

Research Articles

Production of specific pathogen-free larvae from genetically characterized populations of *Anadara tuberculosa* (Bivalvia), for stock enhancement and aquaculture in the Peru Northeast Biosphere Reserve

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ABSTRACT. The pustulose ark, *Anadara tuberculosa*, is considered as an emblematic species of the east Pacific mangrove ecosystem. The decline of its populations is of increasing preoccupation, in particular in Peru. The use of hatchery-produced larvae for stock enhancement or aquaculture is attractive but raises strong criticisms from an ecological point of view, as it could conduce to loss of genetic diversity in wild populations and favor the disease spreading from hatcheries to the environment. In this works, spat production through traditional aquaculture/biotechnology technologies was associated with pathogen molecular diagnostics and with genetic diversity estimation to produce specific pathogen-free (SPF) larvae from genetically characterized populations. All PCR and nested-PCR diagnostics of common bivalve pathogens, such as *Bonamia*, *Martelia*, *Perkinsus* and OsHV1, led to negative results for healthy or moribund adults and spat samples. Microbiological and molecular analyses realized during mortalities led to identifying a pathogenic *Pseudomonas* strain present on culture tank walls, in moribund spats and adults' blood. Population genetic variation was assessed by mitochondrial COI gene sequences which showed 36.7% haplotype diversity. This study with hatchery production, pathology and genetic components for an overexploited native bivalve represents an original model for successful mollusk stock enhancement or/and innovative aquaculture programs.

Keywords: *Anadara tuberculosa*; specific pathogen-free larvae; genetic diversity; stock enhancement; aquaculture

INTRODUCTION

The pustulose ark *Anadara tuberculosa* (Mollusca, Bivalvia) is distributed from the Gulf of California in Mexico to the Tumbes region in northern Peru (Baqueiro *et al.*, 1982). Natural banks of this species develop in muddy sediments, in particular around the roots of the red mangrove, *Rhizophora mangle* (Camacho, 1999). This ark is a symbolic species of the eastern Pacific mangrove ecosystem that has been ancestrally collected by coastal populations as a staple

food, and that is still consumed for traditional meals in several tropical Latin American countries. Extraction is an essential activity for numerous families whose economy relies on *A. tuberculosa* trade. Most of the natural stocks of *A. tuberculosa* are vulnerable, and some populations are close to collapse in several countries (Lucero *et al.*, 2012; Mora *et al.*, 2011). In Peru, the *A. tuberculosa* population of Tumbes region has been reduced by 6.4 fold between 1988 and 2008 in unprotected mangrove areas as well as in the protected area of the National Sanctuary Mangrove of Tumbes

(NSMT) (Vivar, 1996; Ordinola *et al.*, 2010). The diminution of *A. tuberculosa* population is attributed to overexploitation and habitat degradation, but could also result from covert mortalities due to infectious diseases (Gaffney, 2006).

Recuperation strategies generally rely on quota implementation with, on the one hand, permanent collection restrictions based on animal minimum size limits and, on the other hand, occasional collection prohibitions during reproductive seasons to recover depleted aquatic animal stocks. Such regulations are more or less respected and are complicated by their economic consequences for fishers. Another strategy component for retrieving aquatic animal stocks is based on habitat restoration (McCay *et al.*, 2003).

Strategy for restocking and enhancing stocks of natural aquatic populations has also been focused on the mass release of hatchery-produced animals (Bell *et al.*, 2005; Arnold *et al.*, 2005; Arnold, 2008). Such a strategy is attractive for aquatic species, in particular, mollusks, considering their extremely high fecundity and the subsequent possibility to produce in hatchery millions of spat from a numerically limited wild broodstock. However, serious concerns are related to such a strategy, in particular for preventing infectious diseases that could be vertically transmitted and then quickly sprayed from hatchery to the field and for maintaining population genetic diversity that could be rapidly reduced from numerically limited broodstock. The proper management of these pathology and genetics concerns is a priority for successful mollusk stock enhancement and/or new species aquaculture programs.

For several decades, mollusk cultures all around the world have suffered numerous outbreaks caused by infectious diseases with severe productive and socio-economic losses. Such problems result from the lack of preventive consideration for contagious diseases related to several types of pathogens, in particular, some highly pathogenic protozoans, bacteria, rickettsia and viruses (Berthe, 2005; Powell & Hofman, 2015; Guo & Ford, 2016). These mollusk's infectious microorganisms may be vertically or horizontally transmissible from asymptomatic infected broodstocks to their larvae (Arzul *et al.*, 2010; López-Sanmartín *et al.*, 2016).

