RESEARCH ARTICLE

Decline of genetic variability in a captive population of Pacific white shrimp *Penaeus (Litopenaeus) vannamei* using microsatellite and pedigree information

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Abstract

Background: The objective of this study was to estimate the decline of genetic variability and the changes in effective population size in three shrimp populations. One was a wild population collected at several points in the Mexican Pacific Ocean. The other two populations were different generations (7 and 9) from a captive population selected for growth and survival. Microsatellite markers and pedigree were both used to assess genetic variability and effective population size.
Results: Using 26 loci, both captive populations showed a decline in the expected heterozygosity (20%) and allelic diversity indices (48 to 91%) compared to the wild population ($P < 0.05$). The studied captive populations did not differ significantly from each other regarding their expected heterozygosity or allelic diversity indices ($P > 0.05$). Effective population size estimates based on microsatellites declined from 48.2 to 64.0% in cultured populations ($P < 0.05$) compared to the wild population.
Conclusions: An important decline of genetic variability in the cultured selected population due to domestication, and evidence of a further smaller decline in effective population size across generations in the selected population were observed when analyzing pedigree (41%) and microsatellite data (37%). Pedigree keeping is required to prevent the decline of effective population size and maintain genetic variability in shrimp breeding programs, while microsatellites are useful to assess effective population size changes at the population level.

Keywords: effective population size, genetic diversity, heterozygosity, microsatellites, selection, shrimp.

INTRODUCTION

Penaeus (Litopenaeus) vannamei has become the shrimp species most commonly used for culture worldwide (Benzie, 2009). Nowadays, breeding programs play a key role in the increase of shrimp production in these cultured populations.

The decline in genetic variability, when compared to wild reference populations, of advanced generations of cultured breeding populations has been estimated for Atlantic salmon (*Salmo salar*) (Norris et al. 1999), black tiger shrimp (*Penaeus monodon*) (Xu et al. 2001; Dixon et al. 2008), silver-lipped pearl oysters (*Pinctada maxima*) (Lind et al. 2009), and gilthead sea bream, (*Sparus aurata*) (Loukovitis et al. 2012), using different variability indices based on microsatellite data. On the other hand, studies involving the analysis of several generations of selected populations of *P. vannamei* have revealed non-significant declines in genetic variability (Cruz et al. 2004; Luvesuto et al. 2007; Pérez-Enríquez et al. 2009).

The effective population size (N_e) is a crucial parameter for determining the extent of genetic variability that can be maintained in a population (Lande and Barrowclough, 1987). Changes in N_e need to be evaluated during the domestication and artificial selection processes in order to predict the accumulation of inbreeding, which may cause inbreeding depression. Evaluation of N_e also allows determining the potential for further genetic improvement in the population (Hill, 2000).

There are well established methods for assessing N_e based on pedigree analysis (Meuwissen, 2009), but comparisons of those methods with estimates based on genetic markers are not available for aquaculture captive populations and, to our knowledge, are very scarce for any domestic animal population.

The objective of this study was to evaluate, using microsatellite loci, a possible loss in genetic variability and a decrease in the effective population size between two generations (7 and 9) of a captive population of *P. vannamei*, compared with a wild shrimp population. An additional objective was to compare estimates of effective population size for the same population, using microsatellite and pedigree information.

MATERIALS AND METHODS

Populations and sampling procedures

P. vannamei samples were collected from a closed breeding population in a large commercial hatchery in Mexico. This population was started in 1998, incorporating wild shrimps from Sinaloa, Mexico, and domesticated shrimps from Venezuela, Colombia, Florida and Ecuador. Mass, family and within-family selections for growth rate were performed, in the absence of an organized breeding program, from 1998 to 2003. In 2004, a breeding program was initiated based on family estimated breeding values (EBVs) obtained through a mixed model methodology, and on within-family phenotypic selection for body weight. Since 2005, a two-stage selection program has been used to select for: (1) body weight at 28 days of age, and (2) body weight and survival at 130 days of age, based on family EBVs; harvest body weight (130 days of age) was also selected for using within-family data (Castillo-Juárez et al. 2007; Campos-Montes et al. 2013). The generation interval is one year, and the generations are discrete (*i.e.*, they are produced annually). Full pedigree was available for the parents of animals that were born in 2003 and thereafter.

