifera* black-lip pearl oyster larvae. We designed a nucleotide probe targeting 16S rRNA mitochondrial genes, which evolve more rapidly and, therefore, have an increased chance of generating species-specific markers to distinguish between the closely-related species of the *Pinctada* genus: *Pinctada maculata* and *Pinctada margaritifera*. This technique allowed us to accurately discriminate and enumerate these two co-dominant species in plankton samples from French Polynesian atolls, regardless of developmental stage.

2 Materials and methods

2.1 Larvae sampling and fixation

P. margaritifera and *P. maculata* larvae were obtained by experimental rearing conducted at the IFREMER Centre (Vairao, Tahiti, French Polynesia). Spawning was induced with a thermal shock applied on breeders taken from atoll lagoons. A multi-parental pool of trochophore larvae was put into rearing tanks, fed with cultured algae (i.e., *Isochrisis affinis galbana* and *Chaetoceros* sp. *jonquieri*) and sampled on the fifth day of development, corresponding to D-stage larvae, and the thirteenth day (Doroudi and Southgate 2003). At these two stages, larvae were sampled with a 40 μM mesh. The *P. margaritifera* and *P. maculata* larvae of 13 days were first incubated in a 20% $MgCl_2$ solution Samples were then fixed with 72% ethanol and stored at 4 °C. Natural plankton was sampled from the Ahe atoll lagoon (French Polynesia; 146.3 °W, 14.5 °S). Plankton was sampled from a boat by pumping water from the surface to 5 m above the bottom at an average pumping rate of 14 ± 5 L min^{-1}. Pumped water was filtered on a 250 μM mesh and plankton was retained on a 40 μM mesh. Samples were immediately preserved in 72% ethanol and stored at 4 °C. Two samples, S1 and S2, were used, taken in the western part of the Ahe lagoon in May 2007 and November 2007, respectively.

2.2 Design and labelling of DNA probes

2.2.1 *Pinctada margaritifera* 16S specific probe

P. margaritifera-specific probes used for in situ hybridisation (ISH) and dot-blot experiments were designed based on the alignment of *P. margaritifera* and *P. maculata* 16S rRNA gene sequences available in Genbank (accession nos. AB214436 and AB214440, respectively, http://www.ncbi.nlm.nih.gov/Genbank/). The alignment showed that the two sequences are not 100% identical (80% identity, ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/) and consequently revealed sequence stretches specific to *P. margaritifera*, upon which probe primers were designed (Fig. 1A). Forward (Pmarg16S-1F, Table 1) and reverse (Pmarg16S-3R) primers specific to the *P. margaritifera* sequence were synthesized and used (0.2 μM final concentration) in PCR reactions using the iQ^{TM} Supermix (BIO-RAD) and *P. margaritifera* mantle cDNA as template. The 201 bp PCR products generated were purified (mini Quick spin columns, Roche Diagnostics) and digoxigenin (DIG)-labelled. The resulting 201 bp probe, as well as the Pmarg-16S primers, was blasted against eukaryote and prokaryote databases (http://blast.ncbi.nlm.nih.gov/). No significant similarity was shown with other sequences indicating that our probe is highly specific to the Pmarg-16S rRNA gene and that the probe could not produce false positives due to hybridisation with other contaminant organisms, such as symbiotic micro-fauna or seawater contaminants.

2.2.2 Control probes

To develop a positive control probe (Uni-18S) targeting 18S rRNA, universal 18S rRNA primers (Uni1304F and Uni 1670R, Table 1) found in the literature were used (Larsen 2005) (Fig. 1B). The alignment of the two *Pinctada* 18S rRNA gene fragments from Genbank (Access Nos. AB214440, AB214455) showed that the sequences amplified by the Larsen (2005) primers are 100% identical.