For stock enhancement or aquaculture programs, such a pathogen transmission risk must be prevented through pathogen free certification of broodstock in the hatchery. Considering the lack of information on infectious diseases for non-cultivated mollusk species like *A. tuberculosa*, a first approach would rely on specific pathogen-free certification (SPF) of broodstock considering in priority the various pathogens

identified in other bivalve species, such those listed by the OIE (International Office of Epizootics).

Shellfish hatchery-reared larvae may lead to reduced genetic diversity, resulting from a limited number of brooders and/or to higher fitness of some genotypes in artificial culture conditions. Genetic diversity is a crucial component for population adaptive response to environmental conditions and long-term viability of aquaculture or stock enhancement programs. Consequently, the production and release of hatchery-reared larvae must be performed considering the management of genetic diversity at intra- and interpopulation levels. Such considerations have been reviewed with three major genetic concerns relevant to shellfish restoration projects (Gaffney, 2006; Lallias *et al.*, 2010). The broodstock must be identified and selected by genetic characterizations of all the populations in the geographical area of the species. The genetic variability intra- and interpopulation must be conserved as high as possible, and as representative as possible of the different populations to avoid inbreeding or genetic drifts. The actual impact of hatchery-propagated spat introduction on the wild populations must be assessed.

Given all these concerns, suitable and reliable genetic markers are necessary for the proper management of the native genetic pool. Mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I gene (COI) has been successfully used for characterizing interspecific and intraspecific variability in different mollusks, in particular for arks shells in Asia (Cho *et al.*, 2007; Chee *et al.*, 2011a; Feng *et al.*, 2011; Tanaka & Aranishi, 2013, 2014, 2016), and was thus applied to characterize *Anadara tuberculosa* population genetic structure within the NSMT.

The production of *A. tuberculosa* spat in hatchery has been achieved firstly by Robles-Mungaray (1999) with conditioned brooders. Successful seed production has also been reported later by the Centro de Desarrollo de la Pesca y la Acuicultura (CENDEPESCA) (Vásquez *et al.*, 2009). However, these productions have not yet considered the pathology and genetic concerns previously explained.

The present project has been aiming at the development of methodologies for sustainable spat production as a crucial step towards the implementation of an effective conservation strategy for *A. tuberculosa*, fostering alternatives to extraction through social aquaculture and stock enhancement.

The project herein described rested on three core components, integrating *A. tuberculosa* spat production with pathogen-free certification and population genetic characterization.

MATERIALS AND METHODS

Wild animals

Animals were collected from September 2010 to March 2011 by certified extractors within the area of the National Sanctuary Mangrove of Tumbes (NSMT), located from Boca de Capones and International Channel (3°18'S) to Playa Hermosa (3°35'S), and 80°13'08"W to 80°31'53"W (Zarumilla, Peru). Five to 10% of the animals from each batch were sacrificed to evaluate the state of gonadal maturation and to select batches with the highest proportion of ripe animals. The animals were transported to the laboratory, washed and placed into fiberglass tanks. They were then placed in suspended baskets with clean water for 24 h without food to empty their stomach contents from waste.

For genetic analysis, animals of any size were sampled at 7 extraction sites: Z1) La Abeja 3°25'S, 80°16'W, Z2) Chorro de la Almeja 3°24'S, 80°18'W, Z3) El Habilitado 3°25'S, 80°17'W, Z4) La Chalaquera 3°25'S, 80°16'W, Z5) Corral de los Cochets 3°25'S, 80°15'W, Z6) Nacupillal 3°25'S, 80°15'W, Z7) Las Agujillas 3°25'S, 80°13'W, and tissues were collected the same day for analysis.

Spat production

Egg spawning and larval rearing were realized at Marinazul's hatchery (3°54'S, 80°52'W). Maturation of broodstock and induction of gamete emission were performed considering information published by Helm *et al.* (2004) and Vásquez *et al.* (2009). Thermal shock induced gametes emission with a gradual temperature increase (+5°C). When animals started to spawn or ejaculate, they were transferred individually into 1 L recipients where they finished gametes emission. Gametes were easily identified by their color, with white sperms and orange ova being mixed for fertilization. Fertilized eggs were kept in aerated tanks to continue larval rearing.