A restriction on mating between selected broodstock males and females was used, based on the expected inbreeding of future progeny. The expected inbreeding values were obtained from the pedigree-based numerator relationship matrix. The threshold was gradually relaxed over time as the average relationship in the population increased. This procedure is similar in several aspects to that described for tilapia (*Oreochromis niloticus*) by Ponzoni et al. (2010).

Samples were obtained as follows. Generation 7 (GEN07): One individual was sampled from each of 77 full-sib families (progeny from one pair of parents), which were randomly chosen from a total of 208 families of the breeding nucleus of the 2005 cycle. Generation 9 (GEN09): One individual was sampled from each of the 73 full-sib families randomly chosen from a total of 203 families of the breeding nucleus of the 2007 cycle. Wild samples (WILD): Samples were collected from 12 different locations (n = 55) along the northern Pacific coast of Mexico on an official fishing monitoring tour (Figure 1). Adult abdomens were used for WILD samples, while in the case of GEN07 and GEN09, 28-day old post-larvae abdomens obtained during the regular genetic evaluations of the breeding nucleus were used. All samples were stored in 70% ethanol at 4ºC until required for genetic analysis.
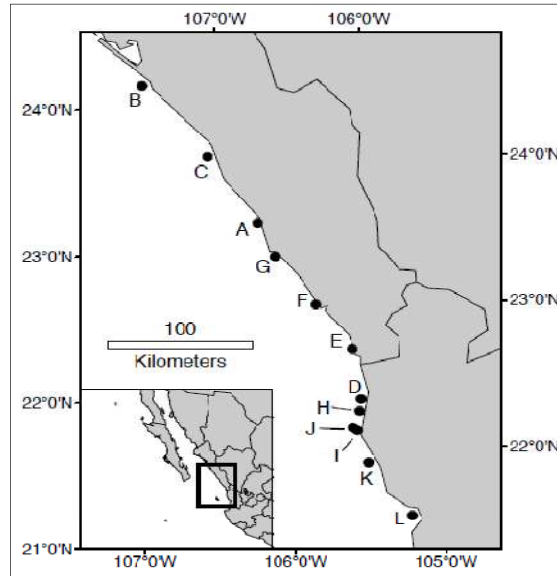
Fig. 1 Map of the sampling locations for the WILD population. Capital letters from A to L represent the sampled locations in the Mexican Pacific Ocean.

Genotyping

The QIAGEN DNeasy Blood and Tissue kit[®] was used for genomic DNA extraction. Each of the extractions was quantified by spectrophotometry and adjusted to 20 ng/µl. A set of 31 microsatellite loci was optimized for PCR amplification from a preliminary list of 72 polymorphic microsatellites for *P. vannamei* available in the literature and in Genbank. From this list, 6 multiplex groups were obtained. Table 1 shows the final composition of each of the multiplex groups and the annealing temperature (Ta) for each PCR reaction performed using the Multiplex QIAGEN PCR kit (Foster City, CA) according to the manufacturer recommendations. The amplified products were separated using capillary electrophoresis in a 3100 ABI PRISM Genetic Analyzer, and the resulting electropherograms were analyzed using ABI's GeneMapper V. 3.7. Five loci were eliminated from further analysis due to technical problems regarding their amplification. Used and discarded microsatellites are shown in Table 1.

