SHORT COMMUNICATION

Development of microsatellite markers for genetic diversity analysis in *Glossogobius giuris* (Hamilton, 1822) and *Rhinogobius giurinus* (Rutter, 1897)

Abriel S. Bulasag*[1], Nathalie T. del Agua[2], Aprill S. Punongbayan[2], and Maria Genaleen Q. Diaz[2]

[1]UP Rural High School, UP Los Baños, College, Laguna
[2]Institute of Biological Sciences, UP Los Baños, College, Laguna

Abstract—Microsatellite-enriched libraries were constructed from *Glossogobius giuris* and *Rhinogobius giurinus*. BLAST analysis revealed significant homology of the clones with microsatellite-rich regions in other teleosts and other members of Gobiidae. Eight microsatellite primers were designed and tested for cross-amplification in two other goby species, *Glossogobius celebius* and *Gobiopterus lacustris*. Four primer pairs amplified putative microsatellite loci in all four species. Four other primer pairs amplified loci in at least two species. Primer Rh18 was the most informative and exhibited the greatest number of polymorphic alleles (PIC=0.7262; N_a=7). Two other primers, Rh51 and Gi47 were also informative markers with PIC values ≥ 0.5. Using five primers, both *G. giuris* and *R. giurinus* samples exhibited moderately high values of genetic diversity based on the number of alleles (~5), percent polymorphism (53%), and observed (61%) and expected (58%) heterozygosities.

Keywords—microsatellites, genetic diversity, subtractive hybridization, *Glossogobius giuris* Hamilton, *Rhinogobius giurinus* Rutter, Gobiidae

INTRODUCTION

Family Gobiidae is one of the largest fish families, with approximately 2,000 species and 200 genera (Froese & Pauly, 2008). Though only few species are commercially important, they play a very important role as prey species for commercially important fishes like sea bass. Some gobies have potential as aquarium fishes. They may be keystone species (having disproportionate importance) in freshwater environments in small oceanic islands because of their prevalence in these habitats (Allen & Robertson, 1994; Helfman et al., 1997).

The diversity in form is a hint of the tremendous genetic variability that exists within the family. Most gobies are adapted to marine environments and are abundant in brackish water and estuarines (lower reaches of rivers, mangrove swamps, and salt marshes). Of 315 Philippine freshwater goby species listed in FishBase, 70 are native, while 16 can only be found in the Philippines (Froese & Pauly, 2010). These figures further present the significance of gobies in Philippine ecosystems. Despite this, there is a dearth of researches about their nature of diversity, genetics and population genetic structure. These parameters are very important in understanding population dynamics, ecology, as well as for conservation studies.

Protection of biodiversity is anticipated to be both crucial and ongoing in the 21st century (Oliveira et al., 2006). Conservation genetics has been given primary importance for avoiding extinction of endangered species alongside the political, economic and ecological aspects of biodiversity protection. With the advent of molecular approaches, the examination of the genetics of species in danger of extinction proves to be a useful tool in conservation research. The impact on genetic diversity is quite critical, as genetic variation is needed for species adaptation and taxa speciation (Hughes et al., 1997). DNA analyses promote increased knowledge on the genetic structure of fish species and their response to environmental changes (Piorski et al., 2008). Several molecular tools have been used to assess genetic variation (Strecker et al., 2003; Barroso et al., 2005), determine population genetic structure (Hatanaka & Galetti, 2003; Spruell et al., 2003) and gene flow among animal species (Mallet, 2005). Traditional molecular markers have, in general, provided insufficient statistical power and accuracy for estimating genetic differences (Oliveira et al., 2006). However, with the discovery of highly variable loci such as microsatellites or simple sequence repeats (SSRs), the statistical power for determining differentiation between species groups at risk of extinction is now usually very high (Hedrick, 2001).

