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Mapping the stranger: genetic diversity of Manila clam in European coastal lagoons

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Abstract: Manila clam *Ruditapes philippinarum* – synonym *Venerupis philippinarum* (Adams and Reeve, 1850) is one of the most successful marine invaders worldwide and represents almost 20% of worldwide mollusc total production. Originally distributed in the Indo-Pacific region, the species was introduced for aquaculture and fisheries in North America, Polynesia, the US Virgin Islands, as well as in Atlantic and Mediterranean coasts of Europe. Due to the commercial value of the species, population genetics and stock composition are of primary importance both in natural and invaded environments. Moreover, molecular genetics can provide valuable data in invasion biology, as, for example, the structure of the exploited populations, the origin of source populations and the routes of invasions. Furthermore, molecular data might be useful for the tracking of seafood products, as requested by the European Commission for European seafood products (see EC laws n° 178/2002, 509/2006, 510/2006 and their improvements). Despite all these considerations genetic data are lacking, especially for introduced populations, and should be urgently provided. In a framework of an European network of researchers involved in Manila clam studies, clams were collected in coastal lagoons and estuaries in both Mediterranean (Northern Adriatic Sea, Italy) and Atlantic (Spain and Portugal) coasts of Europe. Both mitochondrial and nuclear molecular markers were applied to investigate the genetic structure of populations, specifically by the direct sequencing of *16S* rDNA gene fragments and by the genotyping of 7 microsatellite markers previously used in native populations. Results demonstrated the occurrence of multiple haplotypes for *16S* rDNA, even if one main haplotype was identified in the European analyzed populations. However, few sequences were available in Genbank to make comparisons with native populations. Concerning the microsatellite data, the genotyping of Manila clam populations showed low genetic diversity among them, considering the Fst values, and a lack of genetic structuring when analysed by Structure software. Both these data suggest the absence of geographic differentiation among the introduced populations of Manila clam, as a result of introgression due to translocations and other human activities. The levels of genetic diversity within introduced populations were comparable to those described from native regions, suggesting that multiple introduction events and mixed source populations can counterbalance the loss of genetic diversity caused by the founder effect. However, it must be remarked that microsatellites showed a strong occurrence of null alleles when tested by Micro-checker, as previously observed in other bivalves, suggesting that the problem is differently distributed among Manila clam populations or specific loci, thus microsatellites should be carefully checked before used in extensive population studies. In the near future, population

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genetics and stock identification of this species on a large scale should be assessed using new powerful markers such as those obtained by NGS (Next Generation Sequencing) methods.

In conclusion, this research represents the very first extensive genetic investigation on Manila clam's European populations, defining the levels of genetic diversity that will help to inform management practices.

Keywords: *Ruditapes philippinarum,* Europe, *16S*, microsatellites, genetic diversity

Manila clam *Ruditapes philippinarum* – synonym *Venerupis philippinarum* (Adams and Reeve, 1850) is one of the most successful marine invaders worldwide and represents almost 20% of worldwide mollusc total production. The species was introduced in Europe in the early 1970s in North European Atlantic and Mediterranean coastal waters for commercial cultivation, especially in England and France (Gosling, 2003; Bald *et al.*, 2009). Then, from an English hatchery it was introduced in Northern Adriatic Sea in 1983 (Breber, 1985; Pellizzato *et al.*, 1989). As for Portugal, the first introduction is not documented, but it is believed that its introduction dates back to 1984, when it was reported at Ria Formosa. Since then, it was then reported in many estuarine systems all over the country (Gaspar, 2010). Up to know, Italy is the still the leading European country for Manila clam production, whilst China represents the first producer of Asari (Manila) clam in the world.

Despite its commercial value, it is almost surprising that genetic diversity of Manila clam was poorly investigated. As recently reported by Astorga (2014), even if Manila clam is listed as the first species among bivalves for estimated commercial values (FAO, 2013), the existing genetic information in literature is limited. Molecular genetics can provide valuable data (Chiesa *et al.* 2014a) being useful for tracing and tracking seafood products. Genetic profiles of cultivated and harvested clams can certify their geographic origin, preventing illegal selling and labeling (Kim *et al.*, 2014), guarantying consumer's safety and product's quality control (Filonzi *et al.*, 2010; Caldelli *et al.*, 2014), with both economic and social benefits.