Population variability indices

Expected (H_e) and observed heterozygosity (H_o) for all the loci and populations were calculated according to Nei (1987) using the GENEPOP software, version 3.1 (Raymond and Rousset, 1995). The analyses of deviations from Hardy-Weinberg equilibrium and of population differentiation (F_{IS} estimates) were performed with GENEPOP, version 3.1. The number of alleles (A_c) and the unique number of alleles per locus (A_u) were obtained with HP-RARE 1.1, applying a correction for sample size through a rarefaction method (Kalinowski, 2005). In addition, values of the effective number of alleles were obtained (A_e) (Hedrick, 2005). The Fstat program, version 2, was used (Goudet, 1995) to obtain F_{ST} values for all populations (Weir and Cockerham, 1984) and between pairs of populations (Slatkin, 1995). The values of R_{ST} (Slatkin, 1995) were calculated using the RstCalc software (Goodman, 1997). Nei's genetic unbiased distance (NeiD) was estimated for all pairs of populations (Nei, 1978) using the GENALEX 6 software (Peakall and Smouse, 2006).

Estimation of effective population size from microsatellite data

N_e was estimated from the microsatellite data using three different methods for single population data. The first was according to Waples (2006) using the software LDNe (Waples and Do, 2008), an approach based on linkage disequilibrium. Minimum allele frequency was set at 0.006. The estimate of N_e obtained with this method was denoted as $N_{em(1)}$. The second method used was according to Tallmon et al. (2008), using the ONeSAMP software. This software uses summary statistics from simulated populations, approximate Bayesian computation and a Wright-Fisher model to estimate N_e from a single sample of microsatellite data. The estimate of N_e obtained with this method was denoted as $N_{em(2)}$. The third method used was according to Hill (1981), using NeEstimator V1.3 (Peel et al. 2004), which is based on linkage disequilibrium ($N_{em(3)}$).

Table 1. Characteristics of microsatellite loci used in the study.

Multiplex Group	Locus	Ta	Reference
1	CNM-MG-339 CNM-MG-356 CNM-MG-367* CNM-MG-387 CNM-MG 412 CNM-MG-421 CNM-MG-479 Lvan10*	53.3ºC	Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Freitas et al. (2007)
2	Pvan0040* CNM-MG-351 CNM-MG-390 CNM-MG-364 CNM-MG-384 CNM-MG-386 CNM-MG-357*	48.1ºC	Cruz et al. (2002) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005)
3	CNM-MG-430 Lvan07 CNM-MG-369 CNM-MG-383*	46.4ºC	Pérez et al. (2005) Freitas et al. (2007) Pérez et al. (2005) Pérez et al. (2005)
4	CNM-MG-507 HLJN-030 CNM-MG-487 CNM-MG-496	53.2ºC	Pérez et al. (2005) Zhi-Ying et al. (2006) Pérez et al. (2005) Pérez et al. (2005)
5	CNM-MG-371 CNM-MG-380 CNM-MG-354 CNM-MG-362	50.2ºC	Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005) Pérez et al. (2005)
6	CNM-MG-416 HLJN-004 Lvan01 Pvan1758	51.9ºC	Pérez et al. (2005) Zhi-Ying et al. (2006) Freitas et al. (2007) Cruz et al. (2002)

Ta: Annealing temperature.
* Loci excluded from variability analyses.

Estimation of effective population size from pedigree data

Effective population size was estimated in the captive populations by pedigree analysis (N_{ef}) using data from 2002 to 2007 and the software PopReport (Groeneveld et al. 2009). N_{ef} was estimated from the rate of co-ancestry between all selected males and all selected females that produced offspring at generation t (f_t), as: $N_{ef} = 1/(2\ \Delta_f)$; where $\Delta_f = (f_t - f_{t-1})/(1 - f_{t-1})$ (Ponzoni et al. 2010).

