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Cross amplification of *S. paramamosain* microsatellites for use in population structure studies of *S. serrata*

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Abstract: Microsatellites are established means for determining population structure of marine organisms. Development of these markers involve the study of the target organism's genome, determination of working primers for amplification, characterization of successfully amplified markers – all prior to the actual study of the organisms themselves. Primarily species-specific, microsatellites have been known to cross-amplify in closely related organisms and the testing of markers that have been used in these relatives can help save time and other resources.

Thirteen microsatellite markers used for population structure studies of *Scylla paramamosain,* the green mud crab, was tested for cross amplification in *S. serrata*, the king mud crab. Six out of the thirteen were tested and five were able to cross-amplify using Shuelke's nested method of amplification. Only two were consistently labelled with the fluorescent marker. Currently, the amplified sequences are being screened for null alleles, and Hardy-Weinberg equilibrium is being checked.

S. serrata is a highly profitable species due to its capacity to grow bigger and faster compared to other mud crab species. Culturing of mud crabs in the Philippines is still reliant on capturing juveniles in the wild and a proper understanding of its population structure Philippines would help in the development of better management systems for this marine resource.

Key Words: population structure; microsatellites; mud crabs

1. INTRODUCTION

Determination of the connectivity of various populations of an organism within a geographical location has helped in organizing sustainable community-resource management (Armitage, 2005). Following a co-evolutionary framework, it accounts

for socio-political trends affecting actual adaptive systems of the communities affecting the environment under study (Rammel et al, 2007).

Molecular markers have been in use for the determination of gene flow, organism movement, and population structure of various aquatic organisms that prove to be difficult to (Roberts, 1997; Ovenden et al, 1999; Ackiss et al, 2013). Short nucleotide sequences, or variable number tandem repeats (VNTRs), have been frequently used. High variability, the usefulness to detect mutation rate, its capacity to account for homoplasy, and eventually generate an accurate representation of genetic structure, makes the use of microsatellites, or short tandem repeats (STRs), a popular choice (Jarne and Lagoda, 1996; Estoup et al, 2002). The usefulness of microsatellites extend to the possibilities of having them cross-amplify across closely-related species, although this leads to an increased likelihood of having null alleles and a decreased number of successfully amplified loci.

Scylla serrata, the king mud crab, is decapod under Family Portunidae differentiated by the triangular shape of its four frontal lobes, and the presence of two dominant carpus spines (Keenan et al, 1998). It is widely distributed across the Indo-West Pacific (IWP) oceans, with the populations differentiated into two clades based on mitochondrial haplotype (Gopurenko et al, 1999). One clade was found to be widely distributed across the IWP, with the other found exclusively in Australia. Studies of populations within the Indian Ocean, and Australia, show distinct genetic differentiation; hinting at a reduced gene flow of the organisms living within geographically adjacent sites (Hill et al, 1982; Fratini and Vannini, 2002).

In the Philippines, *S. serrata* can increase the productivity of the mud crab industry due to its faster growth rates and bigger maximum sizes compared to the other *Scylla* species (Carpenter and Niem, 1998; Overton and Macintosh, 2002). The latest report from the Bureau of Fisheries and Aquatic Resources (Santos, 2000) show that mud crab harvest has already reached 4,495 tons that makes the country at par with international suppliers such as Indonesia, Taiwan, and China (FAO, 2011). An understanding of its population genetics would aid in the improvement of the management of this marine resource.

Thirteen polymorphic microsatellites have been used for the population structure studies of a closely related species, *S. paramamosain*. Successful cross-amplification of the given loci in *S. serrata* can help save time and resources given to marker development. After amplification, the mode of inheritance of the marker should ideally follow Mendelian inheritance while remaining selectively neutral, and maintain ample variability (Slatkin, 1995).

2. METHODOLOGY

Sampling

Fifty mud crabs were acquired from Dagupan, Pangasinan. Samples were from a grow-out pond, and were initially captured as juveniles at the Dagupan Bay. Species identification was done through the observation of the shape of the six frontal lobes (Fig. 1), and the presence of the two distinct carpal spines.