However, few papers have been published concerning the genetic diversity and structure of Manila clam populations in its native range of distribution (see as examples Sekine *et al.*, 2006; Vargas *et al.*, 2008; Liu *et al.*, 2007; Mao *et al.*, 2011; An *et al.*, 2012; Kitada *et al.*, 2013; Nie *et al.*, 2015a), and in introduced ecosystems (Chiesa *et al.*, 2011; 2014a,b; 2016; Mura *et al.*, 2012; Hurtado *et al.*, 2012).

The present paper will summarize the data obtained on the genetic structure of introduced populations in Portugal, Spain and Italy, investigated by the direct sequencing of *16S* rDNA gene fragments and by the genotyping of 7 microsatellite markers.

Methods

Sampling procedures: Manila clam specimens were collected from both Mediterranean and Atlantic introduced populations, particularly in Italy, Portugal and Spain. See Table 1 for details.

DNA extraction and purification: High molecular

Table 1. Manila clam sampling sites in Portugal, Italy and Spain. The locations and the number of analyzed samples and for each site are provided

Country	Location	N (individuals)
Portugal	Ria de Aveiro Lagoon	24
Spain	Galician Coast	14
Italy	Venice Lagoon	35
Italy	Marano Lagoon	30
Italy	Po River Delta	33

weight genomic DNAs were extracted and purified through Wizard genomic DNA Purification kit (Promega). Ethanol-fixed mantle and foot tissue stored at −20°C were selected for the extraction, to avoid the interferences of the mtDNA "Doubly Uniparental Inheritance" (DUI) (Plazzi and Passamonti 2010). DNA extraction details are reported in Chiesa *et al.* (2011; 2014a).

***16S* rDNA analysis**: Amplification of a *16S* rRNA gene fragment was achieved using primers *16S*ar-ALT (5' GCCTGTTTATCAAAAACATSG 3' and *16S*br-ALT (5' CCGGTCTGAACTCAGATCATGT 3') designed for Veneridae family (Mikkelsen *et al*., 2006). Reaction details and PCR – touch down profile are described in Chiesa *et al.* (2011; 2014a). Fragments sequencing was performed by MACROGEN Europe service (Amsterdam, the Netherlands). The obtained sequences were compared with those available in genomic databases using Blast and multiple alignments of sense and antisense sequences were conducted using software as Clustal X and Sequencer 4.2 (Gene Code Corporation). Original sequences were aligned and compared with those downloaded from GenBank for *R. philippinarum*, *R. decussatus*, *R. variegatus* and *R. bruguieri* (see Chiesa *et al*., 2014a for details). As for outgroups, two were selected: *Glauconome rugosa* and *Corbicula fluminea* (see Chiesa *et al*., 2014a for details).

Phylogeographic analysis was performed through the analysis of aligned fragments of the *16S rDNA*. Haplotype network was performed in TCS v1.21 (Clement *et al*., 2000), with confidence threshold at 95% for *Ruditapes* genus sequences (Hart and Sunday, 2007; Lucentini *et al*., 2011). A median-joining network (Bandelt *et al*., 1999) was constructed using Network 4.611 for *R. philippinarum* haplotypes and outgroups. (see Chiesa *et al*., 2014a for details).

The identification of variable and parsimony informative sites, the translation of nucleotide sequences, the pairwise genetic distances, the nucleotide base composition and the transition/transversion ratios were calculated using MEGA 6.06 (Tamura *et al*., 2013).

Spatial or demographic expansion was estimated through Tajima's D neutrality test (Tajima 1989). Statistical selection of best-fit models of nucleotide substitution was performed by means of jmodeltest (Guindon and Gascuel, 2003; Darriba *et al*., 2012). On the basis of these results, the Jukes-Cantor model was used to assess the evolutionary history among *R. philippinarum*, *R. decussatus* and other outgroups; Maximum Likelihood and Neighbour Joining methods were inferred in MEGA6.06 estimating standard error by a bootstrap procedure (1,000 replicates). See Chiesa *et al*., 2014a for details on statistical analyses.