Microsatellite markers are dubbed as the most versatile molecular markers with various applications in population genetics, conservation biology, and evolutionary biology (Abdul-Muneer, 2014). Microsatellite data sets are important starting points for studies involving parentage analysis, proper identification, and phylogeographic studies. They provide answers to genetic questions that were previously viewed as dead blocks and present insights as to how genetic variation is partitioned among populations, giving reliable estimates of population differentiation that are crucial to understand the connectivity among populations and represent important tools to develop conservation strategies (Balloux & Moulin, 2002). They are the most preferred PCR-based tool for fish population genetic studies (Beckman & Soller, 1990; Shabani et al., 2013) and have been

*Corresponding Author
Email Address: asbulasag@up.edu.ph
Submitted: March 24, 2015
Revised: May 14, 2015
Accepted: May 19, 2015
Published: July 15, 2015

identified as robust and valuable tools in fish genetics (Wright & Benzen, 1994). In the absence of full genome sequences, the most preferred technique is to produce highly enriched microsatellite libraries first described by Edwards et al. (1996). In addition, cross-amplification among related taxa has been found to be commonplace especially among several fish species (Leclerc et al., 2000; Cairney et al., 2000; Vyskocilova et al., 2007).

This study isolated, characterized, and developed microsatellite primers from *Glossogobius giuris* and *Rhinogobius giurinus*, two goby species that are abundant in the Southern Tagalog, Philippines. The developed primers were used for cross species amplification and genetic diversity analysis.

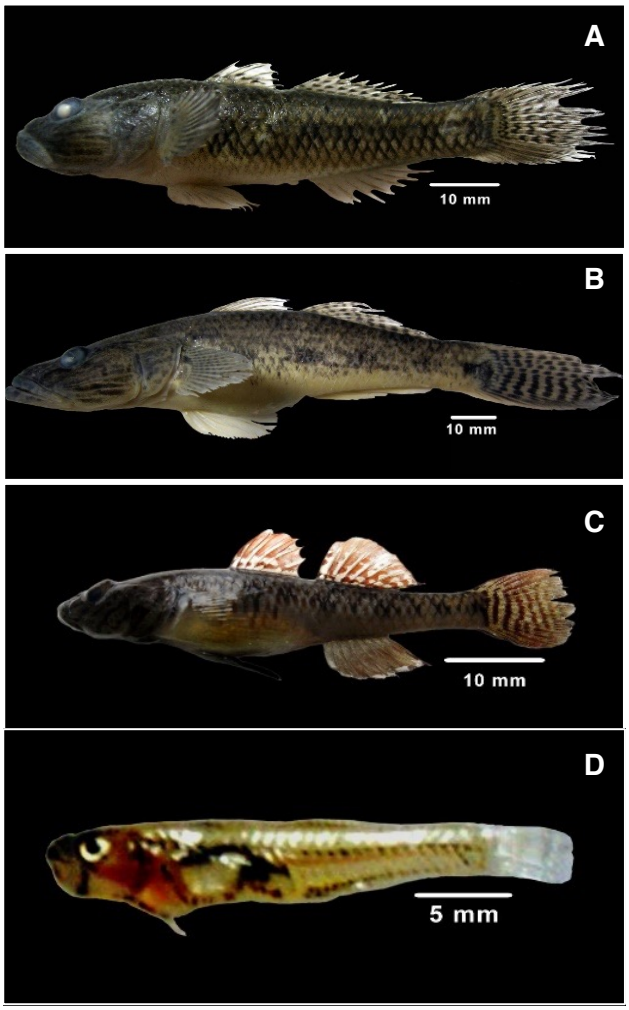

Figure 1. Fish specimens used in the study: A. *Glossogobius celebius* (Valenciennes, 1837); B. *Glossogobius giuris* (Hamilton, 1822); C. *Rhinogobius giurinus* (Rutter, 1897); D. *Gobiopterus lacustris* (Herre, 1927) (Photograph by Labatos, 2013).