RESULTS

Genetic variability of populations

All analyzed loci were polymorphic, with a range of 2 to 26 alleles per locus. Across all populations, 23 out of the 26 loci used were not in Hardy Weinberg equilibrium ($P < 0.05$). The same analysis within populations showed that the WILD, GEN07 and GEN09 populations were not in Hardy Weinberg equilibrium in 22, 16 and 20 loci, respectively ($P < 0.05$). Most of this disequilibrium is attributable to a heterozygosity deficit indicated by positive F_{IS} values. A total of 22, 15 and 17 loci showed a significant deficit in heterozygosity in the WILD, GEN07 and GEN09 populations, respectively.

Using results averaged over all loci, the captive populations (GEN07 and GEN09) did not differ significantly from each other with regard to their expected heterozygosity and allelic diversity (Table 2); however, WILD had a greater expected heterozygosity and greater allelic diversity than GEN07 and GEN09. Using H_e, the decline in estimated genetic variability from WILD to either GEN07 or GEN09 was 20%. Declines were observed when using genetic variability estimated from A_c, A_e, and A_u (45, 47, and 90%, respectively).

Table 2. Summary genetic diversity indices estimated from microsatellite data using 26 loci. Includes H_e: Expected heterozygosity; H_o: Observed heterozygosity; A_c: Number of alleles corrected by rarefaction; A_u: Number of unique alleles corrected by rarefaction; A_e: Effective number of alleles. Heterozygous deficit indicated with positive F_{IS} values. Different letters indicate significant differences between columns ($P < 0.05$).

	WILD	GEN07	GEN09
Total No. of samples	55	77	73
Total No. of alleles	311	165	186
H_e	0.72 ± 0.04^a	0.57 ± 0.04^b	0.58 ± 0.04^b
H_o	0.44 ± 0.05	0.47 ± 0.05	0.45 ± 0.06
A_c	10.2 ± 0.86^a	5.1 ± 0.44^b	5.6 ± 0.47^b
A_u	4.8 ± 0.50^a	0.4 ± 0.11^b	0.5 ± 0.10^b
A_e	5.4 ± 0.63^a	2.8 ± 0.25^b	2.8 ± 0.22^b
F_{IS}	0.36 ± 0.06	0.20 ± 0.07	0.28 ± 0.08

Effective population size

Effective population size was calculated for GEN07 and GEN09 using pedigree data, and for all populations using microsatellite data (Table 3). No estimate was obtained for WILD with $N_{em(1)}$, because the run failed to reach convergence. Nor was it possible to obtain an estimate of $N_{em(3)}$, for GEN09, also due to convergence problems. Hence, an approach in which the data from GEN07 and GEN09 were analyzed together was used instead for obtaining an estimate of $N_{em(3)}$ for GEN09.

Linkage disequilibrium estimates for $N_{em(1)}$ and $N_{em(3)}$ were 65.9-103.5% larger than of N_{ef} for GEN07 and 118-150% larger than for GEN09 respectively. Conversely, $N_{em(2)}$, based on the Bayesian approach of Tallmon et al. (2008), was 50.6 and 38% smaller for GEN07 and GEN09 respectively (Table 3). According to the overlapping of the CL95 values, only the decline between GEN07 and GEN09 for $N_{em(1)}$ (37%) was significant. This estimate was close to the one for N_{ef} (41.2%). $N_{em(2)}$ and $N_{em(3)}$ estimates showed significant declines with respect to WILD in GEN07 (51.2 and 48.9% respectively) and GEN09 (64 and 54% respectively).

Genetic divergence and distance

Results for F_{ST}, R_{ST} and NeiD are shown in Table 4. F_{ST} values indicate that all the populations were significantly different from each other ($P < 0.05$). The divergences between GEN07 or GEN09 and

WILD were 4.4 to 4.7 times those found for GEN07 and GEN09 with F_{ST}; 2.7 to 4.0 times in the case of R_{ST}, and 6 times in the case of NeiD.