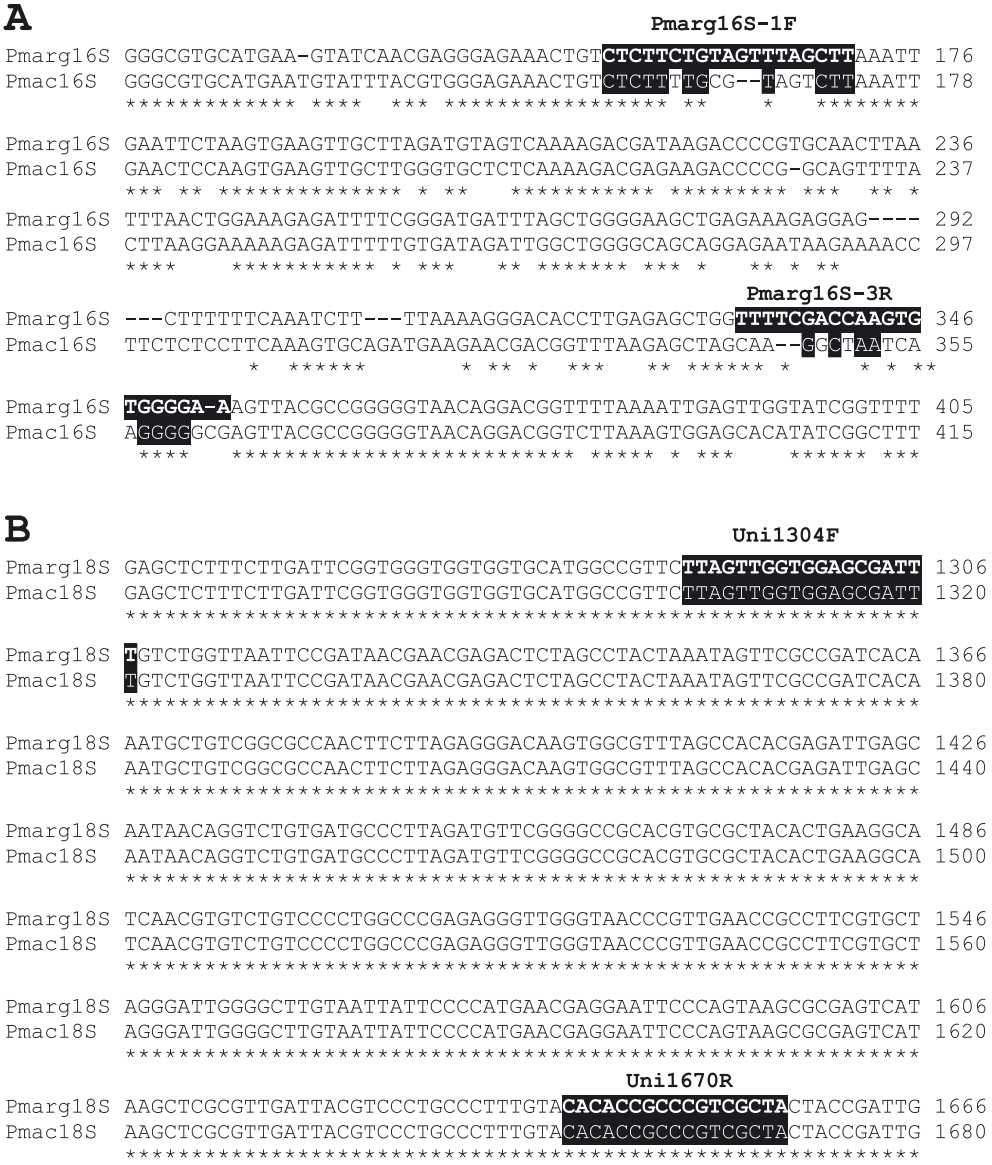

Fig. 1. Design of 16S and 18S rRNA gene-based probes. Sequence alignments of *P. margaritifera* and *P. maculata* 16S (A) and 18S rRNA gene (B) sequences (Genbank accession nos. AN 214436/AN 214451 and 214440/214455.1, respectively) used for probe design with the clustal W2 program. Primers used to generate the Pmarg16S and Uni-18S probes are outlined. Stars (*) indicate nucleotides that are identical between the two sequences. Gaps introduced to improve the alignments are shown as dashed lines. Nucleotide numbers are indicated to the right of the alignments.

