ARTICLE

Genetic diversity within and among natural populations of five gobies based on cytogenetic and isozyme analyses

Riaflor M. Alcabedos*[1], Celia B. Dela Viña[1], Roberto C. Reyes[2], and Rita P. Laude[1]

[1]Genetics and Molecular Biology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, Philippines
[2]Animal Biology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, Philippines

Cytogenetic profiles of five native freshwater gobies from Southern Luzon, Philippines namely, *Glossogobius celebius* (Velenciennes, 1837), *Glossogobius giuris* (Hamilton, 1822), *Redigobius bikolanus* (Herre, 1927), *Redigobius dispar* (Peters, 1868), and *Giuris margaritacea* (Valenciennes, 1837) were studied by using basic cytogenetic analysis. *G. celebius* chromosomes are all telocentric, with a diploid chromosome number of 2n = 50 (FN = 50). Both *G. margaritacea* and *G. giuris* have telocentric chromosomes, diploid chromosome number of 2n = 46, and FN = 46. *R. bikolanus* and *R. dispar* both have metacentric (m), submetacentric (sm), and telocentric (t) chromosomes although they have different diploid numbers: *R. bikolanus* has 2n = 40 (10m + 28sm + 2t, FN = 78) while *R. dispar* has a modal chromosome number of 2n = 44 (24m + 16 sm + 4t, FN = 84). Based on the computed relative lengths of the chromosomes, *G. celebius,* and *R. bikolanus* have slightly asymmetrical karyotypes, while *G. giuris* and *R. dispar* have symmetrical karyotypes. Cytotaxonomically, the results suggest that *G. celebius* and *R. dispar* might be more ancestral than *G. giuris* and *R. bikolanus*, respectively. Genetic variation within and among populations was also analyzed by isozyme electrophoresis using seven enzymes and two proteins. Percent (%) polymorphism (20.00–64.71), heterozygosity (0.05–0.50), and average number of alleles (1.23–1.65) indicating high variation of the individuals within the population, were observed among individuals of *G. celebius* from Social Garden Molawin Creek, *G. giuris* from Lake Bato, *R. dispar* from Bulusan Lake, and *R. bikolanus* and *Giuris margaritacea* from Pansipit River. The dendrogram based on estimates of genetic identity (0.77–0.86) and distance (0.15–0.26) showed that *G. celebius* populations from Laguna, *G. giuris* populations from Lake Bato and Talisay, and *R. dispar* populations from Bulusan and Manapao Lakes, were most related.

*Corresponding author
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INTRODUCTION

The Philippines is home to 361 freshwater fish species, with 181 native and endemic species (Froese and Pauly 2017). Out of these 361 species, 96 species are considered endangered or vulnerable to overfishing, and 83 have aquaculture potential (Froese and Pauly 2017). Of the endemic Philippine freshwater species, International Union for Conservation of Nature (IUCN) (2016) listed 1 species as critically endangered and 8 species being vulnerable. The country also has 56 secondary freshwater species which are now confined to freshwater (Mercene 1997).

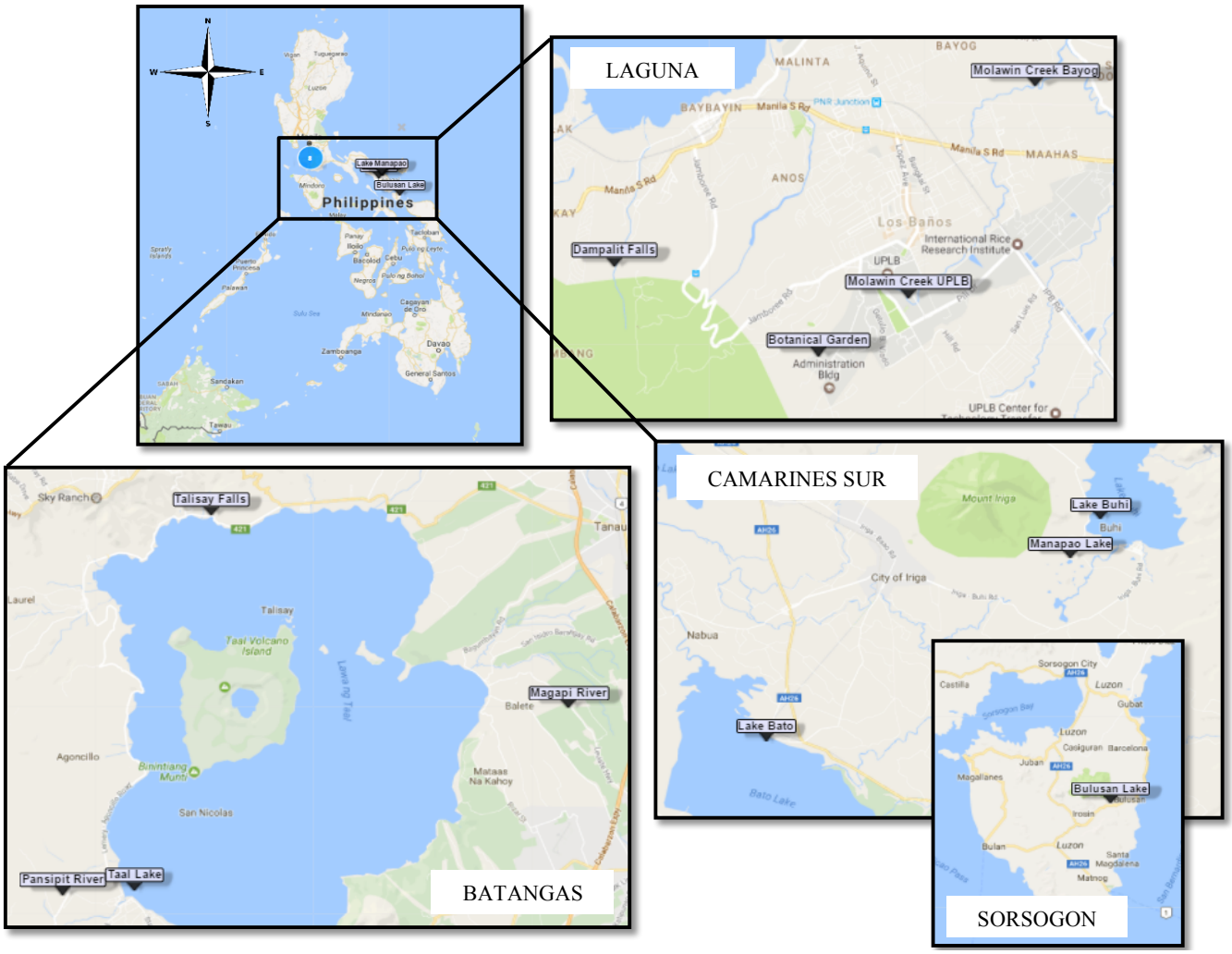


Figure 1: Twelve collection sites of five native Philippine freshwater gobies in Southern Luzon, Philippines.

Family Gobiidae are often the most abundant fish in the oceanic islands. Currently, there are 1,767 species classified under 258 genera (Froese and Pauly 2017), making them the most species-rich family of vertebrates. The smallest fishes in the world belong to this family. Gobiids are poorly known due to their cryptic and secretive nature; thus 10 to 20 new species are described every year (Nelson 1994). They occupy a wide range of habitats from freshwater to estuarine to marine environments, although many species are confined to a single lake or river system or one or a few islands. A number of gobies have been successfully bred in captivity and are used for food, and some are popular in the aquarium trade (Allen and Robertson 1994; Hoese 1998). According to Hoese (1998) and IUCN (2016), there are 5 critically endangered gobies, 18 are vulnerable, and 12 are low-risk. Agricultural practices and introduction of nonnative fishes are some of the causes of their decline.