MATERIALS AND METHODS

Samples and DNA Extraction

R. giurinus and *G. giuris* fish samples were collected from the UPLB Limnological Station and Dampalit River in Los Baños, Laguna and Lake Ticob in Quezon Province. DNA used in library construction was isolated from pooled fish fin samples using DNeasy Blood and Tissue Kit (Qiagen, Germany). For species cross-amplification, two other species were collected (*Gobiopterus lacustris* and *G. celebius*) (Figure 1) and their DNA extracted. For assessment of genetic diversity, thirty-three (33) *R. giurinus* and thirty (30) *G. giuris* individuals were collected. DNA extraction was done from fins and muscles following a standard phenol-chloroform extraction protocol (Wasko et al., 2003). DNA quality and quantity were checked using electrophoretic and spectrophotometric (Nanodrop®) techniques.

Microsatellite Enrichment and Library Screening

*Rsa*I-digested genomic DNA (approx. 10µg) was ligated with 20 µM oligonucleotide linkers (Linker1: 5'- GTTTAGCCTTGTAGCAGAAGC -3', Linker2: 5'- p GCTTCTGCTACAAGGCTAAACAAAA-3') using supplier prescribed conditions (Invitrogen, USA). PCR was performed using primers specific for the linkers. To confirm ligation of linkers to the genomic DNA, PCR was performed in a volume of 20 µL containing 2 µL ligated DNA, 2 µL 10x PCR buffer, 1.6 µL dNTPs (10mM), 1.6 µL of linker (Rsa 21), 0.5 U of *Taq* polymerase in a thermal cycler as follows: 1 cycle for 5 min at 94 ^{0}C, 30 cycles for 30 sec at 94^{0}C, 1 min at 60^{0}C, 1 min at 72^{0}C, and final extension for 7 min at 72^{0}C. Ligation was indicated by the observed smearing at 300-1500 bp in agarose gel (100V for 2 hours). A microsatellite enrichment protocol developed by Glenn & Schable (2005) was used to fish out SSR-rich regions in the genomes of *G. giuris* and *Rhinogobius giurinus* through subtractive hybridization. Repeat-enriched genomic DNA was amplified in 50 µL reaction containing 10 µL repeat-enriched DNA, 5 µL 10x PCR Buffer S (Vivantis, Malaysia), 4 µL dNTPs (10 mM), 4 µL of Rsa21 (10 µM), 0.3 µL of *Taq* pol (5U/µL), and 26.7 µL sterile distilled water was placed in a thermal cycler as follows: 1 cycle for 5 min at 94^{0}C, 30 cycles for 30 sec at 94^{0}C, 1 min at 60^{0}C, 1 min at 68^{0}C, and a final extension for 7 min at 68^{0}C. To avoid high redundancy in the library, the PCR reaction was performed with 20 to 25 cycles. The microsatellite PCR products were cleaned using Qiaquick Purification Kit (Qiagen, Germany). The purified PCR product was ligated to pGEM T-Easy Vector (Promega, USA) and transformed into *E. coli* JM109 competent cells (Promega, USA) according to manufacturer's specifications. Isolated plasmids from 17 different transformants for each species were sent to Macrogen, Inc. (Korea) for sequencing.

Microsatellite Primer Design

The quality of sequences were first analyzed using ChromasPro 2.1 software (Technelysium Pty Ltd, Tewantin QLD, Australia). The presence of specific SSR repeat motifs were analyzed using Microsatellite Finder program, available online (http://biophp.org/minitools/microsatellite_repeats_finder/demo.php). Eight primer pairs were designed using Primer3-BLAST Software Package (Rozen & Staletsky, 2000). The primers were sent to Invitrogen for synthesis. Table 1 shows the designed primers including standard parameters.

Genetic Diversity Assessment and Primer Data Analysis

PCR profile for touchdown protocol was used to enhance the success of the amplifications based on Ghiasi et al. (2009) with minor variations in melting and annealing temperatures. The touchdown PCR profile consists of the following steps: initial denaturation at 94˚C for 5 min; 20 cycles of 94˚C denaturation for 30 sec, annealing with 0.5˚C decrement in temperature every cycle (a 10˚C range) for 45 sec, and extension at 72˚C for 2 min; a final extension at 72˚C for 5 min. Polyacrylamide Gel Elecrophoresis (PAGE) was then used to genotype PCR profiles of the *G. giuris* and *R. giurinus*. Polymorphism Information Content (PIC), a measure of polymorphism for a marker locus used in linkage analysis, was computed for each of the primers used, as well as other parameters like: number of alleles, allele size range, expected product size, number of polymorphic alleles, and observed (H_o) and expected (H_e) heterozygosities. Parameters were computed using Powermarker Version 3.0 devised by Jack Liu from North Carolina State University.