Swimming D larvae were manually harvested and placed in cylindroconical tanks at five larvae mL⁻¹. The tanks were drained daily or at intervals of one day. The temperature was kept between 24-29°C and salinity was kept at 35. A feeding with microalgae was supplied immediately after water changes. Several complementary feedings had permitted to maintain constant food availability. Diets during the first days were based on the flagellated microalgae *Isochrysis galvana* and *Pavlova lutherii*. From the third day, *Chaetoceros calcitrans* and *Chaetoceros gracilis* were included in the diet to reach a rate of 60% gradually.

When larvae exhibit settlement behavior, they were transferred in the black cylindrical settlement at a

density of 100 cm⁻² without substrate until the metamorphosis. The settled larvae were fed with an increasing quantity of microalgae that must be regularly monitored to verify feed availability until they can be harvested (about 1 mm shell length) by scraping the tank bottom delicately. The harvested spats were placed in baskets suspended in black cylindrical tanks (500 L) until they reach a 4-5 mm size that is suitable for transfer for aquaculture or stock enhancement experiments.

Samples collection

The samples for microbiological or DNA analysis were obtained from different methods and tissues according to the purpose. After collection from the NSMT, healthy *Anadara tuberculosa* brooders were brushed to eliminate attached material and placed in 1 L plastic box filled with filtered seawater. After 4 to 12 h, feces were collected using a 1 mL disposable pipette. Thus, brooder's blood was aseptically collected by inserting a 25 gauge needle attached to a 1 mL syringe directly into the posterior adductor muscle. Blood was mix v/v with 10% sodium citrate as an anticoagulant. For moribund broodstock, the animals were carefully opened and samples of gills, mantle or digestive gland were excised. All tissues were deposited in 2 mL microcentrifuge tubes and directly processed. Larvae were obtained by filtering with a mesh size according to the larval stage and washed three times with sterile saline solution (0.2%). Biofilm sample was collected from the tank surface by removing the biofilm with sterile saline water, detached with a sterile cotton swab and suspended in sterile saline solution.

DNA extraction

For molecular analysis, total genomic DNA was individually extracted from approximately 100 mg of tissue that could be spats, gill, and mantle, digestive gland, blood or feces according to the purpose (Table 1) using a standard CTAB protocol (Folmer *et al.*, 1994).

Microbiological analyses

Bacterial isolation was performed from moribund larvae or brooders 'blood'. Bacteria were isolated and purified using TSA and Cetrimide mediums enriched with 2% NaCl and TCBS. Plates were incubated for 18 to 24 h at 28°C, all phenotypically distinguishable colonies were picked, and additionally, about 2-4 per plates were randomly selected to isolate as many different strains as possible. The streaking method and purity realized Gram coloration assessed the purification process. The purification process was repeated until obtaining pure colonies and then cultivated in LB

Table 1. List of primers and target tissue, used in this study for molecular analysis. Primers in bold are used as a Nested or semi Nested PCR primers.