Family Eleotridae, commonly known as sleepers or gudgeons, contains about 34 genera and 179 species (Froese and Pauly 2017). A majority of eleotrids live in brackish or freshwater while only a few species are truly marine (Nelson 1994). They are used for food in many regions (Wheeler 1985). According to the 1994 information gathered by IUCN (2016), 16 species within this family are near-threatened or vulnerable. They have a rather small population in terms of adult individuals or in terms of total area in which they are found, rendering them vulnerable to human exploitation, pollution, hybridization, competitors, or disease.

Chromosomal analysis or karyotyping can be used to study variation as well as to characterize different species. The number of chromosomes indicates relationship of species and interrelationships within families. Studies of these characters help investigate the chromosomal evolution in each species, thereby accurately determining what species are related to one another. This may help facilitate the hybridization among them to improve their strains (Saxena and Vasave 2001).

To date, only a few cytogenetic studies on fish in the Philippines have been done. Vallejo (unpublished observations 1982) had earlier characterized *Mirogobius lacustris,* a species of fish under Family Gobiidae, through karyotyping. His results showed that the chromosome number was 52 and the chromosomal formula based on arm ratio and centromeric index was 2n = 1m + 2sm + 1st + 1t + 21a. In 1985 Formacion and Uwa karyotyped the Philippine medaka *Oryzias luzonensis* (Adrianichthyidae). Their study showed that *O. luzonensis* have a diploid chromosome number of 2n = 48 with a fundamental number (FN) of 96 since all the chromosomes were biarmed. Interspecific hybridizations and karyotype analyses led to the hypothesis that *O. luzonensis* had east Asiatic origin; migration seemed to follow the route China → Formosa → Northern Luzon. In 2003 Masagca and Ordoñez reported a diploid chromosome number and an FN of 46 for *Glossogobius giuris* (Gobiidae) from Taal Lake and some rivers of Cavite, on Luzon island. Four years later, they studied *Ophieleotris aporos* (currently known as *Giuris margaritacea*), also from Taal Lake, and reported the same diploid chromosome number

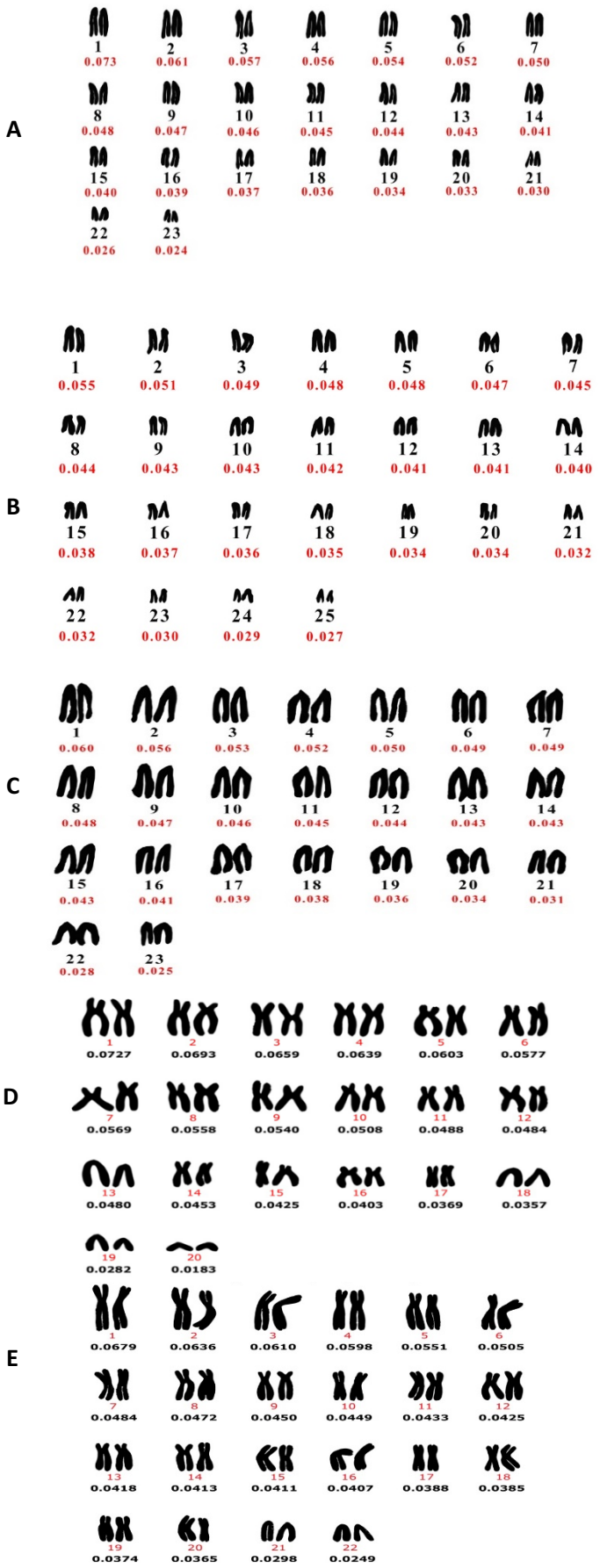


Figure 2: Karyotype of (A) *Giuris margaritacea* Valenciennes, (B) *Glossogobius celebius* Valenciennes, (C) *Glossogobius giuris* Hamilton, (D) *Redigobius bikolanus* Herre, and (E) *Redigobius dispar* Peters, showing relative lengths.

(2n = 46) in which two chromosomes are submetacentric and 44 are acrocentrics resulting in an FN of 48.

For several decades, the successful methods of enzyme electrophoresis have been applied in the analysis of genetic variation as well as genetic characteristics and differentiation of various fish populations (Paulauskas and Lozys 2001). But in the Philippines there are not many isozyme studies done with fish populations. In 2008 Quilang et al. used 12 enzyme systems to study the genetic variation of the silver perch (*Leiopotherapon plumbeus*) in four different locations: Binangonan, Tanay, Sampaloc, and Taal. Of the 12 enzymes analyzed, 22 loci were scored. Only 2 loci were polymorphic by the 99 % criterion and only 1 by the 95 % criterion. Ardestani et al. (2014) also performed isozyme analysis on two native Philippine freshwater fishes, *Glossogobius celebius* and *Glossogobius giuris*, by using 8 enzymes and 2 protein systems. They observed a total of 36 alleles in 19 presumptive loci. A higher level of genetic variation was observed within *G. celebius* populations compared with *G. giuris*.

Because there are as yet only a few studies on native and endemic Philippine fishes, little is known about their genetic diversity and genetic structure. Genetic diversity has been identified as an important factor influencing a population's potential for survival, persistence, long-term fitness, and adaptation, which can be measured in terms of sperm quality, litter size, juvenile survival, and resistance to diseases and parasites (Bouzat 2010; Hedrick and Fredrickson 2010; Ralls et al. 1988; Coltman et al. 1999). According to Markert et al. (2010), populations with low genetic diversity often exhibit an increased rate of extinction. Inbreeding, genetic drift, restricted gene flow, and small population size all contribute to a reduction in genetic diversity. The assessment of native and endemic Philippine fishes will provide baseline information for the development of management and conservation strategies.

The goal of this study is to assess the genetic structure of selected native Philippine freshwater fishes of Southern Luzon by determining the extent of genetic diversity based on their isozyme and cytogenetic profiles among and within populations of *G. celebius*, *G. giuris*, and *G. margaritacea* which are exploited for food, are vulnerable to overfishing and have aquaculture potential, and *R. dispar* and *R. bikolanus*, which are ornamental species. *G. margaritacea* are also maintained as aquarium pets. The chromosome constitution of selected endemic and native Philippine freshwater fish species will also provide information vis-á-vis biodiversity and evolutionary concerns.