Table 1. In situ hybridisation probe specifications. Primer names, sequences and references used to produce digoxigenin (DIG)-labelled probes for whole-mount in situ hybridisation experiments.

Probe (size-bp)	Primers	Sequence	Reference
Pmarg-16S (201)	Pmarg16S-1Fwd Pmarg16S-3 Rev	ctcttctgtagtttagctt ttccccacacttggtcgaaaa	Genbank Access .No._AB214436
Uni-18S (367)	Uni1304 Fwd Uni1670 Rev	ttagttggtggagcgattt tagcgacgggcggtgtg	Larsen et al. (2005)
M13 (243)	M13(-20) Fwd M13 Rev	ctggccgtcgttttac caggaaacagctatgac	pCR® II-TOPO®

P. margaritifera mantle cDNA was submitted to amplification using 40 cycles consisting of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C in 200 μM dNTP, 1 × PCR buffer, 2 mM $MgCl_2$, 0.5 μM of each primer, and 1 unit of Taq DNA polymerase (Promega). The PCR product (367 bp) was purified and labelled by PCR.

The negative control probe was generated using a standard cloning vector sequence, with a sequence that would not be found in our target organisms. A 243 bp sequence of the cloning vector pCR®II-TOPO® (Invitrogen) was amplified using the standard M13 primers. For the design of this negative control (M13, Table 1), purified M13 PCR products (243 bp) were DIG-labelled using above PCR amplification conditions.

2.2.3 Probe labelling

Probes were labelled by PCR in the presence of DIG-dUTP (0.7 mM) in the PCR reaction mixture containing specific or control primers (0.2 μM final) and 2 μl of previously purified PCR fragment as well as a mix of dGTPs-dCTPs-dATPs (200 μM each final), dTTPs (130 μM final), and Taq polymerase (Promega, 2.5 u). After DNA denaturation at 94 °C for 5 min, 35 cycles were run with an MJ-Research thermocycler as follows: 94 °C for 30 s; 55 °C for 30 s; 72 °C for 45 s, ending by a final elongation step at 72 °C for 10 min.

2.3 Dot-blot test

Samples of 1 μg genomic DNA extracted from *P. margaritifera* and *P. maculata* larvae, as well as the specific PCR products (Pmarg-16S, Uni-18S and M13), were dotted onto a nylon membrane (Hybond-N membranes – Amersham Pharmacia Biotech) and probed using DIG-labelled DNA probes. Hybridisations were performed overnight at 42 °C in 50% formamide, 5 × saline sodium citrate (SSC), 1% *N*-lauryl sarcosine and 10% blocking reagent Blotto (Roche Molecular Biochemicals), followed by washing in decreasing concentrations of SSC buffer. The detection steps were performed according to manufacturer's instructions (DIG nucleic acid detection kit, Roche Molecular Biomedicals).

2.4 In situ hybridisation analyses

The ISH technique presented below was adapted from previously published protocols (Montagnani et al. 2001; Fabioux et al. 2004; Pradillon et al. 2007; LeGoff-Vitry et al. 2007). Larvae were first rehydrated for 1 h at 4 °C in a Tris buffered saline – 0.1% Tween 20 (TBS-T) solution containing 50% ethanol 75%, then washed twice in TBS-T. Larvae were then placed in 24-well plates. The 13-day-old larvae were incubated for 30 min in 350 mM EDTA. Samples were treated with proteinase K (10 μg ml^{-1}) in TE buffer (Tris 50 mM, EDTA 10 mM) at 37 °C for 20 min and rinsed twice with TBS-T. Whole larvae were pre-hybridised for 40 min at 65 °C, using warmed hybridisation buffer (4 × SSC, 50% formamide, 100 μg ml^{-1} yeast tRNA, 0.1 mg ml^{-1} salmon sperm DNA, 0.1% Tween 20, 1.5% Blotto Blocking reagent (Roche Molecular Biochemicals), 5 mM EDTA. Probes and samples were denaturated 10 min at 95 °C and 70 °C, respectively. Prehybridisation solution was then replaced with 300 μl of the same buffer containing 20 μl of the DIG-labelled probes and finally incubated overnight at 52 °C for hybridisation. The samples were washed successively 3 times with 2 × SSC prewarmed to 42 °C and twice in 0.4 × SSC at room temperature. The detection steps were performed according to manufacturer's instructions (DIG nucleic acid detection kit, Roche Molecular Biomedicals). After 2 h to 5 h of incubation in detection solution, the samples were extensively washed in washing buffer to stop the reactions. Observations were made using a DM4000B Leica microscope.