Microsatellites analyses: All samples were genotyped for seven microsatellite loci previously used in different studies with Manila clam: *Asari16*, *Asari23*, *Asari24*, *Asari54* (Yasuda *et al*., 2007), and *Ktp5, Ktp8, Ktp22* (An *et al*., 2009), chosen according to their variability among different samples. Polymerase chain reaction (PCR) and microsatellite collection were performed as described in Chiesa *et al.* (2011). The quality of the seven microsatellite markers was tested as suggested by Vignaud *et al.* (2013): Micro-checker v2.2.3 (van Oosterhout *et al*., 2004) was used to check genotyping errors and null alleles potentially present. Linkage Disequilibrium between each pair of loci was tested though GenePop (Rousset, 2008).

To evaluate allelic richness, private alleles and test for the Hardy-Weinberg equilibrium for each locus both in the entire dataset and in each pool (Adriatic or Atlantic), the software GenAlEx 6.4.1 (Peakall and Smouse, 2006) was used. Since the presence of null alleles is a compromising-factor in the evaluation of genetic distances, the ENA (Excluding Null Alleles) method implemented in FreeNA (Chapuis and Estoup, 2007; http://www.montpellier.inra.fr/URLB/) was performed and Fst values were computed using both corrected and uncorrected data. Correlation analysis and both t-test and F-test were performed to evaluate the correlation between corrected and observed frequencies. (See Chiesa *et al*., 2016 for details). Furthermore, to investigate whether the presence of null alleles influenced the allelic diversity, a per locus dispersion graph and a correlation analysis were performed. (See Chiesa *et al*., 2016 for details).

Using Structure 2.3.3 program (Pritchard *et al*., 2000) a Bayesian approach was performed with the aim to estimate the a posteriori probability of a given number of stocks. To detect the K-value that best fit the data, both approaches were used: the mean posterior probability of the data [Ln(K)] and the Evanno *et al*. (2005) method of delta K using STRUCTURE HARVESTER (Earl and VonHoldt, 2012). Structure assignment test was performed for the whole dataset or removing all samples affected by null alleles at three or more loci.

Results

mtDNA: The *16S*rDNA sequences were successfully sequenced for 133 individuals of *R. philippinarum* with a final alignment of 438 bp (see Chiesa *et al*., 2014a). Twenty haplotypes were identified including outgroups, clustering into 5 separate haplogroups. One haplogroup included 12 haplotypes for *R. philippinarum* (Chiesa *et al*., 2014a). The original Italian, Spanish and Portuguese samples (GenBank A.N. KF736199–211) were included within haplotypes Rphap1–10 and Rphap12, whilst Rphap11 contained only a Chinese sequence (GenBank A.N. DQ356383) (Chiesa *et al*., 2014a). Rphap1 (weight =0.174) had the biggest haplotype probability among *R. philippinarum* haplotypes; other haplotypes were rare (weight ranged from 0.0006 to 0.162). (Chiesa *et al*., 2014a). The Tajima's Neutrality Test performed on all *R. philippinarum* sequences showed the occurrence of 12 segregating sites (S) and a significant ($p<0.05$), Tajima statistics test ($D = -1.914$). (Chiesa *et al*., 2014a).

If considering only European sequences, S was 10 and Tajima statistics test D was −1.789 ($p<0.05$). Considering only Adriatic or Atlantic samples, the values changed as S was 6, whether D was −1.755 for Adriatic samples and for Atlantic samples D −1.487 for Atlantic ones, both with $p>0.05$. (See Chiesa *et al*., 2014a for details). J modeltest identified JC as the best model. Bootstrap NJ and ML phenograms performed with this model showed almost the same topology, with a clear separation of *R. philippinarum* from congeneric species. A significant node separation (99/100 bootstrap) was observed within *R. philippinarum* haplotypes, separating the Chinese haplotype Rphap11 from all the European haplotypes (Chiesa *et al*., 2014a).