MATERIALS AND METHODS

Sample Collection

A total of 434 individuals at 111, 131, 56, 63, and 73 of *Glossogobius celebius* (N = 111), *Glossogobius giuris* (N = 131), *Redigobius dispar* (N = 56), *Redigobius bikolanus* (N = 63), and *Giuris margaritacea* (N = 73), respectively, were collected from two to four different locations in Southern Luzon, Philippines, in coordination with Ocampo et al. (2010) under the project entitled "Fish Ark Philippines: Direction for the Conservation of Native and Endemic Philippine Freshwater Fishes" (tbl. 1 and fig. 1). The project is funded by the Philippine Government Department of Science and Technology's Philippine Council of Agriculture, Aquatic and Natural Resources Research and Development (DOST-PCAARRD). A set of samples is maintained live at the University of the Philippines Los Baños (UPLB) Limnological Research Station until use for cytogenetic analysis. Another set was stored at -80°C until use for isozyme studies.

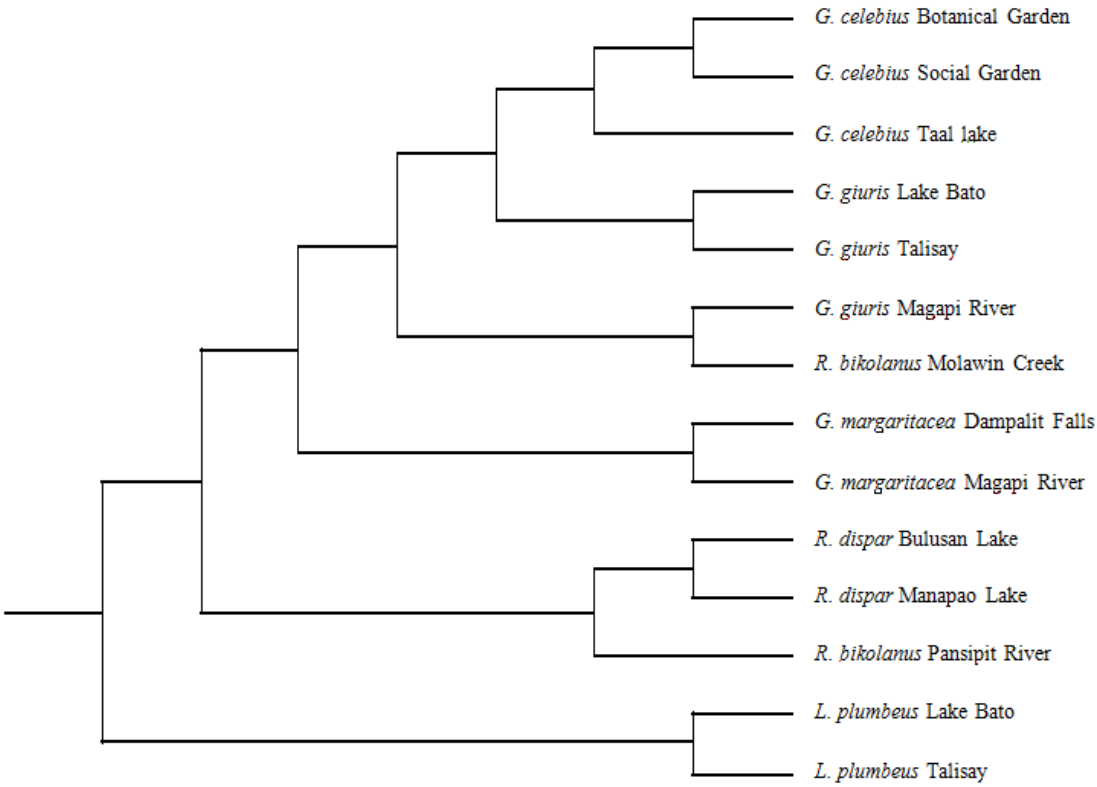


Figure 3: Dendrogram showing the relationships of the different Philippine endemic and native freshwater fish species studied under the Fish Ark program based on 17 presumptive isozyme loci.

Field documentation of collected fish samples and initial taxonomic identification were done by the UPLB Limnological Station research personnels following the methods in the national training course on freshwater fish identification held in October 2007 at the Southeast Asian Research Center for Graduate Study and Research in Agriculture (SEARCA), UPLB, and WorldFish (Vidthayanon 2007). Field identification was initially done through standard morphometric measurements given by FishBase in 1995. Taxonomic nomenclature mainly follows the current systematic status presented in FishBase in 2007, but additional references were consulted, including Conlu (1986), Herre (1953), and Vidthayanon (2007).

Cytogenetic Analysis

C-metaphase chromosome preparations were obtained from regenerating caudal fins of at least three individuals of each of the five native Philippine freshwater fish species using a Vallejo modified protocol (Vallejo, unpublished protocol). The caudal fin of the fish was cut 2mm from its border and was allowed to regenerate for three days. To avoid infection, the water in the aquarium was changed daily. After fin regeneration, the fish was allowed to swim in a 0.01% colchicine solution for 3 to 5 hours. The regenerated fins were cut and placed in a hypotonic solution of 0.46M KCl for 1.5 to 2 hours to allow the liquid to enter the cells, resulting in cell swelling and expansion (tbl. 2). Slides were stained with 2% acetoorcein and examined by using Zeiss Axioskop research microscope under the high power (HPO) and oil immersion magnification (OIO).

Photomicrographs of *G. giuris* C-metaphase cells were taken using Zeiss Axioplan 2 with DP70 Olympus digital camera in the International Rice Research Institute (IRRI), Los Baños, Laguna. C-metaphase spreads of the other fish species were taken by using Zeiss Axioskop and Canon EOS 550D camera in the UPLB Institute of Biological Sciences' Genetics and Molecular Biology Laboratory.

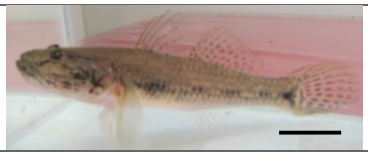
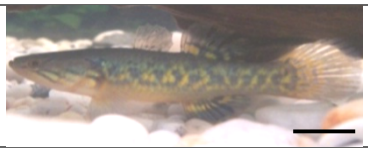
The best C-metaphases were used to prepare karyotypes. The chromosome pairs were classified into metacentric (m), submetacentric (sm), acrocentric (a), and telocentric (t), with arm ratio ranges of 1-1.7, 1.7-3, 3-7 and 7-∞, respectively following the criteria of Levan et al. (1964). The FN, which corresponds to the number of visible chromosome arms (m and sm are biarmed; a and t are single-armed) in a karyotype, was also determined. FN would be less than or equal to 2 x 2n, the difference depending on the number of single-armed chromosomes present.

Isozyme Analysis

One gram of skeletal muscle tissue was obtained from the whole body or midsection of the body of each of the 434 fishes (depending on their size) collected, macerated in 2 ml of homogenizing buffer ratio (50 ml of 0.5M Tris-His pH 8.0 and 0.01 ml of 2-mercaptoethanol), and ran in Mupid electrophoretic setup using 11 % starch gel, 50 volts, and 15ºC for 1.5 to 2 hours. Acetate buffer (pH 4.65) was used for acid phosphatase (ACP), and Tris-HCl (pH 8.0) for esterase (EST), glutamate oxaloacetate (GOT), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), malic enzyme (ME), α-glycerophosphate dehydrogenase (GDP), transferrin (TRF) and albumin (ALB). Analysis of these enzymes was done following the protocol adapted from Shaw and Prasad (1970) and Harbison (1992). The enzymes were used because they showed polymorphism in previous fish studies (Ardestani et al 2014). The enzyme commission (E.C.) number of each enzyme is summarized in Table 3.