TABLE 1. Characteristics of eight (8) SSR primer pairs based on microsatellite-enriched libraries of *Glossogobius giuris* and *Rhinogobius giurinus* used in species cross-amplification and genetic diversity analysis.

PRIMER	SEQUENCE (5'TO 3')	REPEAT TYPE	LENGTH (bp)	EXPECTED PRODUCT SIZE (bp)	MELTING TEMP/T_m (^{0}C)	ANNEALING TEMP/T_a (^{0}C)
Gi21-F	AGTGCATACCGACGCCAGGAC	$(GA)_{19}$	21	339	58.93	56
Gi21-R	CGGATGATGCGGGCGTTTG		20		59.11	
Gi47-F	CTGTCTCTCTCCCGTTGCGCACG	$(CA)_{13};(AT)_4$	23	76	61.88	54
Gi47-R	AGCCGCTAGCCGAACAGAGC		20		59.22	
Gi51-F	CCAGCTACGAAGAGAAGAG	$(TG)_{27}$	19	123	48.55	45
Gi51-R	CTCTTTAGAAGGAAAACGC		19		46.00	
Rh4-F	AGCCGGAGTCTGGGTAGTCC	$(GA)_{12}$	20	343	56.91	54
Rh4-R	GCTCAAAGGGCAACCACCAAC		21		56.85	
Rh18-F	ATACCGCTGCACACACACAC	$(CA)_5;(CA)_{15}$	20	194	55.51	52
Rh18-R	GATACGTCAGGGCGTGAGAT		20		55.58	
Rh37-F	CAGGAATTATCACTCCATCC	$(TGC)_3;$ $(GA)_{21}$	20	314	47.08	45
Rh37-R	ACAGTATGATCATGTCGAC		19		46.11	
Rh23-F	ACTGATCATGTGACAGCAGCTG	$(AT)_3;(CA)_6$	22	263	55.09	51
Rh23-R	TGGATACAATACGTTGCCGAC		22		55.79	
Rh51-F	TGCCAGAAGCCCTCTCATTG	$(GT)_{12}$	20	140	55.04	53
Rh51-R	TCACCACGATAATGAAACAGCC		22		53.18	

RESULTS AND DISCUSSION

Isolated Microsatellite Loci and Species Cross-amplification

Seventeen sequences (100%) from *G. giuris* and 14 of 17 (82.4%) from *Rhinogobius giurinus* contained microsatellites. There were more GA/CT clones (58.8%) than CA/GT (41.2%) clones despite previous reports (Stallings et al., 1991) stating CA/GT repeats are more common among animals. Since only a fraction of the total library clones were screened, there is still a possibility that the other clones contain different motifs.

Almost all sequences had BLAST hits with microsatellites from the model zebrafish (*Danio rerio*) genome, with maximum identity of ≥75% (Table 2). There were also a great number of BLAST hits with microsatellite sequences from carp (*Cyprinus carpio*) and other teleosts such as perch (*Perca schrenkii*), salmon (*Salmo salar*), and Chinese perch (*Siniperca chuatsi*). These suggest the robustness of the procedure employed in selecting microsatellite-containing DNA fragments and microsatellites' ubiquity among teleosts. Rico et al. (1996) provided evidence that sequence homology of the flanking regions of microsatellites are quite conserved in fishes and that this might be due to the low rate of base substitution observed in aquatic organisms compared to terrestrial animals. This was further corroborated by the observations on the family Cyprinidae by Zardoya et al. (1996).

TABLE 2. BLAST hits of the different microsatellite sequences from different teleosts in the NCBI GenBank.