The haplotype Rp hap1 was the most frequent, commonly distributed among all populations. On the opposite, the other 10 haplotypes were rare and locally distributed, showing a specific geographic structuring (Chiesa *et al*., 2014a).

Microsatellites: As for microsatellites, all loci were affected by missing amplifications. At the locus *Asari16*, 5% of samples did not amplify. The percentage was higher at the other loci: 25%, 27% and 60% for loci *Asari23*, *Asari24*, and *Asari54* respectively; 54%, 35%, 45% for loci *Ktp5*, *Ktp8*, *Ktp22*, respectively. Considering the geographic distribution, the occurrence of missing amplifications ranged from 27% in the Venice population to 49% in Marano population, 29% within the Spanish population, 39% for Delta Po and 43% for the Ria de Aveiro populations. These observations are in accordance with Micro-checker results and FreeNA tests, showing that each locus could be globally affected by null-alleles, except for locus *Asari16* (Chiesa *et al*., 2016). In the Marano lagoon population, high frequency of missing amplifications resulted in data deficiency of Micro-checker tests (Chiesa *et al*., 2016). The comparison of gene frequencies corrected by means of FreeNA with original ones, underlined a correlation of up to 80% and t-student and F tests showed no statistical significant differences between these two series. The graph and the correlation analysis performed to investigate whether the presence of null alleles influenced the allelic diversity indicated differences among loci (Chiesa *et al*., 2016).

Exact test for genotypic Linkage Disequilibrium was significant at 0.05 level for 17 out of 21 comparisons. The same LD test was performed for each couple of loci, removing samples affected by null alleles: results underlined the absence of linkage among different loci.

The expected heterozygosity (He) across populations differed among the seven analyzed loci ranging from 0.47 ± 0.08 for *Asari54* to 0.85 ± 0.02 for *Ktp22*; the observed values (Ho) of heterozygosity ranged between 0.23 ± 0.05 for *Ktp5* and 0.82 ± 0.04 for *Asari16*. (See Chiesa *et al*., 2016 for details).

Per population, F ranged from −0.02 (Po Delta

population locus *Ktp8*) to 0.93 (population Po Delta locus *Ktp22)* (See Chiesa *et al*., 2016 for details); Hardy-Weinberg (HW) analysis results underlined that *Asari16* was under equilibrium, in contrast the others were in equilibrium, not under equilibrium or monomorphic, showing variations across populations. HW results across populations underlined that only *Asari 16* was under equilibrium, and the remaining loci were not (Chiesa *et al*., 2016).

Fst values calculated with or without ENA corrections did not change significantly (Chiesa *et al*., 2016). Exploratory structure runs demonstrated that a burn-in period of 100,000 steps, followed by 500,000 steps of data collection, for each of the combined conditions mentioned in materials and methods, ensure the convergence of the Monte Carlo Markov Chain (MCMC). When estimating K, a discrepancy emerged between Pr (X/K), corresponding to LnP(D), and ΔK; the highest LnP(D) was found for K= 5 but ΔK clearly indicated the uppermost K=2 (Fig. 1). It was demanding to choose a single K to performed a structure's cluster, in fact if Evanno *et al*. (2005) found that LnP(D) might fail, is also true that Evanno's method might underestimate K (Waples and Gaggiotti, 2006). Therefore, the structure's bar plot was performed both with K=2 and K=5 (Fig. 2). In both cases, a geographical pattern was lacking; for K=2 almost all individuals were assigned to both genetic clusters, analogously, for K=5 almost all individuals were assigned to three or more clusters (Fig. 2). Genetic structure performed removing the entire Marano population, or removing all samples affected by null alleles at three or more loci, presented the same results of the global pool.

Discussion

Concerning mtDNA analyses, the direct sequencing of a *16S*rDNA fragment showed interesting results: European introduced populations were characterized by one common haplotype, Rphap1, whilst the other 10 haplotypes were rare and represented by only 1 or 2 sequences (Chiesa *et al*., 2014a). When comparing the two macro areas Atlantic vs Adriatic, haplotype frequencies and the haplotype minimum spanning tree underlined a biogeographic differentiation among regions (Chiesa *et al*., 2014a). Apart from the common haplotype Rphap1, the other haplotypes differently occurred depending on the sampling sites. Haplotype Rphap1 could represent an haplotype characteristic of the ancestor lineage introduced in Italy, Spain and Portugal (Chiesa *et al*., 2014a). However, the occurrence of rare haplotypes with a specific geographic distribution suggested multiple founder effects (Chiesa *et al*., 2014a).