Intrapopulation and interpopulation estimates of genetic variation were assessed by using POPGENE 32 (Yeh and Yang 1999). On the one hand, intrapopulation estimates of genetic variation considered were percent polymorphism (%P), average observed heterozygosity (Ho), and average number of observed alleles (A). On the other hand, interpopulation estimates of genetic variation, which served as the bases of the dendrogram

Table 1: Summary of native fish samples indicating the species collection site and sample size

Family	Scientific Name	Sample Photograph	Collection Site	Province	Sample Size
Gobiidae	*Glossogobius celebius*		BG Molawin Creek	Laguna	31
			SG Molawin Creek	Laguna	29
			Dampalit Falls	Laguna	25
			Taal Lake	Batangas	26
				TOTAL	**111**
	Glossogobius giuris		Lake Bato	Camarines Sur	28
			Lake Buhi	Camarines Sur	27
			Talisay Falls	Batangas	46
			Magapi River	Batangas	30
				TOTAL	**131**
	Redigobius dispar		Manapao Lake	Camarines Sur	36
			Bulusan Lake	Sorsogon	20
				TOTAL	**56**
	Redigobius bikolanus		Pansipit River	Batangas	33
			Bayog Molawin Creek	Laguna	30
				TOTAL	**63**
Eleotridae	*Giuris margaritacea*		Dampalit Falls	Laguna	30
			Pansipit River	Batangas	17
			Magapi River	Batangas	26
				TOTAL	**73**
			OVERALL TOTAL		**434**

Note: BG – Botanical Garden; SG – Social Garden

constructed, were F-statistics (F_{ST}), gene flow (*Nm*), Shannon Information Index (SI), genetic identity (I) and genetic distance (D). The electrophoretic data were also subjected to Arlequin ver. 3.5 (Excoffier et al. 2005) for interspecific genetic variation analysis: analysis of molecular variance (AMOVA) to evaluate the population genetic structure ($P<0.05$).

RESULTS AND DISCUSSION

Cytogenetic Analysis

Karyotypes of the gobiid species studied ranged from 2n = 40 of *R. bikolanus* to 2n = 50 of *G. celebius*, with differences in FN. All species in this study, except for *G. giuris* and *G. margaritacea*, were analyzed for the first time.

From at least five good C-metaphase spreads, *G. margaritacea* showed a diploid chromosome number of 2n = 46 and an FN of 46 (fig. 2A). In this study, all chromosomes of *G. margaritacea* are telocentric; this differs from the report of Masagca and Ordoñez (2007) with *G. margaritacea* having 2 submetacentric and 44 acrocentric chromosomes (FN = 48). However, both findings revealed that the said species has a chromosome number of 2n = 46.

Five well-spread C-metaphase cells of *Glossogobius celebius* from Dampalit Falls had a diploid chromosome number of 2n = 50 (fig. 2B) and FN = 50. The same number of cells of *G. giuris* (fig. 2C) from Laguna de Bay had a chromosome number of 2n = 46 and FN = 46. All chromosomes of both species are telocentric. The chromosome number obtained from *G. giuris* in this study was similar to the findings of Kaur and Srivastava (1965), Manna and Prasad (1974), and Masagca and Ordoñez (2007). However, Masagca and Ordoñez's samples, which were collected from Taal Lake and rivers in Cavite, had different karyotype formula and FN due to the presence of two acrocentric chromosomes and 44 submetacentric chromosomes in the somatic cells, while Kaur and Srivastava (1965) and Manna and Prasad (1974) showed that all chromosomes of *G. giuris* are acrocentric.

Differences in FN in the same species of fish with the same chromosome number, as observed in *G. margaritacea* and *G. giuris*, suggest that there is intraspecific karyotypic variation in the said species. Urbina-Sanchez et al. (2016) karyotyped the silverside fish (*Chrirostoma humboldtianum*) from four different locations and found different cytotypes. All four populations have the same chromosome number (2n = 48) but different FN (50, 54, and 58) and different chromosome formula. They detected morphological variation, but the length was not affected. Their results supported their hypothesis that orthoselection–i.e., only one type of chromosome rearrangement occurs repeatedly within a species–may have occurred. Their resolved karyotypes specifically showed pericentric inversions. A cytogenetic analysis and chromosomal characterization of the *Haliotis discus hannai* by Wang et al. (2015) attributed the same differences to chromosomal rearrangements as shown by the results of their Ag-NOR staining and fluorescence *in situ* hybridization (FISH) treatment of mitotic cells. They also suggested that the extreme environmental conditions in terms of salinity, O_2 concentration and water temperature, in which *H.*

Table 2: Optimum duration of exposure to Colchicine solution and KCl of the fish species used in this project

Fish species	Duration of swimming in 0.01% colchicine solution (h)	Duration of exposure to 0.46M KCl (h)
Glossogobius celebius	4 – 4.5	1.5
Glossogobius giuris	4 – 4.5	1.5
Redigobius dispar	3.0	2.0
Redigobius bikolanus	2.5 – 3.0	2.0
Giuris margaritaceae	4 – 4.5	2.0

discus hannai was exposed, might contribute to the promotion of the observed intraspecific variation.

The karyotypes of *R. bikolanus* and *R. dispar* (figs. 2D and 2E) had different kinds of chromosomes as indicated by the various locations of their respective centromeres. Seven good C-metaphase cells were chosen and used for the karyotyping of *R. bikolanus*. The number of chromosomes ranged from 32 to 40 with a mode of 40. Specimens of *R. bikolanus* from Bayog Molawin Creek had a diploid chromosome number of 2n = 40 with 10 m + 28 sm + 2 t and an FN of 78. The counts of chromosomes in *R. dispar* ranged from 42 to 44 per C-metaphase with a mode of 44. In six good C-metaphase spreads, five C-metaphases have the diploid chromosome number of 2n = 44. Karyotypes of *R. dispar* from Bulusan Lake have a diploid chromosome number of 2n=44, with 24 m + 16 sm + 4 t and an FN of 84.

Based on the computed relative lengths of the chromosomes (tbl. 4), *R. bikolanus* had the widest range (0.021–0.071), while *G. celebius* had the narrowest range (0.027–0.055). The presence of some short chromosomes in the set of long chromosomes in *G. celebius, G. margaritacea*, and *R. bikolanus* indicates that they have slightly asymmetrical karyotypes (figs. 2A, 2B, and 2D). Subtle gradations were observed in the chromosome sizes in *G. giuris* and *R. dispar*, giving symmetrical karyotypes (figs. 2C and 2E).