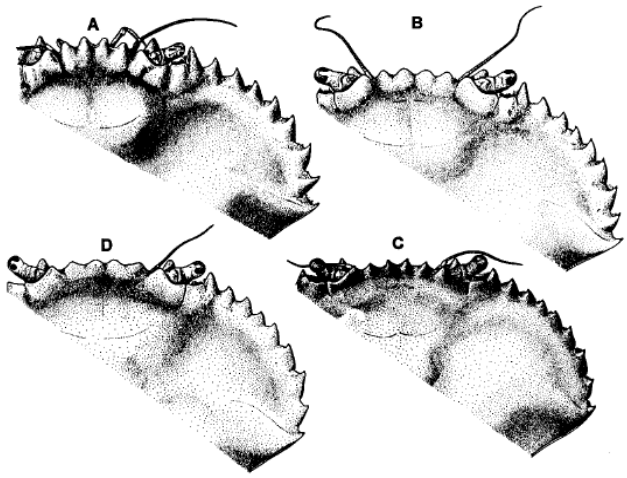

Figure 1. Frontal lobe shape, as a means to determine *Scylla* species identity. A – triangular – *S. serrata*, B – rounded – *S. tranquebarica*, C – blunted – *S. olivacea*, D – pointed – *S. paramamosain* (Keenan et al 1998).

DNA Extraction

DNA extraction was done on muscle tissue using 10% Chelex after a 3-hour incubation with Proteinase K. at 65°C. Samples were heated to 90°C for 20 minutes in two intervals separated by a 15-minute incubation at -20°C.

PCR Amplification

PCR Amplification of the microsatellite loci followed the modified method of Shuelke (2000), making use of fluorescent markers attached to an M13 universal sequence. The primers were modified to attach to fluorescent markers (Table 1), through nested PCR runs.

Table 1. Sample modification of the forward primer used for microsatellite amplification.

Loci	Modified Forward Primer	Reverse Primer
Scse 15-1	5' - TGT AAA ACG ACG GCC AGT TCT CCC TTC CTG ACT ACT – 3'	5' AAA CTT TGT CCT GCC ATC 3'
Scse 43-1	5' - TGT AAA ACG ACG GCC AGT GAA ATC TGA GCT GCC AAT C – 3'	5' CAC CCA TCC AAG TAC CAA 3'
Scse 53-1	5' - TGT AAA ACG ACG GCC AGT CCG TCA CTT CAC AGT ATA – 3'	5' GTT TTC ATT TGA GTT TCC 3'
Scse 109-1	5' - TGT AAA ACG ACG GCC AGT AAT AGC CAT ACT GGA AGC – 3'	5' AAT CAG ACC AAG GAG GTT 3'
Scse 96-1	5' - TGT AAA ACG ACG GCC AGT CTT CCT CAC CGT CCC TAT – 3'	5' CTCTGTTGCCTAATTC CTC 3'
Scse 97-1	5' - TGT AAA ACG ACG GCC AGT AAA AGC AGT TCG TTG TTA – 3'	5' TAG ACT GGT GGA AGG ATG 3'
Scse 118-1	5' - TGT AAA ACG ACG GCC AGT CCT AAT CCA ATC CAA CCT – 3'	5' TCT CCC CAC ATT CTC ATA 3'
Scse 72-2	5' - TGT AAA ACG ACG GCC AGT GGT CCA AAT CGA ATG TCC – 3'	5' ATA AGC CAA GGT TCT ACT C 3'
Scse 85-1	5' - TGT AAA ACG ACG GCC AGT AAA CAG ATT GGC GTC CTC – 3'	5' CCA CGA TTT ACC GAG AAG 3'
Scse 99-1	5' - TGT AAA ACG ACG GCC AGT ATT CAG CGG GAA TGG GAT G – 3'	5' ACG AGC CAC AGC AAG AGC C 3'
Scse 101-1	5' - TGT AAA ACG ACG GCC AGT GTA TTT TGC CTG TCT GCC – 3'	5' ACC GCT ATT ATC CTC CAC 3'

A 10 uL PCR mix was prepared with an initial recipe of 0.2 mM dNTPs, 1 uL template DNA, 1 U Kappa Taq, 0.5 uM of forward primer, and 0.5 uM of the reverse primer, with 1X Kappa MgCl- free buffer. The initial conditions for PCR were 94°C for 5 minutes, 30 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 1 minute, and a final extension of 72°C for 10 minutes. Table 2 shows the corresponding tag for each microsatellite loci used.

Table 2. Fluorescent label used for microsatellite loci used.

Fluorescent Tag	Microsatellite Loci
FAM	Scse 15-1
	Scse 118-1
VIC	Scse 43-1
	Scse 53-1
	Scse 72-2
PET	Scse 96-1
	Scse 97-1
	Scse 101-1
NED	Scse 109-1
	Scse 85-1
	Scse 99-1

Negative Control

A negative control was used for both sets of PCR optimization. The control had distilled deionized water as replacement for the corresponding volume of template DNA.