ISH was first applied on monospecific samples of *P. margaritifera* and *P. maculata* larvae at two development stages: 5 (L5) and 13 (L13) days old. Second, ISH was applied on mixed samples of the two species at the L5 stage, at five different relative proportions: 100/0, 75/25, 50/50, 25/75, 0/100. Finally, ISH was applied on the two natural plankton samples S1 and S2. Another specific ISH was performed with addition of L5 *P. margaritifera* larvae to the S1 sample, at a known proportion corresponding to 50% of the larvae.

3 Results

3.1 Probe specificity assay

A *P. margaritifera*-specific probe was designed using 16S rRNA (Fig. 1A). This choice was made because the alignment of the entire *P. margaritifera* and *P. maculata* 18S rRNA sequences that revealed 99.5% identity, which demonstrated the impossibility of designing species-specific primer based on it (Fig. 1B). Moreover, previous studies had already described the use of the 16S gene in discriminating bivalve molluscs (O'Foighil et al. 1995; Jozefowicz and O'Foighil 1998).

Probe specificity was assayed using a dot-blot experiment on genomic DNA extracted from *P. margaritifera* and *P. maculata* larvae, and a control consisting of PCR products used for probe labelling based on the following primers: Pmarg 16S-1F/3R, uni1304 fwd/uni1670 rev and M13 forward and reverse (Table 1, Fig. 2). The Pmarg-16S probe showed a specific hybridisation with *P. margaritifera* genomic DNA and no cross-hybridisation with *P. maculata* genomic DNA and Uni-18S or M13 PCR products. The Uni-18S probe appeared to be a good positive control, showing cross-reaction with the genomic DNA of both species. Finally, the M13 probe appeared to be a good negative control, no hybridisation signal being noted on the genomic DNA of either species (Fig. 2).

3.2 *Pinctada margaritifera* larvae identification through whole mount ISH

3.2.1 Mono-specific samples

The designed probes were evaluated in ISH experiments on both *P. margaritifera* and *P. maculata* larvae at two different ages: L5, corresponding to a very early planktonic stage

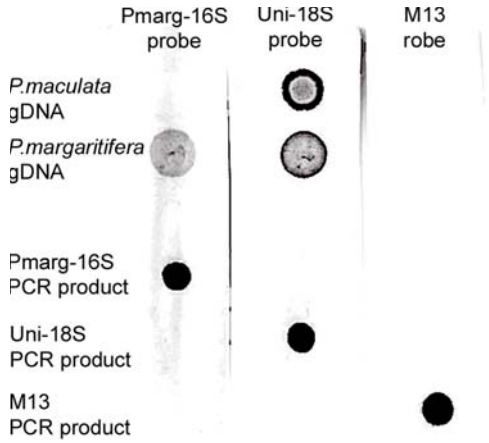

Fig. 2. Probe specificity checking by dot-blot. Nylon membranes were spotted with *P. margaritifera* and *P. maculata* genomic DNA as well as control DNA PCR products. Pmarg-16S PCR product refers to the Pmarg16S-1F and Pmarg16S-3R amplification product used to label the Pmarg16S probe; M13 PCR product refers to the M13(–20) forward and M13 reverse amplification product used to label the negative control probe. Membranes were independently hybridised for 12 h with the Pmarg-16S probe, the Uni-18S probe as a positive control and the M13 probe as a negative control. The colour precipitate reaction was complete after one hour but the reaction was stopped after 24 h, when the membrane picture was taken.