As for microsatellite markers, they seemed to be useful and promising to characterize fisheries and aquaculture Manila clam stocks (Chiesa *et al*., 2011; An *et al*., 2012; Kitada *et al*., 2013; Nie *et al*., 2014; 2015a,b). However, our investigation clearly showed that missing amplifications and null alleles are

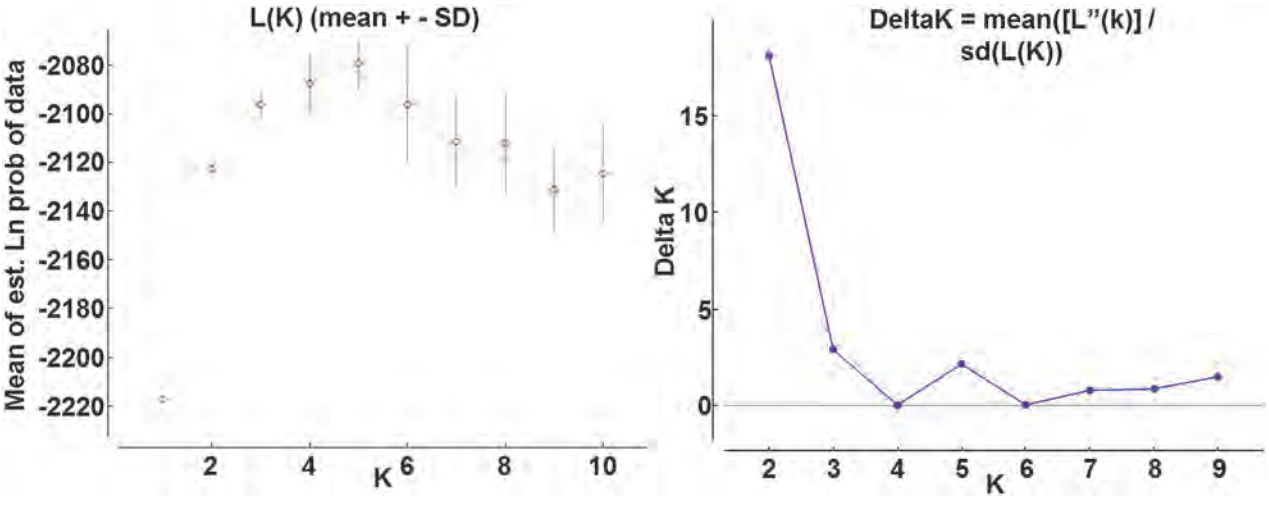

Fig. 1 Inference of true K. a. Use of Ln(K); b. Use of an ad hoc quantity [ΔK].