The number and morphology of chromosomes within family Gobiidae (*G. celebius*, *G. giuris, R. bikolanus,* and *R. dispar*) varied. Nogosa (1960) and Kaur and Srivastava (1965) reported that most gobiids had a chromosome number of 2n = 44, 46, or 42, but mostly 46. With the exception of *G. celebius*, two species studied had a diploid chromosome number of 2n = 46, and two species had a chromosome number of less than 46. The differences in the karyotype formula suggest the occurrence of chromosomal rearrangement or microstructural changes during the karyotypic evolution of these fishes. Cytogenetic studies in Perciformes (Poletto et al. 2010), Loricariidae fishes (Artoni and Bertollo 2001; Martinez et al. 2011), and stickleback species (Urton et al. 2015) through conventional technique and FISH, suggest that centric fissions, fusions, and pericentric inversions were the main mechanism contributing to changes in the basal chromosome arm sizes. Nirchio et al. (2014) proposed a mechanism for the evolution of fishes by studying the karyotypes of 103 neotropical species of fish. Their data indicated that the most ancestral fishes tended to have a higher chromosome number (values greater than or near 2n = 60) with a high number of biarmed chromosomes compared to the more derived fishes (2n = 48) with acrocentric chromosomes. Their data revealed a trend toward simplification of karyotypes from the more basal to the more derived lineages of the studied fishes perhaps because of fusions and deletions. Their study conforms with the observations of Liu et al. (2012) in two species of minifish, *Paedocypris carbunculus* (2n = 30) and *Paedocypris* sp. (2n = 34). Chromosome behavior in first meiosis revealed the presence of a chromosomal ring consisting of two metacentrics in *P. carbunculus*, suggesting that centric fusion was responsible for the karyotypic evolution from *Paedocypris* sp. to *P. carbunculus*. However, more pairs of metacentrics and fewer pairs of acrocentics are found in *P. carbunculus* compared to *Paedocypris* sp.; this is in contrast with the general observations of Nirchio et al. (2014).

In the case of the *Redigobius* species, *R. dispar* has a higher chromosome number (2n = 44) and more biarmed chromosomes compared to *R. bikolanus* with a lower chromosome number (2n = 40) and more single-armed chromosomes. Hence, it could be suggested that the diploid number 2n = 44, with the presence of many biarmed chromosomes is a more basal karyotype than the 2n = 40. This would further suggest that *R. dispar* is possibly a more basal species than *R. bikolanus*. Centric fusions may have caused the lower number of chromosomes in *R. bikolanus.* The same mechanism could also explain the difference of chromosome number in *G. celebius* (2n = 50) and *G. giuris* (2n = 46). Fusions and deletions may have occurred and resulted in a lower chromosome number in *G. giuris*. Thus *G. celebius* may be a more basal species than *G. giuris.* Maake (unpublished observations 2009) investigated the relationships among *Glossogobius* taxa in Southern Africa by using molecular markers, and the phylogram revealed that *G. celebius* appeared to be more basal in the genus than the Southern African species including *G. giuris*.

The preliminary results and suggestions of this study require validation and further investigation. Structural rearrangements are undetectable when using conventional cytogenetic techniques but can be made visible through molecular banding techniques. The application of differential staining techniques and FISH with several deoxyribonucleic acid (DNA) probes allows the detection of structural changes in the karyotype. It is also necessary to compare cytogenetic data of the species studied with molecular and genomic data.

Isozyme Analysis

Analyses of seven enzyme systems (ACP, EST, GOT, GPD, LDH, MDH, and ME) and two protein systems (ALB and TRF) revealed 17 loci with 33 alleles resolved, of which 16 loci were polymorphic and one (ALB-1) was monomorphic relative to all populations studied. Table 5 shows the genotype frequencies of all loci.

A. Intrapopulation Genetic Variation

Table 6 summarizes the intrapopulation estimates of genetic variation of the five Philippine freshwater gobies studied. Polymorphism provides a measure to demonstrate that a gene is showing variation. Polymorphism in enzymes increases individual plasticity of the varying environment, and this could lead to higher *Glossogobius celebius* populations from Social Garden (SG) Molawin Creek showed the highest percentage of polymorphic loci (%P = 64.71) and the highest average number of alleles (A = 1.65) while Dampalit Falls showed the lowest (%P = 47.06). The observed heterozygosity (Ho) within the population ranged from 0.043 to 0.063 with the Dampalit Falls population as the highest (Ho = 0.063). Although SG Molawin

Table 3: Names of enzyme systems and their E.C. number (number of loci observed is enclosed in parenthesis)

Names of enzymes/Proteins	Abbreviation	E.C Number
Acid Phosphatase (2)	ACP	E.C. 3.1.3.2
Esterase (3)	EST	E.C. 3.1.1.2
Glutamate oxaloacetate transaminase (1)	GOT	E.C. 2.6.1.1
Lactate dehydrogenase (1)	LDH	E.C. 1.1.1.27
Malate dehydrogenase (2)	MDH	E.C. 1.1.1.37
Malic Enzyme (2)	ME	E.C. 1.1.1.39
α-glycerophosphate dehydrogenase (2)	α-GPD	E.C. 1.1.1.8

has the highest %P, its heterozygosity of 0.053 has an increment of 0.01 compared to the heterozygosity of the Dampalit Falls population. A higher degree of heterozygosity, polymorphism, and number of alleles at a locus (Paulauskas and Lozys 2001) permits adaptation and a greater chance of survival of the population under different environmental conditions.

Glossogobius giuris populations from Lake Bato showed the highest percentage of polymorphic loci (%P = 47.06) and the highest average number of alleles (A = 1.57) but the lowest observed heterozygosity while Magapi River populations showed the lowest %P (17.65) and the lowest A (1.21) but the highest observed heterozygosity (0.139) despite having only three polymorphic loci (EST-2, MDH-2, and α-GPD-1). The inverse relationship in the observed Ho with %P and A may imply that the populations are possibly affected by the environment. Almost all of the *G. giuris* populations have a relatively low heterozygosity (0.026–0.139). The collection sites, Lake Buhi and Lake Bato are actually isolated lakes. A study by Furlan et al. (2012) on platypus (*Ornithorhynchus anatinus*) reported that an isolated population might have a greater possibility of inbreeding that might lead to a lower genetic diversity. Furthermore, genetic drift may also be a possible cause of lower heterozygosity within the isolated lakes, and this, in turn, leads to lower variation in the populations. Mamuris et al. (2005) studied the genetic variation in an endangered fish *Ladigesocypris ghigii*, and found a low level of intrapopulation genetic variation resulting from successive bottleneck events that correlated to shrinkage and expansion of the population over a long time.

For *Redigobius dispar* populations, the Bulusan Lake population had a higher %P (33.33), Ho (0.408), and A (1.42) than the Manapao Lake population with its %P of 26.67, Ho of 0.312 and A of 1.33. Bulusan Lake is larger than Manapao Lake (Ocampo et al. 2010). Among the watersheds of Bulusan Volcano Natural Park (BVNP), Bulusan Lake is one of Bicol's few major areas that harbor a diverse ichthyofauna. Manapao Lake has been recorded to have the lowest diversity index among the Buhi watersheds. The differences between the two populations could be traced to the conditions of the two bodies of water. Manapao Lake is more basic (9.1 pH) than Bulusan Lake (7.1 pH) (Ocampo et al. 2010).

Redigobius bikolanus populations from Pansipit River showed a higher %P (46.67), Ho (0.500), and A (1.58) than the populations from Bayog Molawin Creek with a %P of 26.67, Ho of 0.180 and A of 1.29. The differences of genetic variation could be traced to the collection sites. Samples from Batangas were collected from different parts of the river, while samples from Bayog Molawin Creek were collected from one area only. Thus *R. bikolanus* from Pansipit River has greater distribution which leads to greater diversity within the population compared to the Bayog Molawin Creek individuals.

In *Giuris margaritacea* populations, the Pansipit River population showed the highest %P (20.00), Ho (0.105), and A (1.23), while Magapi River showed the lowest %P (6.67), Ho (0.022), and A (1.08), consistent with the findings of Paulauskas and Lozys (2001).