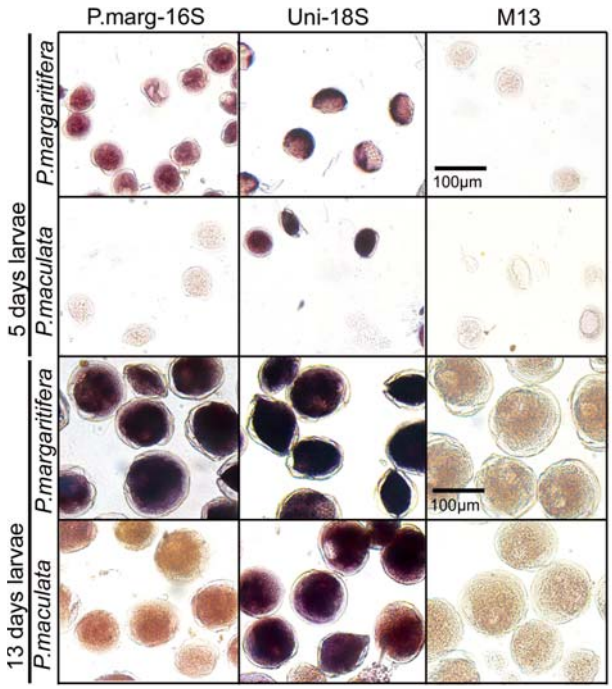

Fig. 3. Whole-larvae in situ hybridisation probe specificity testing on mono-specific *Pinctada* sp. larvae samples of different ages. The *P. margaritifera* specific probe (Pmarg-16S), positive control probe (Uni-18S) and negative control probe (M13) were applied on *P. margaritifera* and *P. maculata* larvae at two ages: L5 (5 days) and L13 (13 days) using whole mount in situ hybridisation (ISH). Pictures show representative observations made on three independent experiments. No hybridisation could be observed in the M13 probed samples nor in the *P. maculata* samples probed with the Pmarg-16S probe. Scale bars are presented on pictures for 5-day-old and 13-day-old samples, respectively.

(D stage), and L13, corresponding to an evolved umbo stage (Fig. 3). Both *P. margaritifera* and *P. maculata* larvae were strongly hybridised by the universal probe (Uni-18S) while no signal was detected with the non-specific (M13) probe. The Pmarg-16S probe showed a specific hybridisation signal on *P. margaritifera* larvae at both ages, with unlabelled larvae representing less than 10% of the sample, and no signal on *P. maculata* larvae at any developmental stage. This result, observed in three independent experiments, demonstrates the efficiency of our set of ISH probes.

3.2.2 Bi-specific samples

The same approach was applied on bi-specific samples. We serially diluted *P. margaritifera* L5 larvae in *P. maculata* L5 larvae samples to obtain 5 different proportions: 100% *P. margaritifera* larvae, 75% *P. margaritifera* larvae, 50% each, 25% *P. margaritifera* larvae and 0% *P. margaritifera* larvae (Fig. 4). *P. margaritifera* larvae could be specifically identified in all samples using the Pmarg-16S probe, with no labelled larvae observed in the 100% *P. maculata* samples. Only a small proportion of larvae appeared unstained in the 100% *P. margaritifera* sample. In addition, the percentage of identified *P. margaritifera* larvae observed in two independent experiments was in accordance with the expected proportions (λ^{-2} test; $p < 0.0001$, Fig. 5).

3.2.3 Planktonic samples

The identification method was applied on natural plankton samples, with and without addition of *P. margaritifera* D-stage larvae (L5) (Fig. 6). We first added a known proportion (50%) of *P. margaritifera* L5 to a plankton sample (S1) that revealed the presence of 53% labelled larvae when 90% labelled larvae could be observed in L5 samples and 17% in S1 alone (Fig. 7). These results show that the developed method is applicable to planktonic samples and that we were able to retrieve the initial ratio of *P. margaritifera* larvae, since no cross-hybridisation could be observed with other plankton species. This test demonstrated that we could successfully identify *P. margaritifera* larvae in plankton in the expected proportions and, thus, validated the Pmarg-16S probe specificity for wild plankton samples. Interestingly, the Uni-18S probe hybridised with a large number of species, even some outside the *Pinctada* genus.