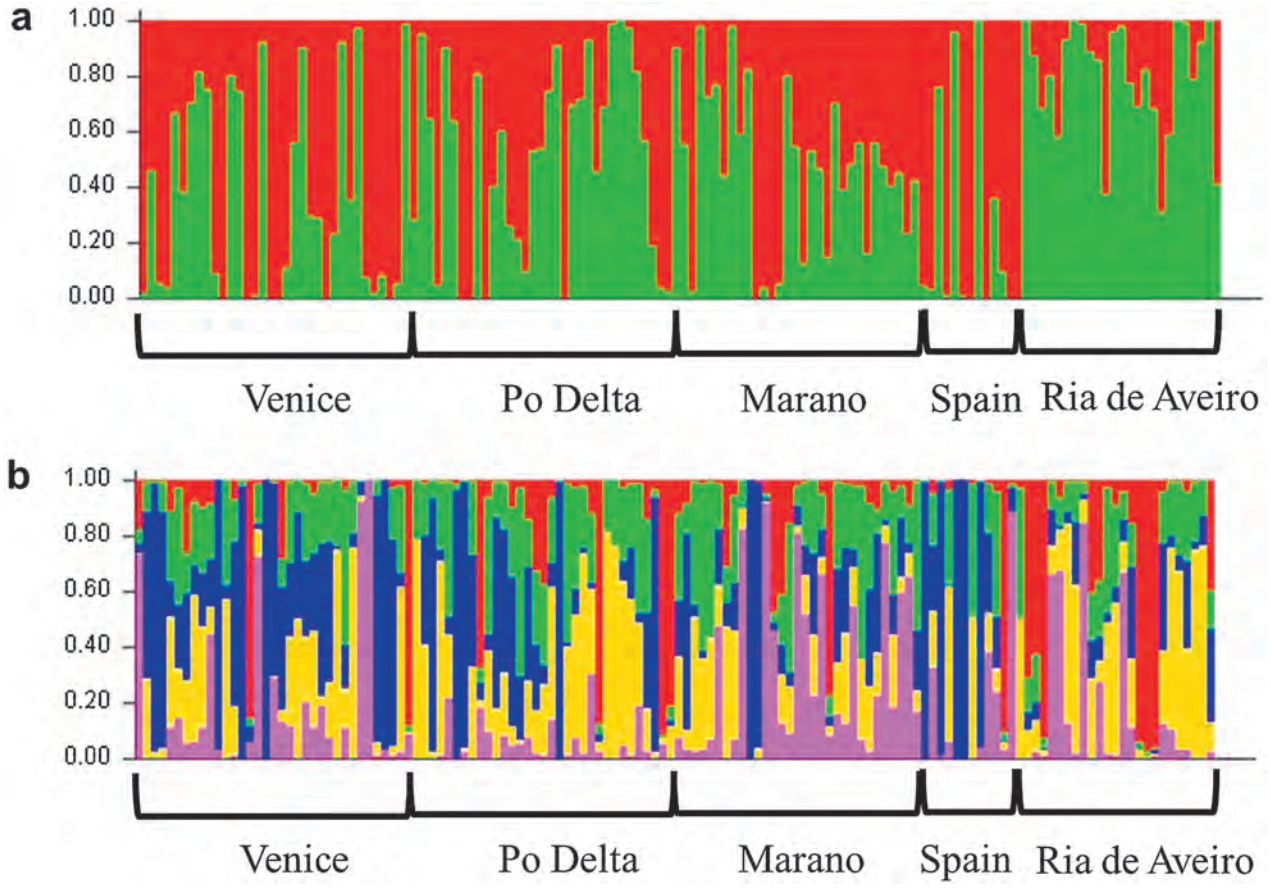

Fig. 2 Genetic structure revealed by 7 microsatellite markers in *R. philippinarum* for (a) K=2 and (b) K=5. Each vertical bar represents a single individual divided into color segments, proportional to its membership within the K genetic clusters.

critical points in this species genotyping, especially when comparing different populations. The results herein presented showed both the presence of missing amplifications in the Manila clam populations herein analyzed, and null alleles in six of the seven investigated loci, previously used in other Manila clam studies for stock analyses and conservation genetics. The occurrence of null alleles is very common in animal populations, especially in molluscs (see as examples Arias-Pérez *et al.*, 2012; Marin *et al.*, 2012). Null alleles were likely to be encountered especially in populations with a large effective size, with an unusually high mutation rate in the flanking regions, and that have diverged from the population from which the cloned allele state was drawn and the primers designed (Chapuis and Estoup, 2007). To date, several statistical approaches were reported for correcting allele frequencies when null alleles are occurring (van Oosterhout *et al.* 2004; Chapuis and Estoup 2007; Wagner *et al.*, 2006). These methods can be reliable to evaluate levels of population differentiation, as Fst, but they are not useful when performing assignment tests. As reported by Carlsson (2008) "they rely on accurate information about the multilocus genotype of individuals" . Furthermore, the frequencies correction seems to be particularly powerful when null alleles are related to an incorrect scoring of a/anull genotypes as an homozigote a/a. Unfortunately, our data showed an anull/anull pattern in several cases, and it is difficult to understand the feasibility of frequencies correction methods in this condition (Chiesa *et al.*, 2016). It is remarkable that, even though in our data several loci were affected by null alleles showing an anull/anull pattern, globally the number of observed alleles was comparable to those observed in other populations. Exact test for genotypic Linkage Disequilibrium, were non significant for all

comparisons when removing samples affected by null alleles, showing that the statistical values were reinforced. The influence of null alleles on Linkage Disequilibrium was already demonstrated (Sved *et al*., 2013).