Microsatellite	NCBI Accession (Gene ID)	Organism	Max. Identity
Gi47	JN757775.1	*Cyprinus carpio* clone 650568 microsatellite sequence	87%
	CR388371.8	Zebrafish DNA sequence from clone DKEY-78N12	96%
Gi52	CR396586.8	Zebrafish DNA sequence from clone DKEY-184A18	99%
	Y08605.1	*S.salar* DNA segment containing GT repeat, 348bp	97%
	JN765143.1	*Cyprinus carpio* clone 730033 microsatellite sequence	92%
	DQ487866.1	*Siniperca chuatsi* clone Sc31 microsatellite sequence	93%
Rh18	EU780049.1	*Perca schrenkii* clone Y01 microsatellite sequence	91%
	JN765601.1	*Cyprinus carpio* clone 735479 microsatellite sequence	93%
	AF470013.1	*Oncorhynchus mykiss* microsatellite OMM1233 sequence	90%
Rh23	FP085435.5	Zebrafish DNA sequence from clone CH73-228D8	70%
	JN784454.1	*Cyprinus carpio* clone 965050 microsatellite sequence	92%
Rh51	JN761082.1	*Cyprinus carpio* clone 681606 microsatellite sequence	94%
	CU861887.12	Zebrafish DNA sequence from clone CH211-91M11	94%
	AY543923.1	*Salmo salar* clone Rsa225 microsatellite sequence	93%

The eight primer pairs successfully amplified the microsatellite-containing loci in the DNA samples from four species (*G. celebius, G. giuris, Gobiopterus lacustris*, and *Rhinogobius giurinus*). In some interspecies amplifications, PCR products were not uniform in size, suggesting variation in the number of tandem repeats in the DNA template.

Results from cross-amplification implies that there is diversity in the characteristic length of allelic forms of microsatellite loci. Table 3 summarizes the results of cross-amplification across the species. Inter-specific cross-amplification of DNA segments may provide a more cost-effective approach in population genetics. Cross-species amplification, which was evident in this study, is an indication that the primer sequences used are greatly conserved across the family as suggested by Moore et al. (1991) and Peakall et al. (1998) and that they may be quite useful in investigating other goby species' population genetic structure.

Half of the primers (Gi47, Rh23, Rh4, and Rh18) were successful in amplifying DNA fragments across all four species used. This suggests that these primer sequences are effectively conserved across the four species. Two primer pairs (Rh51 and Gi51) amplified DNA fragments in three out of four species.

While two other primer pairs (Rh37 and Gi21) were successful in amplifying DNA fragments for two out of four species. Gi21, on the other hand, amplified DNA fragments only for the two *Glossogobius* species. As expected, all eight primers amplified microsatellite DNA of the respective source organisms, *G. giuris* and *R. giurinus*. The primers were effective in amplifying not only the source DNA but also DNA from close relatives. This is in support of previous studies on the effectiveness of heterologous primers across species in the same family (Larmuseau et al., 2007; Lin et al., 2007; Yaraguntappa et al., 2007).

TABLE 3. Cross-amplification of microsatellite-containing loci across four species of Family Gobiidae.

PRIMER	SPECIES			
	G. celebius	*G. giuris*	*Gobiopterus lacustris*	*Rhinogobius giurinus*
Gi21	+	+	-	-
Gi51	-	+	+	+
Gi47	+	+	+	+
Rh23	+	+	+	+
Rh4	+	+	+	+
Rh18	+	+	+	+
Rh37	-	+	-	+
Rh51	+	+	-	+

Genetic Diversity Assessment

Table 4 summarizes the result of the diversity analysis on *G. giuris* based on five microsatellite loci. Amplification with primers Gi51 and Gi47 exhibited SSR loci with lowest numbers of alleles observed (N_a=2) and correspondingly had lowest PIC values (0.0905 and 0.3610, respectively). These two were the least informative or not at all informative among the five primers used. In contrast, Rh18, which produced seven polymorphic alleles, had the highest PIC value of 0.7981. Two primers, Rh18 and Rh51 are considerably informative (PIC≥0.5) markers for the *G. giuris* population. Deviations from expected heterozygosities may indicate important population dynamics. Based on this premise, three markers (Rh18, Gi47, and Rh51) provide promising insight into the diversity that exist within the *G. giuris* population. The mean observed heterozygosity approximates the value of the expected heterozygosity. Moreover, heterozygosity values are greater than 0.5 suggesting a high value of genetic diversity. For the loci considered, genetic diversity is quite evident in the *G. giuris* population.