Finally, we tested a second plankton sample (S2), which revealed the presence of numerous *P. margaritifera* larvae (41%), mostly at early stages, which may be evidence of a recent spawning event. Thus, this method has proven its effectiveness to monitor for the presence of *P. margaritifera*, as it detected different stages of larvae in wild plankton samples.

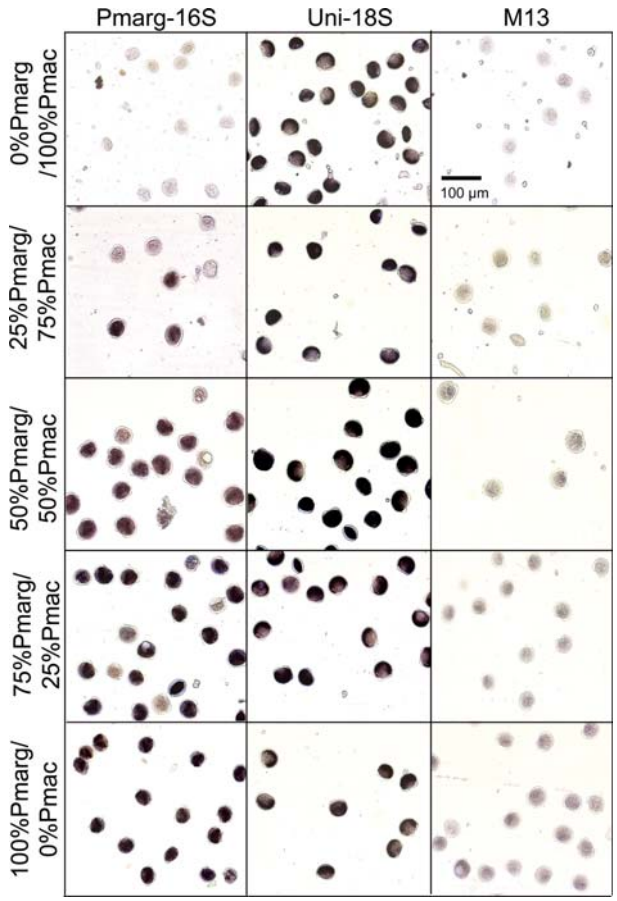

Fig. 4. *P. margaritifera* larvae identification in *Pinctada* sp. bi-specific samples. Various *P. margaritifera* vs. *P. maculata* larvae concentration samples containing 2000 larvae each were tested through in situ hybridisation with Pmarg-16S specific probe and control probes (Uni-18S, M13). The same scale applies for each image, as indicated by a scale bar. These analyses are representative of the observations made in two independent experiments.

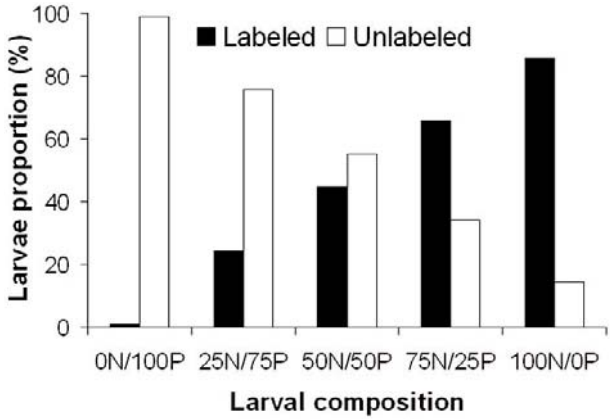

Fig. 5. Enumeration of labelled and unlabelled larvae in bi-specific samples. The bar graph represents *P. margaritifera* (N) and *P. maculata* (P) percentage of labelled and unlabelled larvae tested through ISH with Pmarg-16S specific probe on samples with five different proportions of the two species: 0N/100P, 25N/75P, 50N/50P, 75N25P and 100N/0P.