The mean values of observed heterozygosity (Ho) were in agreement to those reported for *Asari16* in other Manila clam populations by Yasuda *et al*. (2007), Mura *et al*. (2012) and Kitada *et al*. (2013), but lower for other loci (see Yasuda *et al*., 2007; An *et al*., 2012 for comparisons).

Fst values calculated with or without ENA corrections did not change significantly (Chiesa *et al*., 2016). These data underlined a very low genetic pairwise differentiation of the investigated populations (Fst < 0.05) (Chiesa *et al*., 2016).

Finally, concerning the structure analysis, two main issues must be highlighted. First, it is interesting that the discordance between reliable K values suggested by LnP(D) (K=2) and ΔK (K=5). Such discordance might be related to the presence of null alleles; as also reported in the Structure's manual "A serious problem arises when data are missing in a systematic manner, as with null alleles" . Only few discordant information are available about the effects of null alleles on genetic assignment testing, even if Carlsson (2008) clearly underlined that null alleles cause a reduction in the power to correctly assign individuals.

Conclusions

Biogeographic and phylogenetic analyses obtained by *16S*rDNA sequencing showed a complex scenario for Manila clam introduced populations, dominated by multiple introductions of individuals coming from different sources (Chiesa *et al*., 2014a). Microsatellite genotyping of Manila clam populations introduced in Italy, Spain and Portugal showed low genetic diversity among them, considering the Fst values, and a lack of genetic structuring when analysed by Structure (see Fig. 2). Both these data suggest the absence of genetic differentiation among the introduced populations of Manila clam, as a result of introgression due to translocations and other human activities. This conclusion is in agreement with those obtained by *16S* rDNA analyses (see Chiesa *et al*., 2014a).

Although, the results herein reported showed a strong occurrence of null alleles; even thought evidences of null alleles emerged in previous studies on Manila clam (Chiesa *et al*. 2011a; Mura *et al*. 2012; An *et al*. 2012, Kitada *et al*. 2013; Nie *et al*. 2014) where Micro-checker/FreeNA tests were largely missing, or used only to confirm their occurrence (An *et al*., 2012). The present work underlined not only the diffuse presence of null alleles, but particularly suggested that such problem is differently distributed among populations or loci (Chiesa *et al*., 2016).

The authors cannot exclude that a limited sampling session may have affected the results here presented, both in terms of genetic structuring and of null alleles influence. Nevertheless, as already suggested by Kelly *et al*. (2011), genotyping errors should be reported to increase awareness of problematic markers in the scientific community, especially when deviations from HWE are detected (DeWoody *et al*., 2006). This is particularly important, and generally applicable to all studies that employ microsatellites, independently from the taxon involved, to allow a careful selection of microsatellite loci before using them for population genetics studies (Kelly *et al*., 2011).

Considering that microsatellites are both money- and time- consuming, an accurate selection of the genetic markers might be fundamental. Case by case, a reliable panel of loci should be selected before being applied to population genetics studies. However, few papers underlying genotyping errors are encouraged to be published by scientific journals. In cases where microsatellites seemed to be problematic, the possibility to use other markers should also be considered for stock identification and population genetics.

MtDNA sequence analysis – previously used in other valuable species of conservation or aquaculture interest (Chiesa *et al*., 2014c; Lucentini *et al*., 2011; 2014) – or new powerful NGS (Next Generation Sequencing) derived SNPs (Single Nucleotide Polymorphisms- see for example Kim *et al*., 2014) can be alternative solutions. Particularly SNPs could supply new genetic informations based on a wider dataset, hopefully less affected by technical problems as

microsatellites, meanwhile being highly informative.

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