TABLE 4. Genetic diversity analysis of *Glossogobius giuris* population using the following parameters: number of alleles (N_a), allele size range, no. of polymorphic alleles, polymporphism information content (PIC), and observed (H_o) and expected (H_e) heterozygosities.

PRIMER	N_a	ALLELE SIZE (bp)		EXPECTED SIZE RANGE (bp)	NO. OF POLYMORPHIC ALLELES	PIC	H_o/H_e
		Min	Max				
Gi47	2	60	120	70-100	2	0.3610	0.77/0.47
Gi51	2	108	124	100-140	2	0.0905	0.10/0.10
Rh18	7	108	220	160-200	7	0.7981	0.57/0.82
Rh23	3	250	280	250-280	3	0.4345	0.50/0.52
Rh51	5	110	190	120-160	5	0.6011	0.83/0.66
Total	19				19		
Mean	3.8					0.4570	0.55/0.51

Table 5 summarizes the results for the genetic diversity analysis on *R. giurinus*. Number of alleles ranged from two to five. Rh51 was the most informative marker (PIC=0.6987), followed by Gi47 (PIC=0.6345). Rh23 and Gi51 were the least informative having PIC values lower than 0.4. This may be in part due to the low number of polymorphic alleles observed in *Rhinogobius* population using these two markers. However mean PIC still approximates the value 0.5, which means the markers are still informative and hence may be used in diversity studies. Mean observed heterozygosity is slightly higher than expected and may be attributed to inherent variability of the population. A high level of average heterozygosity in terms of the observed heterozygosity is expected to correlate with high genetic variation at loci which are important for adaptive response to environmental stresses (Kotze & Muller, 1994; Lieutenant-Gosselin & Bernatchez, 2006). Hence, this would strengthen the effectivity of the markers

developed for future genetic diversity studies not only of important freshwater goby species but of other important endemic and native freshwater species.

TABLE 5. Genetic diversity analysis of *Rhinogobius giurinus* population using the following parameters: number of alleles (N_a), allele size range, no. of polymorphic alleles, polymporphism information content (PIC), and observed (H_o) and expected (H_e) heterozygosities.

PRIMER	N_a	ALLELE SIZE (bp)		EXPECTED SIZE RANGE(bp)	NO. OF POLYMORPHIC ALLELES	PIC	H_o/H_e
		Min	Max				
Gi47	4	60	120	70-100	4	0.6245	0.79/0.68
Gi51	2	108	124	100-140	2	0.3713	0.70/0.49
Rh18	3	108	220	160-200	3	0.4442	0.70/0.51
Rh23	2	210	280	250-280	2	0.2078	0.27/0.24
Rh51	5	110	190	120-160	5	0.6987	0.88/0.74
Total	16				16		
Mean	3.2					0.4693	0.67/0.53

CONCLUSION

Microsatellite loci can be effectively isolated from Philippine freshwater fish species through microsatellite-enrichment protocols. SSR primers can be designed and utilized in future investigations on genetic diversity of native and endemic Philippine freshwater goby species. They can also be potentially utilized for other applications such as studies on parentage analysis, phylogenetic relationships, breeding, and others.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Pablo P. Ocampo of the UPLB Limnological Station, the program leader of the Fish Ark Program which provides direction for the conservation of endemic and native Philippine freshwater fishes, and DOST-PCAMRD, for funding the research.

CONFLICT OF INTEREST

The authors report no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

ASB drafted the manuscript. ASB, ASP and NTDA conducted all experiments. ASB and ASP participated in the field collection of the fish specimens. MGQD conceptualized the research and helped write the manuscript.

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