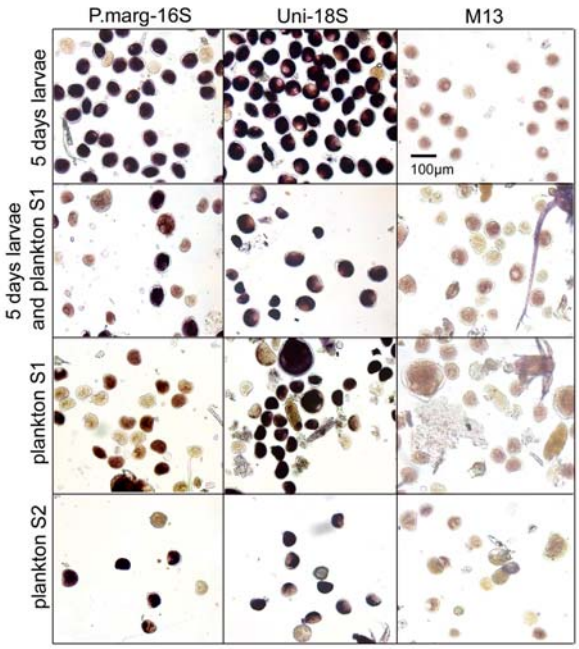

Fig. 6. *P. margaritifera* larvae identification in natural planktonic samples. *P. margaritifera* 5-day-old larvae (L5), Plankton sample 1 (S1), and a 50:50 mix of the L5 and S1 samples, and a plankton sample (S2) were tested through in situ hybridisation with the Pmarg-16S specific probe and control probes (Uni-18S, M13). Images are representative of observations made in separate duplicate samples. The same scale applies for each image, as indicated by a scale bar.

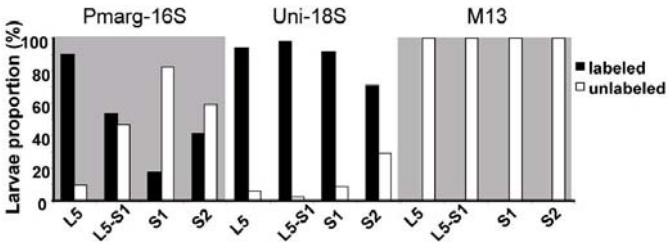

Fig. 7. Enumeration of labelled and unlabelled larvae in natural planktonic samples. The bar graph represents *P. margaritifera* 5 day old larvae (L5), Plankton sample 1 (S1), or a 50:50 mix of the L5 and S1 samples, and a second plankton sample (S2), with the percentage of labelled and unlabelled larvae tested through ISH with the Pmarg-16S specific probe and control probes.

4 Discussion

Studies on bivalve ecology have been constrained in their efforts to describe the composition of natural plankton communities using traditional methods. Few bivalve larvae have sufficiently distinctive morphology to be recognized by microscopy. As a result, current detection methods rely on molecular techniques and a growing number of studies are beginning to reveal the power of these methods to identify larvae (Demers 1993; Toro 1998; Garland and Zimmer 2002; Larsen et al. 2005; Vadopalas et al. 2006; Bott et al. 2010). A range of DNA-based techniques has been used to discriminate between marine species, using a variety of DNA markers such as

nuclear ribosomal genes and spacers or mitochondrial genes (reviewed in Bott et al. 2010). In situ hybridisation (ISH) has been reported to be a reliable identification tool for bivalve larvae (Le Goff-Vitry et al. 2007; Mountfort et al. 2007; Pradillon et al. 2007). Even though other molecular-based assays (PCR) may appear more suited to high-throughput applications, the whole-larvae colorimetric ISH method is non destructive and allows identification to the species level without damaging morphology, which helps in providing data on the size and shape of labelled larvae. However, as reported by Pradillon et al. (2007), ultrastructural details of the larval shell may be lost. This whole-larvae identification technique also offers an alternative method for enumeration compared with quantitative PCR methods, which have some limitations related to the individual cell number, PCR inhibitors or manipulation errors (Vadopalas et al. 2006).