G. celebius exhibited the highest proportion of polymorphic loci ($\%P_{ave}$ = 55.88), while *G. margaritacea* had the lowest ($\%P_{ave}$ = 13.33). The observed heterozygosity was highest in *R. dispar* (36%) and lowest in *G. celebius* (5%). This suggests that even if the species had more polymorphic loci, there were only a few heterozygous individuals at each locus. The average number of alleles per locus ranged from 1.08 to 1.65 in all populations, indicating the presence of from 1 to 2 alleles per locus.

Low values for the intrapopulation estimates indicate low variation among individuals of a population. This is detrimental to fish populations exposed to unfavorable environmental conditions. Low genetic variation in a population should be critically considered in its management and conservation.

B. Interpopulation Genetic Variation

Tables 7 and 8 summarize the interpopulation estimates of genetic variation that compare the *Glossogobius* species and the *Redigobius* species, and among various populations for each species, respectively.

F_{ST} values are useful estimates of the amount of genetic differentiation between species. Wright (1978) provided the following groupings for the evaluation of F_{ST} values: 0 to 0.05 is considered to reflect little genetic differentiation; 0.05 to 0.15 is indicative of moderate differentiation; 0.15 to 0.25 indicates great genetic differentiation; and values greater than 0.25, reflect great genetic differentiation. The gene flow parameter, *Nm*, is a measurement of the effective population number and rate of migration among populations (Larson et al. 1984). If $Nm > 1$, there is enough gene flow to negate the effects of genetic drift, and if $Nm > 4$, then local populations belong to one panmictic (randomly mating) population (Wright 1931). The SI is a parameter used to characterize species diversity in a community.

G. giuris and *G. celebius* have F_{ST} values of 0.52 and 0.30, respectively, reflecting great genetic differentiation. However, the effective population number in *G. celebius* populations is higher (Nm = 0.59) than in *G. giuris* populations (Nm = 0.23); the numbers suggest that neither has enough gene flow to negate the effects of genetic drift. The lower F_{ST} value and higher *Nm* of *G. celebius* may be accounted for by the shorter distance between Laguna and Batangas, their collection sites, compared to the distance between the Bicol Region and Batangas, the collection sites for *G. giuris*. The SI is found to be significantly higher in *G. celebius* than in *G. giuris* ($I = 0.44 > I = 0.30$, $p = 0.0209$), thereby supporting the higher level of polymorphism within this species.

Both *R. dispar* and *R. bikolanus* gave F_{ST} values of 0.34, indicating a great degree of genetic differentiation. On the other hand, *R. dispar* and *R.bikolanus* have *Nm* values of 0.50 and 0.49, respectively, which also suggest that there is not enough gene flow to negate the effects of genetic drift. The SI is higher in *R. bikolanus* (0.39) compared to *R. dispar* (0.35), validating the higher level of polymorphism within the said species. The

Table 4: Average relative chromosome lengths of *Giuris margaritacea*, *Glossogobius celebius*, *Glossogobius giuris*, *Redigobius bikolanus*, and *Redigobius dispar* (The number of best C-metaphase cells observed is enclosed in parenthesis)

Chromosome Pair	*Giuris margaritacea* (N≥5)	*Glossogobius celebius* (N≥5)	*Glossogobius giuris* (N≥5)	*Redigobius bikolanus* (N=7)	*Redigobius dispar* (N=6)
1	0.073	0.055	0.060	0.071	0.070
2	0.061	0.051	0.056	0.067	0.065
3	0.057	0.049	0.053	0.065	0.061
4	0.056	0.048	0.052	0.062	0.059
5	0.054	0.048	0.050	0.060	0.057
6	0.052	0.047	0.049	0.058	0.053
7	0.050	0.045	0.049	0.057	0.052
8	0.048	0.044	0.048	0.056	0.051
9	0.047	0.043	0.047	0.054	0.048
10	0.046	0.043	0.046	0.053	0.047
11	0.045	0.042	0.045	0.050	0.046
12	0.044	0.041	0.044	0.049	0.045
13	0.043	0.041	0.043	0.047	0.044
14	0.041	0.040	0.043	0.045	0.042
15	0.040	0.038	0.043	0.043	0.040
16	0.039	0.037	0.041	0.041	0.039
17	0.037	0.036	0.039	0.039	0.036
18	0.036	0.035	0.038	0.035	0.034
19	0.034	0.034	0.036	0.028	0.032
20	0.033	0.034	0.034	0.021	0.030
21	0.030	0.032	0.031	-----	0.027
22	0.026	0.032	0.028	-----	0.022
23	0.024	0.030	0.025	-----	-----
24	-----	0.029	-----	-----	-----
25	-----	0.027	-----	-----	-----

data show that *R. bikolanus* exhibits greater heterozygosity, but its population in Bayog Molawin Creek is rather small. According to IUCN (2012), this species was considered in 1996 as a threatened species but progressed to a least-concern species in 2012. However, populations in the Philippines and Singapore may be more at risk due to development and clearing. Damage to coastal rivers and mangrove habitats through habitat degradation and destruction are also potential threats to this species and may hasten the decline in the number of individuals (IUCN 2012). A smaller population size could lead to a faster decline in heterozygosity. In a few more years, the heterozygosity of this species might decrease as a consequence of the increase in frequency of one of the alleles' near fixation possibly due to the drastic effects of environmental changes to include diseases.

Genetic identity (I) and genetic distance (D) measure the amount of genetic divergence that has occurred between two species and that may have caused a hypothetical separation point sometime in their evolutionary past. Based on the distance decay theory (Nekola and White 1999), an increase in the geographic distance between sites lowers the degree of gene flow, hence more variation between the populations. Among the *G. celebius* populations, the highest genetic identity was observed between Botanical Garden (BG) Molawin Creek and SG Molawin Creek populations (I = 0.90), which belong to the same river system and are located at a distance of 1.24 kilometers from each other, hence the lowest genetic variation of 0.15. The observed lower genetic variation between the two sites may be due to their having common ancestors as a result of their geographic proximity. Moreover, the observed lower level of interpopulation genetic diversity may suggest that their historical population sizes are small and/or of only recent dispersal occurrence (Davey et al. 2003). More definitive studies on the historical population sizes of these Philippine freshwater species are necessary. All populations of *G. celebius* clustered together (fig. 3).

The populations of *G. giuris* from Lake Bato and Lake Buhi, which are 20.31 kilometers apart, show the lowest genetic distance (D = 0.09) and the highest genetic identity (I = 0.91), suggesting the existence of a high level of identical alleles between the two populations. The two locations have nearly the same environmental conditions, suggesting that the environment has no effect on the alleles in the population (tbl. 9). Aquaculture can also explain the situation between the two populations. The rise of aquaculture in Philippine lakes, including Lake Buhi and Lake Bato, led to the introduction of exotic fishes that could alter the existing fish population through inbreeding, predation, and competition for food, space, and habitat (Araullo 2001). To a certain extent, genetic pollution can occur. There is a history of entry of migratory fishes in Lake Bato and Lake Buhi (Gindelberger 1981). Tilapia culture, motorized push nets, and fish corrals also led to the changes in these lakes' fauna. In Lake Bato there is a high abundance of three introduced species, *Trichogaster pectoralis*, *Oreochromis niloticus*, and *Trichogaster trichopterus*, while in Lake Buhi the introduced *Oreochromis niloticus* is highest in abundance (Ocampo et al. 2010). The invasiveness of these species, their high reproductive success, and their aggressive behavior and feeding habits might be the causal agent in the diversity decline of other native fish faunas including *G. celebius* (Ocampo et al. 2010). By contrast, *G. giuris* populations from Magapi River and Talisay Falls, which are separated from each other by 11.48 kilometers and inhabit the Taal Lake watershed region, showed the highest genetic distance (D = 0.55). The inverse relationship of the geographic distance and genetic variation in *G. giuris* samples from Lake Bato and Lake Buhi, and from Magapi River and Talisay Falls, suggests that environmental factors in neighboring ecosystems could significantly restrict gene flow. *G. giuris* populations also clustered together (fig. 3).