Target	Primer	Sequence	Samples used for analysis	Annealing temp.	Reference
ITS of <i>Perkinsus marinus</i>	PerkITS-85	CCGCTTTGTTGGATCCC	Spat batch, gill and mantle or feces and blood	55°C	Audemard <i>et al.</i> (2004)
<i>Perkinsus olseni</i>	PerkITS-750	ACATCAGGCCTTCTAATGATG11111		55°C	This study
<i>Perkinsus</i> spp.	Pk-ITS-Fw1	AAAACGAAATTCAAACTCTCAAC		55°C	
	Pk-ITS-Rev1	GTGCAAAACCCGACAAGCGTG		55°C	
	Pk-ITS-Fw2	ATATCAGTGTGGCTCTCTTCTCC		55°C	
	Pk-ITS-Rev1	GTGCAAAACCCGACAAGCGTG		55°C	
SSU of <i>Bonamia exitiosa</i> ,	Bo	CAITTAATTGGTCGGGCCCG	Spat batch, gill and mantle or blood	55°C	Coehennec <i>et al.</i> (2000)
<i>Bonamia ostreae</i>	BoAs	CTGATCGTCTTGGATCCCCC		55°C	
<i>Bonamia</i> spp.	Cf	CGGGGGCATAATTCAGGAAC		55°C	Carnegie & Coehennec-Laureau (2004)
	Cr	CCATCTGCTGGAGACACAG		55°C	This study
	CF Fw2	GTGCTCAAAGCAGGCTCG		55°C	
	CR Rev2	GCCCAATGCTTAGTTGACATC		55°C	
<i>Martelia refrigens</i>	ITS1 4	CCGCACACGTTCTTCACCTC	Spat batch, digestive gland or feces	55°C	Le Roux <i>et al.</i> (2001)
ITS1	ITS1 5	CTCGCGAGTTTCGACAGAG		55°C	This study
<i>Martelia</i> spp. ITS1	Ma-ITS1-Fw	GAAGGCTTCGAAGTAGCAG		55°C	
	Ma-ITS1-Rev	GGAAAGGGCAACGAGAGC		55°C	
<i>Martelia</i> spp. IGS	Ma-IGS-Fw1	ACTACGACCGTAGCCTTTCC		55°C	This study
	Ma-IGS-Rev1	AGACACGCCTCTACTCTTCTCC		56°C	Arzul <i>et al.</i> (2001)
OSHV1	C2	CTCTTTACCATGAAGATACCCACC	Spat batch, gill and mantle or blood	57°C	
	C6	GTGCACGGCTTACCAITTTT		57°C	
	C2	CTCTTACCATGAAGATACCCACC		57°C	
	C4	GCAGTTGTGGTATACTCGAGATTG		57°C	
OsHV1, AVNV, Malacoherpesvirus	Fw1	CTATTGAAAAGGTTGCGGG	Spat batch, gill and mantle or blood	57°C	This study
	Rev1	TTCTCTTCATCTTTTGACCTCC		57°C	
	Fw1	CTATTGAAAAGGTTGCGGG		57°C	
	Rev2	CTCTCTTTTTCCTTCTTATCC		50°C	Folmer <i>et al.</i> (1994)
COI	LCO 1490	GGTCAACAATCATAAAGATATTGG	Blood or spat batch	50°C	
	HCO 2198	TAAACTTCAGGGTGACCAAAAAATCA		58°C	Guisande <i>et al.</i> (2004)
16SRNA amplification	27F	AGAGTTTAGTCMTGGCTCAG	Individual pure colony	58°C	
	1492R	GGYTACCTTGTACGACTT			

supplemented with 2% NaCl. Pure strains were identified based on their sequences of the 16S rRNA gene as described by Guisande *et al.* (2004). Briefly, 16S rRNA was partially amplified using 27F and 1492R primers (Table 1). PCR products were sequenced using internal primers 518F and 800R at Macrogen Inc. (USA). Only high-quality sequences were used to reconstitute the 16S rRNA sequences which were then compared with GeneBank entries using BlastN to identify bacterial genus. The classification level was attributed according to their respective match with the database; species; 99-100%, Genus; 97-98%, family; 95-96%.

Pathological analyses

Pathological analyses were performed on samples of moribund spats and adults, but also healthy adults and their offspring. Molecular biology diagnostics were realized using both primers and protocols recommended by the OIE for the detection of various pathogens of bivalves such as *Bonamia exitiosa*, *Bonamia ostreae*, *Marteilia refringens*, *Perkinsus marinus*, *Perkinsus olseni*, OSHV1 μ var or others specially designed in this study (Table 1). PCR controls assays were performed using primers specific for the COI gene for checking the quality of extracted DNA and the reagents (Table 1).

For OIE's recommended primers, diagnostic protocols were performed as detailed by their authors (Table 1). In the case of primers designed in this study, reactions were realized in a final volume of 50 μ L, using the Platinum Taq DNA Polymerase kit (Invitrogen) and following supplier recommendations with the addition of Bovine serum albumin (0.04X). The PCR reactions were carried out under the following conditions: an initial denaturation for 5 min at 95°C, then 35 cycles of denaturation for 30 s at 94°C, annealing for 45 s at their respective temperature (Table 1), followed by extension for 1 min at 72°C and a final extension step of 7 min at 72°C. When possible, primers for nested PCR assays were designed to increase the sensibility of the diagnostic.

Population genetic analyses

For COI amplification, primers used were as proposed by Folmer *et al.* (1994). PCR was performed in a total volume of 50 μ L including 1.5 U High fidelity Platinum Taq DNA polymerase (Invitrogen), 100 ng template DNA, 20 pmol of forward and reverse primers (LCO 1490 and HCO 2198), 0.2 mM of each dNTP, 1X PCR buffer and 1.5 mM MgCl₂. The PCR reaction was carried out under the following conditions: an initial denaturation for 5 min at 95°C, then 35 cycles of denaturation for 30 s at 94°C, annealing for 45 s at

50°C, followed by extension for 1 min at 72°C and a final extension step of 7 min at 72°C. PCR amplicons were sent to sequencing at Macrogen Inc. (USA). Sequences were edited with the software Mega5 (Tamura *et al.*, 2013) and aligned by Muscle (Edgar, 2004).