Ribosomal genes are the most commonly used for diagnostics and phylogenetic studies due to their high level of conservation through the animal kingdom (Sanchez et al. 2003; Bendezu et al. 2005). Within the mitochondrial genome, the 16S ribosomal RNA (16S rRNA) gene is commonly used (Amann et al. 1990; Evans et al. 1998; Lindeque et al. 1999) and has been reported to be useful when analysing species and populations (Kamke et al. 2010; Li et al. 2010; Geller et al. 1994; O'Foighil et al. 1995; Jozefowicz and O'Foighil 1998; Schneider and O'Foighil 1999; Garland and Zimmer 2002). In addition, mitochondrial genes are known to evolve more rapidly than nuclear ones, meaning that they can be used to discriminate between species of the same genus, while the slower-evolving nuclear 18S rRNA is more suitable for resolving deep branching orders (Moon-van der Staay et al. 2001; Pradillon et al. 2007). In the present study, our goal was to discriminate between larvae of the two sympatric pearl oysters of the same genus co-occurring in Polynesian lagoons, *P. margaritifera* and *P. maculata*, which exhibit highly similar morphological characteristics (Paugam et al. 2006). These closely-related species were indistinguishable from one another by 18S rRNA gene sequence analysis. Therefore, the 16S rRNA gene was selected as an alternative phylogenetic marker. To develop a highly specific probe, we designed a probe of several hundred base pairs, which was much longer that the probes used in previously described bivalve ISH studies. We showed that, using this methodology, we could successfully identify *P. margaritifera* larvae in mixed samples of the two species, while preserving larval morphology. However, complications were observed at the later 13 day-old stage, mainly due to the stronger and thicker shell, which makes the permeabilization step less efficient (Pradillon et al. 2007). The latter difficulty was overcome by applying a pre-treatment with a chelating agent (EDTA) to slightly decalcify the shell and ensure efficient probe penetration (Jackson et al. 2007). Results from multiple ISH experiments using the 16S rRNA probe gave a moderate unlabelled rate of *P. margaritifera* larvae of less than 10% for all development stages. This labelling discrepancy might be explained by several technical aspects of the method that require further improvement: probe penetration efficiency, as seen for the older samples; the presence of undesirable dead larvae with closed shells, which would appear as unlabelled larvae; poor homogenization during the hybridisation step, or the stringency of experimental conditions that could be improved. A low probe concentration to larvae number ratio might also play an important role in increasing this error rate. The Uni-18S probe did not, as expected, show any species specificity. When testing mixed bi-specific samples, we were also able to discriminate *P. margaritifera* from *P. maculata* larvae with respect to the different proportions tested. Thus, we demonstrated the feasibility of not only identifying *P. margaritifera* larvae, but also enumerating them in heterogeneous species samples. On mixed environmental samples, we confirmed that the Pmarg-16S probe was extremely efficient in targeting *P. margaritifera* larvae. As demonstrated on *P. maculata* larvae, no other plankton components were marked. Through the application of the whole-mount ISH method on *P. margaritifera* and *P. maculata* larvae with the highly-specific 16S rRNA gene probe, we showed for the first time the possibility of effectively discriminating between species within the same genus in bivalve larvae, allowing the planktonic life stages to be identified.

In conclusion, this method has been successfully adapted for detecting specific organisms, even when they occur in complex natural communities, and applied to extensive field collections using a simple and safe fixation procedure in ethanol, allowing high-throughput identification and facilitating the serial analysis of large samples. Our technique is comparable to other methods (e.g. PCR) in that the procedure is highly specific, although it appears much slower (approximately 2 days from receipt of sample to identification). The method can, however, preserve larval morphology, accurately enumerate the numbers of individual larvae in each sample, and be developed to fit high-throughput demands. Our work provides a new method for in situ larval monitoring and overcomes previous technical limitations of research focusing on the role of the larval stages in population, community and ecosystems ecology (Garland and Zimmer 2002). Whole-larvae ISH allowed us to solve a critical issue in determining processes governing *P. margaritifera* larval ecology. It represents a promising tool that offers new opportunities to monitor *P. margaritifera* larval development and cohort dispersal in French Polynesian lagoons and across their geographical distribution as a whole. This first step in the study of black-lip pearl oyster larval ecology will enable a better understanding of factors affecting larval growth and dispersal, and provide data to increase spat collection efficiency, while insuring its sustainable development.

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