Table 5: Genotype frequencies of the 17 presumptive loci of the seven enzymes and two proteins observed in five native Philippine freshwater fish species from four different locations in Southern Luzon, Philippines.

Locus	Genotype	*G. celebius*				*G. giuris*				*R. dispar*		*R. bikolanus*		*G. margaritacea*		
		BG (N=31)	SG (N=29)	DF (N=25)	TL (N=26)	Bt (N=28)	Bh (N=27)	TF (N=46)	Mgp (N=30)	Mnp (N=36)	Bul (N=20)	Pst (N=33)	Byg (N=30)	DF (N=30)	Mgp (N=17)	Pst (N=26)
ACP-1	SS	0.64	0.62	0.92	1.00	0.93	0.89	0.36	1.00	--	--	--	--	--	--	--
	SF	--	--	--	--	0.07	--	0.64	--	--	1.00	1.00	--	0.79	--	--
	FF	0.36	0.38	0.08	--	--	0.11	--	--	--	--	--	1.00	0.21	1.00	1.00
ACP-2	SS	0.28	0.59	0.55	1.00	--	--	1.00	1.00	--	--	--	1.00	0.20	1.00	1.00
	SF	0.31	--	--	--	--	--	--	--	--	--	--	--	0.80	--	--
	FF	0.41	0.41	0.45	--	--	--	--	--	--	--	--	--	--	--	--
EST-1	SS	1.00	0.93	1.00	1.00	0.89	1.00	--	1.00	--	--	--	1.00	--	1.00	--
	SF	--	--	--	--	--	--	--	--	0.89	1.00	0.91	--	--	--	1.00
	FF	--	0.07	--	--	0.11	--	--	--	0.11	--	0.09	--	1.00	--	--
EST-2	SS	0.58	0.34	0.28	0.19	0.86	0.37	0.59	--	--	1.00	1.00	--	1.00	1.00	1.00
	SF	0.16	--	--	--	0.03	--	--	0.9	--	--	--	1.00	--	--	--
	FF	0.26	0.66	0.72	0.81	0.11	0.63	0.41	0.1	1.00	--	--	--	--	--	--
EST-3	SS	0.87	0.86	--	1.00	1.00	1.00	1.00	--	--	--	--	--	--	--	--
	FF	0.13	0.14	--	--	--	--	--	--	--	--	--	--	--	--	--
GOT-1	SS	0.79	0.50	1.00	0.77	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	FF	0.21	0.50	--	0.23	--	--	--	--	--	--	--	--	--	--	--
αGPD-1	SS	1.00	0.21	0.56	0.19	0.74	0.55	--	--	0.06	0.10	--	0.97	1.00	1.00	1.00
	SF	--	0.07	--	--	0.26	0.41	--	1.00	0.94	0.90	1.00	--	--	--	--
	FF	--	0.71	0.55	0.81	--	0.04	--	--	--	--	--	0.03	--	--	--
αGPD-2	SS	0.90	1.00	--	1.00	0.90	1.00	1.00	--	--	--	--	--	--	--	--
	FF	0.10	--	--	--	0.10	--	--	--	--	--	--	--	--	--	--
LDH-1	SS	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	--
	SF	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1.00
MDH-1	SS	0.40	--	0.79	0.27	0.79	0.67	0.82	--	--	--	0.14	--	1.00	1.00	1.00
	SF	--	0.55	--	--	--	0.15	0.11	--	--	--	0.57	--	--	--	--
	FF	0.60	0.45	0.21	0.73	0.21	0.18	0.07	1.00	1.00	1.00	0.29	1.00	--	--	--
MDH-2	SS	1.00	1.00	1.00	0.38	1.00	1.00	0.76	0.81	0.03	1.00	--	--	1.00	1.00	1.00
	SF	--	--	--	0.54	--	--	0.22	0.04	0.92	--	0.52	0.52	--	--	--
	FF	--	--	--	0.08	--	--	0.02	0.15	0.05	--	0.48	0.48	--	--	--
ME-1	SS	0.39	0.31	0.52	0.96	0.21	0.07	--	1.00	1.00	--	1.00	--	1.00	0.83	0.76
	SF	--	--	0.36	--	--	--	0.07	--	--	1.00	--	--	--	--	--
	FF	0.61	0.69	0.12	0.04	0.79	0.93	0.93	--	--	--	--	1.00	--	0.17	0.24
ME-2	SS	1.00	0.33	1.00	1.00	1.00	1.00	1.00	1.00	1.00	--	1.00	1.00	1.00	1.00	1.00
	FF	--	0.67	--	--	--	--	--	--	--	--	--	--	--	--	--
ALB-1	SS	1.00	1.00	1.00	1.00	--	--	--	1.00	--	--	--	1.00	1.00	--	1.00
ALB-2	SS	1.00	1.00	0.73	0.50	0.36	--	0.98	--	--	--	--	--	--	1.00	--
	SF	--	--	--	--	--	--	--	--	1.00	1.00	1.00	--	--	--	--

	FF	--	--	0.27	0.50	0.64	1.00	0.02	--	--	--	--	--	--	--	--
TRF -1	SS	0.18	--	--	0.33	--	0.36	--	1.00	1.00	--	--	--	1.00	--	1.00
	SF	0.03	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	FF	0.79	1.00	--	0.67	--	0.64	--	--	--	1.00	--	--	--	--	--
TRF -2	SS	0.71	0.38	0.28	0.23	1.00	0.67	0.78	1.00	1.00	--	--	--	1.00	1.00	--
	SF	0.23	0.28	0.52	0.38	--	--	--	--	--	--	1.00	1.00	--	--	--
	FF	0.06	0.34	0.20	0.38	--	0.33	0.22	--	--	1.00	--	--	--	--	--

Note: BG-Botanical Garden Molawin Creek; SG-Social Garden Molawin Creek; DF-Dampalit Falls; TL-Taal Lake; Bt-Lake Bato; Bh-Lake Buhi; TF-Talisay Falls; Mgp-Magapi River; Mnp-Manapo Lake; Bul-Bulusan Lake; Pst-Pansipit River; Byg-Bayog Molawin Creek

R. dispar populations from Bulusan and Manapao Lakes, which are about 180 kilometers apart, showed a relatively high genetic identity (I = 0.77) and low genetic distance (D = 0.26), conforming with the observed grouping of the two populations in one cluster (fig. 3). Bulusan Lake is a crater lake that is a part of a national park and thus far from human settlements. This lake is a safe haven for *R. dispar*, where they are found in high abundance. Manapao Lake is also a crater lake, smaller than Bulusan Lake, but its surroundings are also uninhabited by humans or any agricultural settlements. *R. dispar* also thrives here but not as many as the endemic sinarapan. The physicochemical properties of both lakes (tbl. 9) have a close resemblance, which could explain the relatively high genetic identity between the two populations.

Pansipit River and Bayog Molawin Creek populations of *R. bikolanus* exhibited a genetic identity of 0.69 and a genetic distance of 0.37. These *R. bikolanus* populations were observed in different clusters. Pansipit River is pristine and protected and maintained by the community. By contrast, a slaughterhouse, a research center, and a residential area are within the vicinity of Bayog Molawin Creek, which may be contaminated due to improper waste disposal. *R. bikolanus* individuals collected from this creek may have adapted to suboptimal conditions, which in turn might have contributed to the observed genetic distance of the two populations. Another possible reason for these genetic differences is the varying mode of collection. Samples from Pansipit River were collected from different areas, while samples from Bayog Molawin creek were collected in one area only, where they were concentrated. However, there is a need for physicochemical data in these two locations to support the assumption that environment affects the gene frequencies of the isozyme data.