Genealogical relationships among different haplotypes were investigated by applying maximum parsimony as implemented in TCS software (Clement *et al.*, 2000). Haplotype (gene) diversity was calculated as in Nei (1987), by equations 8.4 but replacing 2n by n. Nucleotide diversity (Nei, 1987), the average number of nucleotide differences per site between two sequences (π), was also calculated. Haplotype and nucleotide diversities were estimated by using DnaSP v.5.10.01 (Librado & Rozas, 2009).

RESULTS

Reproduction and spat production

From August 2010 to January 2011, 463 animals between 30 and 62 mm length were collected in the protected area of NSMT and kept in the hatchery as previously described. Gamete emission was successfully induced for 55 females and 54 males, *i.e.*, 23.5% of the selected animals. Gametes recovered from these animals permitted to obtain 27 million of D larvae at 48 h after fertilization that means an average of 470,940 larvae D per female. The culture from planktonic trochophore stage to semi benthonic pediveliger stage lasted from 14 to 18 days at 25°C and 12 to 16 days at 29°C, and lead to the obtaining of 17.2 million of pediveliger larvae (64% survival).

The recovered pediveliger larvae were seeded at a density of 100 per cm² and cultivated 20 to 25 days to obtain 0.6 to 1 mm spat. Survival from pediveliger larvae to spat ranged between 4 and 25% with an average of 11.8% and a total number of 1,932,000 spats.

Pathology

Infectious pathology has been considered, on the one hand, as a crucial component of broodstock and spat certification for the production of pathogen-free spats permitting reliable aquaculture or stock enhancement operations and, on the other hand, to identify opportunistic and pathogenic bacteria present in a hatchery that could affect production.

PCR diagnostics were performed on the recommended tissue by the OIE, respectively gill and mantle (*Bonamia* spp., *Perkinsus* spp. and malacoherpesvirus) or digestive gland (*Marteilia refringens*) for moribund animals (21 individuals) and blood

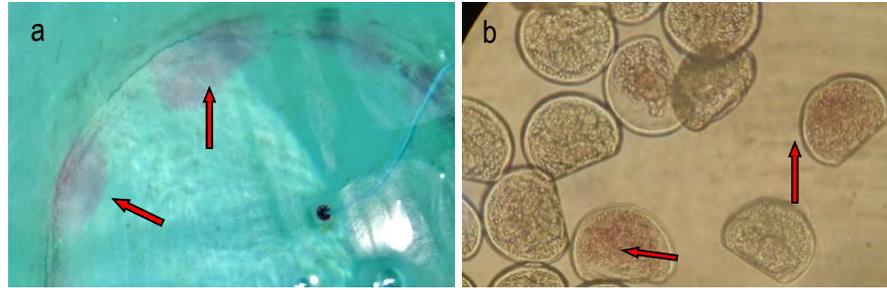


Figure 1. Massive mortalities in *Anadara tuberculosa* larval rearing tanks, in the presence of a pinkish biofilm. a) Pinkish biofilms at the bottom of the tank with moribund pink larvae; b) D larvae with pinkish tissues.

(*Bonamia* spp., *Perkinsus* spp. and malacoherpesvirus), and feces (*Marteilia refringens*, *Perkinsus* spp.) for healthy mollusks (60 individuals). All led to negative results. Similarly, all PCR analyses realized with homogenates of six healthy spat batches and three moribund spat batches (10-15 individuals per sample) were negative. In all the cases, amplicons were obtained for the PCR positive controls with primers specific of COI gene that showed the quality of extracted DNAs and reagents.

Putatively pathogenic bacteria have been isolated from moribund adults and larvae during a mortality event that occurred in January 2011. In this outbreak, the presence of a pinkish biofilm has been noticed at the bottom of larval tanks and the tissue from moribund larvae also exhibited the same pinkish color (Fig. 1).

Blood samples from eight moribund adults were collected along with pink D larvae and biofilm. A total of 36 bacterial strains were isolated; four from biofilm samples, eight from D larvae and 24 from adults (Table 2). Those strains were grouped in five clusters by their 16SrRNA sequences where *Vibrio* and *Pseudomonas* genus were dominant. Microbiological analysis indicated that the pink biofilm observed in tanks with moribund adults and pink spat was related to the presence of *Pseudomonas*. Such pink biofilm was also seen in rearing tanks with moribund larvae and juveniles of the whiteleg shrimp *Penaeus vannamei* within the hatchery during this study.