Agarose Gel Electrophoresis

PCR products were run in 3% agarose for 25 minutes at 100 V.

3. RESULTS AND DISCUSSION

Identification of S. serrata

Initial identification using the frontal median lobe shapes and the carpus spines indicate 24 out of the 50 samples were *S. serrata* (Table 3).

Difficulties were encountered due to unexpected morphological characteristics in the samples. Some had damaged frontal spines while others have inconsistencies in the number of carpus spines in the left and right primary chelipeds. These observations have been reported in relation to bioaccumulation of heavy metals (Krishnaja et al, 1987; Kamaruzzaman et al, 2012) and in hybridization of the four species (Imai, 2005).

Table 3. Samples from Dagupan, Pangasinan identified as *S. serrata.*

S. serrata samples based on morphological features		
MI 00028	MI 00042	MI 00060
MI 00029	MI 00047	MI 00066
MI 00030	MI 00049	MI 00067
MI 00031	MI 00051	MI 00068
MI 00032	MI 00054	MI 00070
MI 00033	MI 00055	MI 00071
MI 00038	MI 00058	MI 00075

MI 00040	MI 00059	MI 00078

PCR Conditions

Initial PCR conditions did not result to successful amplification. An increase in the $MgCl_2$ concentrations, and the forward primer concentrations (Table 4) were done that resulted to consistent amplification of five out of the thirteen possible microsatellite loci. No adjustments in the PCR conditions were done so far.

Table 4. Resultant PCR Master mix concentration.

Component	Concentration
PCR Buffer	1x
$MgCl_2$	0.7 mM
dNTPs	0.2 mM
Forward primer	0.05 uM
Reverse primer	0.2 uM
Fluorescent tag	0.2 uM
Template DNA	1 uL
Taq polymerase	1 U
Total volume	**10 uL**

The sensitivity of PCR to the concentrations of its various components is often the cause for most reactions to fail. Myriad of possible adjustments can be done such as the adjustment of $MgCl_2$ concentrations to increase the affinity of primers to anneal to the template DNA, adjustment of the volume of Taq polymerase, and the improvement of the DNA template quality (Palumbi et al 1991). Adjustments in PCR conditions are also viable option. Annealing time or temperature for the primers are changed, though this may result to a decrease in specificity.

In the study, the concentration of the template DNA posed the problem. DNA concentration after extraction could not be determined using spectrophotometry due to the nature of the method used. Chelex does not require the separation of the extracted DNA from the substrate preventing accurate measurement of absorbance since the solution will not contain pure DNA (Walsh et al 1991).

In order to adjust DNA concentration for the PCR mix, the volume of the added template is changed and diluted to 1 uL. Successful amplification was observed when 0.25 uL of template DNA was used. A high concentration of template DNA has been noted to decrease efficiency in PCR since excess template end up blocking the action of the polymerase, preventing successful amplification (Kramer and Coen 2001). Another possibility is the presence of Chelex additive in the extract that interferes with the PCR. Dilution would decrease the concentration of these substances enabling the reactions to proceed. Speedy DNA extraction methods resulting pure extracts are being tested.

Amplified loci

Currently, 5 out of the thirteen possible microsatellite loci have been successfully amplified. These are Scse 15-1, Scse 85-1, Scse 43-1, Scse 96-1, and Scse 118-1. Clear bands have been observed in the electrophoretic gels run after PCR of both sets of samples (Fig. 2).

Increase in annealing time has been attempted on Scse 53-1 but it has failed to amplify, excluding it from the remaining microsatellites to be tested.

4. CONCLUSIONS

Five of the thirteen markers show potential for use in the population structure studies in *S. serrata*. Current trends in the use of microsatellite markers for such kinds of study require at least 10 markers for the study to be publishable. If five more markers are successfully amplified then the need for marker development is reduced. If none of the markers are amplified, microsatellite marker studies might be needed or the use of other VNTRs, such as SNPs, may also be considered. This would require, however, a whole genome or transcriptome assembly of the organism.

Fluorescent screening of the available PCR products will determine if the poor man's method of labelling has been successful. A larger sample size would be required for use in population genetics studies.

Errors in species identification due to malformations, and unexpected morphological methods, may require molecular methods of species identification prior to inclusion in population genetics studies.

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