Another possibility is the occurrence of genetic drift. In each generation some individuals, by chance, may leave behind a few more descendants (and genes) than other individuals due to interactions with humans or other natural phenomena. Thus, the next generation will be composed of the genes of the surviving individuals, which are not necessarily the "better" individuals. The genes from the other individuals drift out and are gone for good. This will lead to fixation of genes of the surviving individuals, loss of fitness (vigor, viability, fecundity, resistance to disease), loss of adaptive genetic variation, and increase in inbreeding depression (Charlesworth and Charlesworth 1999; Crnokrak and Roff 1999; Hedrick and Kalinowski 2000; Lacy

Table 6. Summary of the intrapopulation estimates of genetic variation of five native Philippine freshwater gobies. %P (Percentage of polymorphic loci), H_O (Observed heterozygosity), and A (Average number of alleles)

Species	Location	% P	Ho	A
G. celebius	BG Molawin Creek	58.82	0.043	1.59
	SG Molawin Creek	64.71	0.053	1.65
	Dampalit Falls	47.06	0.063	1.57
	Taal Lake	52.94	0.054	1.53
	Average	**55.88**	**0.053**	**1.59**
G. giuris	Lake Buhi	41.18	0.037	1.47
	Lake Bato	47.06	0.026	1.57
	Talisay Falls	41.18	0.081	1.54
	Magapi River	17.65	0.139	1.21
	Average	**36.77**	**0.071**	**1.45**
R. dispar	Bulusan Lake	33.33	0.408	1.42
	Manapao Lake	26.67	0.312	1.33
	Average	**30.00**	**0.360**	**1.37**
R. bikolanus	Pansipit River	46.67	0.500	1.58
	Bayog Molawin Creek	26.67	0.180	1.29
	Average	**36.67**	**0.340**	**1.44**
G. margaritacea	Dampalit Falls	13.33	0.069	1.14
	Magapi River	6.67	0.022	1.08
	Pansipit River	20.00	0.105	1.23
	Average	**13.33**	**0.065**	**3.45**

1997; Lynch 1996). However, this hypothesis should be tested. Just recently *R. bikolanus* was reviewed by Larson (2010), who reported that this is possibly a species-complex. A genetic and morphological study covering all habitat types and geographic localities may help clarify if there are cryptic species within *R. bikolanus* (Larson 2012). Moreover, the *Redigobius* genus is actually being revised especially the *bikolanus* group that should be split into several taxa, leading to a revision of the group distribution (Keith et al. 2011). The possibility that there are cryptic species within *R. bikolanus* may help explain why the two populations did not cluster together. The Bayog Molawin Creek population might be of a different variant of the said species.

Table 7: Summary of the interpopulation estimates of genetic variation used to compare *Glossogobius celebius* and *G. giuris*, and *Redigobius dispar* and *R. bikolanus*. F_{ST} (F-statistics), *Nm* (Gene flow), SI (Shannon Information Index)

Species	F_{ST}	*Nm*	SI
G. celebius	0.30	0.59	0.44
G. giuris	0.52	0.24	0.30
R. dispar	0.34	0.50	0.35
R. bikolanus	0.34	0.49	0.39

High genetic identity (I = 0.80–0.89) and low genetic distance (D = 0.12–0.13) were observed from the *G. margaritacea* populations (tbl. 8). Pansipit River and Magapi River are both in Batangas, while Dampalit Falls are located in Laguna, a nearby province. Due to the proximity of the three collection sites, the environmental conditions are relatively similar; hence a uniform response is possible. Although the physicochemical properties of Dampalit Falls are available (tbl. 9), the data of Pansipit River and Magapi River are not; hence complete physicochemical data of all collection sites are needed to support the assumption above.

While highest genetic relatedness was observed for the *G. margaritacea* populations from Dampalit Falls and Pansipit River, populations from Dampalit Falls and Magapi River were those observed in one cluster (fig. 3). Pansipit River population was not included in the dendrogram because the software (POPGENE 32) can accommodate only a certain number of populations, and, among the three populations, Pansipit River has the smallest sample size. The isozyme loci studied may have only reflected the expression of similar enzymes under the same environmental conditions.

RECOMMENDATIONS

The genetic variation among and within species should be further investigated by using DNA markers. In order to deduce clearly the evolutionary relationships between species, more sensitive techniques of karyotyping (e.g., differential staining, molecular banding, and FISH) should be done. An effort is needed to fill in the gap between chromosome and genomic data on fishes and to integrate cytogenetics and genomics in comparative and evolutionary studies. Morphometric analysis should be conducted to determine if the variation at the cellular and molecular level corresponds to the phenotype. Further investigation and taxonomic work should be done on *R. bikolanus* to confirm the result of the cluster analysis. An increase in the number of samples and collection sites is recommended. Complete physicochemical data from all collection sites should be gathered to strengthen the claim that environment has an effect on the genetic variation. Future studies, however, should take into consideration the current status of fish populations, especially those which are already dwindling in their natural habitat.

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Table 8: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) values based on 17 presumptive loci among the populations of *Glossogobius celebius, G. giuris*, *Redigobius dispar*, *R. bikolanus*, and *Giuris margaritacea*.

Species		Locations			
G. celebius		BG Molawin Creek	SG Molawin Creek	Dampalit Falls	Taal Lake
	BG Molawin Creek	***	0.90	0.84	0.84
	SG Molawin Creek	0.11	***	0.77	0.87
	Dampalit Falls	0.17	0.26	***	0.83
	Taal Lake	0.18	0.14	0.19	***
G. giuris		Talisay Falls	Magapi River	Lake Bato	Lake Buhi
	Talisay	***	0.5751	0.86	0.78
	Magapi River	0.55	***	0.65	0.64
	Lake Bato	0.15	0.42	***	0.91
	Lake Buhi	0.25	0.44	0.09	***
R. dispar		Bulusan Lake	Manapao Lake		
	Bulusan Lake	***	0.77		
	Manapao Lake	0.26	***		
R. bikolanus		Bayog Molawin Creek	Pansipit River		
	Bayog Molawin Creek	***	0.69		
	Pansipit River	0.37	***		
G. margaritacea		Dampalit Falls	Magapi River	Pansipit River	
	Dampalit Falls	***	0.86	0.89	
	Magapi River	0.15	***	0.80	
	Pansipit River	0.12	0.23	***	

Table 9: Physicochemical properties of the eight freshwater sites in Southern Luzon Philippines (Ocampo et al. 2010; Paller et al. 2011)

Location	BG	SG	DF	Bt	Bh	TF	Mnp	Bul
Dissolved O_2	6.00	6.30	5.93	4.50-8.50	6.20-6.80	--	6.00-6.70	7.00-7.30
pH	8.50	8.53	6.11	8.60-10.00	8.90-9.10	8.50	8.90-9.30	7.10-7.30
Water Temperature (^oC)	25.4	25.63	26.79	25.45-29.30	26.00-33.00	23.00-23.80	28.20-33.50	26.30-28.10
Air Temperature (^oC)	26.79	26.93	27.03	24.20-30.40	27.80-30.5	24.80-27.00	27.50-28.60	27.50-31.50

Note: BG-Botanical Garden Molawin Creek; SG-Social Garden Molawin Creek; DF-Dampalit Falls; Bt-Lake Bato; Bh-Lake Buhi; TF-Talisay Falls; Mnp-Manapao lake; Bul-Bulusan Lake.

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