A single strain, CNLS1P, has been isolated from the pink biofilm and was identified as *Pseudomonas* sp. (based on 99% percentage of similarity with *Pseudomonas* 16SRNAr JN684003.1 sequence in Genbank). This strain has also been found on infected D larvae.

Samples extracted from blood samples of moribund *Anadara tuberculosa* showed a higher bacterial diversity and six genetically distinct strains had been isolated. Interestingly, the same *Pseudomonas* strains CNLS1P was found in all blood samples. Another

distinct *Pseudomonas* strain CNAs10 was also identified but could be found only in one adult. Additionally, four different members of the Vibrionaceae family were isolated, with the strains CNLS2V that was also found on D larvae. Strains CNLS2V and CNAs4 closest match belonged to *Vibrio* genus but with only 96% of similarity and were thus classified as Vibrionaceae. Two additional strains were identified as *Vibrio shilonii* CNAV11 and *Vibrio shilonii* CNAV2 with 99% correspondence.

Population genetic analyses

In this study, we have evaluated *A. tuberculosa* population diversity based on their COI gene. Data were obtained from 111 animals collected in seven areas of the NSMT. All sequences obtained were processed by Blast (NCBI) and showed 99 to 100% homology with the *A. tuberculosa* COI reference sequence. Of the 111 sequences, 39 haplotypes were identified. Haplotype and nucleotide diversity are reported in Table 3. Overall haplotype diversity was 0.898 (standard deviation, SD = 0.021). Haplotype diversity ranged from 0.826 ± 0.073 (Z2) to 0.958 ± 0.036 (Z7). These two locations also exhibited lower and higher nucleotide diversity ($\pi = 0.004 \pm 0.001$ for Z2, and 0.008 ± 0.001 for Z7).

Figure 2 represents genealogical relationships between the 39 haplotypes of *A. tuberculosa* from the NSMT. The size of circles and square is proportional to the frequency of the haplotype.

Some haplotypes were more prevalent than others, such as haplotype 1 (31 ind), 26 (13 ind), 13 (7 ind), 17, 28 and 33 (6 ind each), that represent 27.9%, 11.7%, 6.3%, and 5.4%, respectively, of the total population. According to Clement *et al.* (2000), for its high frequency, haplotype 1 could be considered the "ancestor" haplotype. Also, it is the only haplotype found in all areas, with a rate varying from 6.25 (Z7) to 40.0% (Z2). 44.7% of haplotypes were found only in a

Table 2. Cultivable bacterial strains isolated from *Anadara tuberculosa* rearing tanks and moribund adults and larvae. Strains were identified based on their 16S rRNA sequences.

Sample	Number of bacterial isolates per group	Strain name	16S rRNA identification gene nearest neighbor	Sequence similarity	Closest GenBank number
Biofilm	4 out of 4	CNLS1P	<i>Pseudomonas</i> sp.	99%	JN684003.1
Larva	4 out of 8	CNLS1P	<i>Pseudomonas</i> sp.	99%	JN684003.1
Larva	4 out of 8	CNLS2V	Vibrionaceae	96%	AP014635.1
Adult	2 out of 24	CNLS2V	Vibrionaceae	96%	NR_146027.1
Adult	13 out of 24	CNLS1P	<i>Pseudomonas</i> sp.	99%	JN684003.1
Adult	1 out of 24	CNA510	<i>Pseudomonas</i> sp.	99%	KT695822.1
Adult	2 out of 24	CNA54	Vibrionaceae	96%	KP713778.1
Adult	3 out of 24	CNAV11	<i>Vibrio shilonii</i>	99%	HF541940.1
Adult	3 out of 24	CNAV2	<i>Vibrio shilonii</i>	99%	AY911395.1

Table 3. Haplotype and nucleotide diversity of *Anadara tuberculosa* from the National Sanctuary Mangrove of Tumbes based on mitochondrial COI sequences.