DISCUSSION

An average deficit of heterozygotes, indicated by positive F_{IS} values, was found in all populations. Surprisingly, F_{IS} was higher in the WILD population, which may be taken as an indication of a higher 'inbreeding coefficient' estimate for that population; however, other authors have also found inconsistent results using this statistic in *P. vannamei* (De Lima et al. 2008) and in different species of invertebrates (*e.g.* Bierne et al. 2000; Xu et al. 2001; Goyard et al. 2003; Dixon et al. 2008; Lind et al. 2009; Meng et al. 2009), including *P. vannamei* (Cruz et al. 2004; Pérez-Enríquez et al. 2009). The presence of null alleles could be a better explanation of these values (Xu et al. 2001; Ball and Chapman, 2003; Goyard et al. 2003). Attempts to infer inbreeding levels of shrimp populations using F_{IS} statistics may be therefore misleading, particularly when used with small numbers of loci and individuals. On the other hand, other studies showed that even when using as many as 200 markers loci, estimates of inbreeding coefficients have shown to be biased (Alves et al. 2008).

Table 3. Effective population size for single sample methods by population using microsatellite (N_{em}) and pedigree information (N_{ef}). Includes $N_{em(1)}$ = Microsatellite data, method by Waples and Do (2008), $N_{em(2)}$ = Microsatellite data, method by Tallmon et al. (2008), $N_{em(3)}$ = Microsatellite data, method by Peel et al. (2004), N_{ef} = Pedigree data, method based on average coancestry change.

	$N_{em(1)}$	CL95[a]		$N_{em(2)}$	CL95		$N_{em(3)}$	CL95		N_{ef}
WILD	[b]	-	-	86	64	154	272	219	358	-
GEN07	173	138	227	42	35	54	141	122	168	85
GEN09	109	94	129	31	26	42	125[c]	118	134	50

[a]CL95 = Ninety five per cent confidence limits.
[b]No results were obtained (infinite/not converged).
[c]Estimates for GEN09 did not converge, therefore the data form GEN07 and GEN09 were analyzed together.

The F_{IS} value in the WILD population (0.36) was smaller than the one reported by Valles-Jiménez et al. (2004), who obtained an F_{IS} value of 0.53 for a sample of wild *P. vannamei* in different locations of the Pacific Ocean from Panama to Mexico. These differences may be the result of sampling errors, or may be caused by differences in the population structure (*i.e.* Wahlund effect) expected in samples from wild populations of several groups of *P. vannamei*.

Previous studies in *P. vannamei* suggested there was no loss of genetic diversity when they weren't able to find differences in H_e between wild and cultured populations when using small numbers of microsatellite loci (Cruz et al. 2004); however, in other species, such as Atlantic salmon, Norris et al. (1999) found reductions in H_e when comparing cultured with wild populations using 15 microsatellite loci. We attribute the differences in the results to the number of loci evaluated. Several authors have proved that the number of loci is important when comparing populations; preliminary analysis (data not shown) indicate that at least 15 microsatellites and 50 individuals are required to obtain a standard deviation of $H_e \leq 0.03$ using microsatellite data typical for *P. vannamei*. Koskinen et al. (2004), using simulated data, showed that the standard deviation of the Nei distance (NeiD) decreased as the number of loci increased, changing from 0.25 when only 6 loci were used, to less than 0.05 when 17 loci were used, for a NeiD value of 0.3. In accordance with these findings, some authors such as Xu et al. (2001) suggest that allelic diversity indices are more sensitive in detecting changes on the population's structure than H_e values. Luikart et al. (2010) showed that H_e is less sensitive than allelic diversity when assessed soon after a bottleneck. This should be taken into account when drawing conclusions from this kind of studies, as the number of loci influences the kind of indices that should be used and the interpretation of the results.

Effective population size estimates based both on microsatellites and pedigree showed a decline between populations GEN07 and GEN09 (Table 3), even when non-significant statistical differences were found, but this trend was not observed for any of the other genetic variability indices (Table 2).