Extraction site	# of sequences analyzed	# of different haplotypes	Haplotype diversity	Nucleotide diversity (π)
Z1 La Abeja	18	9	0.889 ± 0.049	0.007 ± 0.001
Z2 Chorro de la Almeja	20	9	0.826 ± 0.073	0.004 ± 0.001
Z3 El Habilitado	19	13	0.877 ± 0.074	0.006 ± 0.001
Z4 La Chalaquera	14	9	0.912 ± 0.059	0.004 ± 0.001
Z5 Corral de los Cochis	12	8	0.894 ± 0.078	0.005 ± 0.001
Z6 Nacupillal	11	7	0.909 ± 0.066	0.007 ± 0.002
Z7 Las Agujillas	16	12	0.958 ± 0.036	0.008 ± 0.001
Total	111	39	0.898 ± 0.021	0.006 ± 0.0005

single site, with Z6 being the more diverse site (61.5%) and Z3 the lesser one (28.6%).

DISCUSSION

Spat production

In this study, brooders with sizes between 45-60 mm were successfully induced to spawn by a progressive heat shock. Spawning prevalence (23.5%) and D larvae produced per female (470,000) were lower than those reported by Vásquez *et al.* (2009) with 40% and 2-3 billion D larvae, probably due to the bigger size of their brooders (50-80 mm). Such sizes are nowadays almost inexistent in the NSMT due to overexploitation.

Genetics

Hatchery produced shellfish larvae may lead to reduced genetic diversity resulting from the utilization of a limited number of brooders and to higher fitness of some genotypes in artificial culture conditions. Genetic diversity is a crucial component for populations' adaptive response to environmental conditions and long-term viability of aquaculture or stock enhancement

programs. Consequently, the production and release of hatchery-reared larvae must be performed considering the management of genetic diversity at intra- and inter-population levels. In this study, we used the mtCOI gene as a barcoding marker for the evaluation of *Anadara tuberculosa* genetic diversity in the NSMT.

Results indicate that the NSMT population presents a very high level of haplotype diversity which is essential considering the limited size of the sampled area (10 linear km between the two farthest extraction points). The star-like shape of the TCS network and the shallow length of branches (the maximum distance between the ancestor haplotype and the most divergent haplotype corresponds to only seven nucleotides) would suggest a recent population expansion (Clement *et al.*, 2000). As a point of comparison, haplotype diversity of *A. tuberculosa* from the NSMT was higher (36.7 vs 28.5%) than the genetic diversity reported for the same species extracted from Nariño region (Colombia) by Chamorro & Rosero (2016) or from *Anadara granosa* from Malaysia (14.7%) (Chee *et al.*, 2011b).

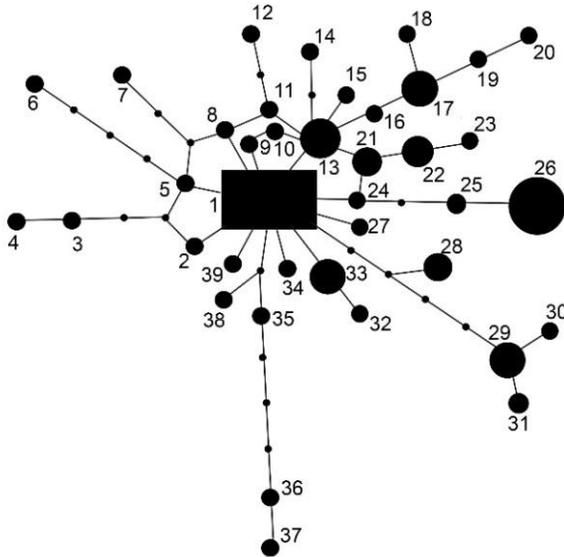


Figure 2. Maximum Parsimony Network. It represents the genealogical relationships between the 39 haplotypes of *Anadara tuberculosa* from the NSMT, based on COI-gene partial sequence. The size of circles is proportional to the number of individuals that share the same haplotype. "Ancestor" haplotype is symbolized by a square base and corresponds to the most frequent haplotype (1). Every nucleotide switch generates a difference that is represented by a line (branch) between circles. Smaller circles correspond to missing haplotypes that could exist but have not been sampled or extinct haplotypes.

The extension of *A. tuberculosa* genetic study to other South American population would help to gain information on intraspecific genetic flow and would help to understand the poorly understood patterns of larval dispersion of this bivalve. Research in this regard has started, indicating the importance of oceanic currents and palaeoclimatic events in shaping patterns of genetic variation of the species (Diringer *et al.*, 2019). This information would have significant implications for fishery management, conservation, translocation policies for aquaculture and stock enhancement programs.