This suggests that N_e estimates obtained from a relatively large number of polymorphic DNA markers may be more sensitive to relatively small genetic changes in the true genetic variability of the population than the other genetic variability indices used in this study (Table 2).

Absolute N_e values using microsatellite information were different to pedigree estimates (Table 3). Nevertheless, the relative change in the pedigree estimate of N_e between populations followed the same general trend for all methods. Cervantes et al. (2011) also found differences in absolute estimated values of N_e, but similar trends between methods based on microsatellites. Therefore, microsatellite estimates of N_e may be more useful for monitoring the evolution of the genetic variability of the populations than for predicting actual effective population sizes. This is what can be expected from the properties of the methods (Wang, 2005). Similar to what was found in this study, Ne estimated from microsatellite data with different methods in a study with sheep were different (Álvarez et al. 2008).

Population subdivision statistics revealed differences between the three studied groups when using F_{ST} and R_{ST} (Table 4). Similar findings were reported by Xu et al. (2001), Dixon et al. (2008), Zhang et al. (2010) for F_{ST} in breeding populations of Penaeus, which indicated a decline in genetic variability over time, although only 6 microsatellite loci were used in the latter study. Results for NeiD were similar to those for F_{ST} and R_{ST}. This suggests that these statistics may distinguish smaller genetic differences between populations better than either allelic indices or H_e when a limited number of loci is available; however, in this study the divergence values found between WILD and GEN07 were slightly larger than the divergence values between WILD and GEN09, which again seems to indicate a better sensitivity of N_e.

Table 4. Paired divergences and Nei unbiased distance values between populations.

Populations		F_{ST}	R_{ST}	NeiD
WILD	GEN07	0.086*	0.044*	0.157
WILD	GEN09	0.080*	0.030*	0.155
GEN07	GEN09	0.020*	0.011*	0.026

*$P < 0.05$.

In our study, we were able to detect important reductions of genetic variability due to domestication in the cultured selected population, and some evidence of a further decline in genetic variability over time in the breeding population, although a slower one. The estimated size of the decline in genetic variability depended on the statistics used.

Our results also suggest that the initial reduction of population size while the population was being established and during initial selection process has more importance for the reduction of the genetic variability of the population than the selection between generations 7 and 9. This implies that most of the reduction in the genetic variability occurred during domestication, as happened in cattle (The Bovine HapMap Consortium, 2009), therefore, maintaining an N_e as large as possible during the initial generations of domestication has important implications for the long-term viability of a selective breeding program. It is likely that H_o and F_{IS} indices based on microsatellites are not useful to monitor changes in the genetic variability of shrimp populations due to the presence of null alleles.

The theory indicates that keeping a minimum effective population size of about 50 is a requirement for the maintenance of genetic variability in a closed breeding population (Meuwissen, 2009). The loss of genetic variability can be monitored using effective population sizes estimated from pedigree or from microsatellites. The methods for estimating effective population size based on linkage disequilibrium tended to give inflated values compared to estimates of coancestry obtained from pedigree, while the Talmon Bayesian method tended to slightly underestimate effective population sizes. DNA markers and pedigree information may be combined (*e.g.* Fernández et al. 2005) for the appropriate management of genetic variability on selected shrimp populations.

CONCLUDING REMARKS

We conclude that using microsatellites to assess the decline of genetic variability in breeding populations of *P. vannamei* is possible at the population level, but data from a single generation may not be informative enough, and absolute effective population sizes values are likely to be under or overestimated, even with the use of relatively large number of individuals and loci. Estimation of changes in effective population sizes was an efficient way to assess the decline in genetic variability in shrimp populations, superior to either expected heterozygosity, allelic diversity indices or genetic divergence measurements. Practical management of genetic variability and inbreeding in aquaculture nucleus populations at the individual level may still require the use of relationships estimated from pedigree; otherwise, these relationships should be calculated using SNP chips containing thousands of markers (Vignal et al. 2002).

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