Pathology

Diseases are a primary threat to mollusk aquaculture growth and sustainability, to the extreme that some have led that rearing activity stopped and that natural stocks have been decimated (Guo & Ford, 2015). Like for other invertebrates, such as shrimp, transfers of hatchery-produced larvae to grow-out areas are probably crucial in disease dissemination. Nevertheless, hatchery productions may also be a way to provide disease-free juveniles and therefore be a critical tool to prevent the transfer of infected stocks to disease-free

areas. In that sense, the present work relies on early molecular diagnostic to avoid disease transmission. Such a strategy is nowadays common in other aquaculture activity such as shrimp farming (Lo *et al.*, 1998; Motte *et al.*, 2003). Despite the fact that *A. tuberculosa* rearing is an emerging activity and that no infectious pathogen has been documented, early disease diagnostic is justified because: 1) three OIE listed pathogens are present in Latin America (Cremonte *et al.*, 2005; Wetchateng, 2008), 2) other ark species are known to be infected by *Perkinsus* species (Goggin & Baker, 1993) or OsHV1 (Xia *et al.*, 2015).

During this study, mortalities have happened during culture, but none could have been associated with one of the infectious disease sought by molecular diagnostic in either broodstock or spats. However, microbiological analyses revealed the presence of some cultivable bacterial pathogens, in particular, a *Pseudomonas* sp. and Vibrionaceae strains during outbreaks in both larvae and broodstock.

Members of the genus *Pseudomonas* have been occasionally described as pathogens for bivalve larvae, (Brown, 1973, 1981; Lodeiros *et al.*, 1992; Romalde *et al.*, 2014), but were generally co-isolated with representatives of the genus *Vibrio* that have been frequently incriminated to cause important losses in hatcheries and in native populations (Travers *et al.*, 2015; Petton *et al.*, 2015; Dubert *et al.*, 2017). Nevertheless, it is often difficult to distinguish between pathogenic bacteria and the native microbiome of the bivalve. In fact, *Vibrio* species predominate in the cultivable microbiota in bivalves (Garnier *et al.*, 2007; Romalde *et al.*, 2014) and arks (Kueh & Chan, 1985; Bilung *et al.*, 2005; Ahmad *et al.*, 2014; Sanchez *et al.*, 2015), while *Pseudomonas* is also commonly found in arks (Romanenko *et al.*, 2008; Ahmad *et al.*, 2014). In this study, the presence of *Pseudomonas* sp. strain CNLS1P was correlated with the presence of a pink biofilm and significant mortalities in all spat and adult samples which suggest its implication with those outbreaks. Also, no ubiquitous strain of *Vibrio* or Vibrionaceae could be identified as it is the case with the CNLS1P strain. Experimental infection realized *per os* or by injection on healthy adults of *Anadara tuberculosa* and *Penaeus vannamei* juveniles indicates that this *Pseudomonas* strain is particularly pathogenic to both organisms with or without association with *Vibrio* species (data not shown).

All isolated strains were molecularly characterized by sequencing their 16SRNAr gene which is considered as the gold standard for phylogenetic analysis of bacteria (Klindworth *et al.*, 2012). Nevertheless, few strains were able to be identified to the species level, and two strains were only classified at the family level

(CNAs4 and CNLS2V as Vibrionaceae). Multilocus sequence analyses (MLSA) could be realized for further genetic discrimination within *Vibrios*' clades (Travers *et al.*, 2015). The use of next-generation sequencing tools to study the metagenome of bivalves' native or pathogenic bacteria appears also as an interesting option, with the possibility to detect cultivable, co-cultivable and uncultivable strains (King *et al.*, 2012; Trabal *et al.*, 2012; Wegner *et al.*, 2013; Wendling *et al.*, 2014), in particular by characterizing bivalves' microbial composition following experimental infection with *Vibrio* sp. (Lockmer & Wegner, 2015).

CONCLUSIONS

This project, developed in the particular context of a biosphere reserve, aimed to pave the way for the sustainable production of spats of the pustulose ark, for aquaculture and stock enhancement purposes, considering three components: 1) spat output by classical aquaculture/biotechnology methods, 2) early pathology diagnostic so that vertical and horizontal disease transmission could be prevented or at least diagnosed, and 3) pustulose ark' genetic diversity estimation in order to perform responsible genetic management.

The results presented in this study have shown the feasibility to produce specific disease-free spat and also highlighted the relatively high genetic diversity of natural populations from the NSMT.

Altogether, this work illustrates the need to integrate classical biotechnologies with modern molecular biotechnology and offers a model for future stock enhancement strategies. The sustainability of such projects will require building collaborations among government institutions, environmental groups, aquaculture private-sector and the scientific community.

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