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Development of Microsatellite Markers for the Assessment of Genetic Diversity in *Leiopotherapon plumbeus* Kner 1864

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Abstract

Microsatellite loci from a Philippine-endemic silver perch, *Leiopotherapon plumbeus* Kner 1864, were isolated, characterised and used in the development of simple sequence repeat (SSR) markers for genetic diversity analysis. Ten primer pairs were designed from a microsatellite-enriched library and polymorphic markers were used in the diversity assessment of populations from three Philippine lakes (Laguna de Bay, Taal Lake and Sampaloc Lake). Seven primers detected a total of 33 alleles and obtained polymorphism information content (PIC) values ranging from 0.12–0.75. The overall H_o and uH_e values across the populations were 0.247 and 0.494, respectively. Pair-wise population F_{ST} values showed low but significant differentiation while unweighted pair group method with arithmetic mean (UPGMA) cluster analysis revealed a closer relationship between populations from Taal and Sampaloc lakes. The markers used in this study can be used to jumpstart genetic-based breeding programmes and conservation strategies to save the dwindling population of this species.

Keywords: Genetic diversity, microsatellite-enriched library, SSR markers, *Leiopotherapon plumbeus*

Introduction

The Philippines is very rich in aquatic resources. It is home to several native and endemic marine and freshwater fish species. Among these is the Philippine silver perch (*Leiopotherapon plumbeus* Kner 1864), a small silver-coloured fish locally known as "ayungin" (Fig 1). *Leiopotherapon plumbeus* is considered as one of the tastiest and most expensive freshwater fish species and was once the most abundant fish in Laguna de Bay, the largest inland water body in the Philippines. Unfortunately, high fishing pressure and intensive misuse of the watershed led to the collapse of the whole Laguna de Bay fishery. In 2012, the Bureau of Fisheries and Aquatic

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Resources reported the minimal but consistent decline in the silver perch population. This threat in the fish population is posed not only by the continuous degradation of the water quality of the lake but also the proliferation of invasive fishes (Yap 2012).

Fig. 1. *Leiopotherapon plumbeus* Kner (Paller, Corpuz and Ocampo, 2011)

Many populations and species of fish have become extinct due to pollution, destruction of habitats and exploitation. Two commonly used approaches in fishery management to reduce risk of extinction are translocation and captive breeding. Transplantation and translocation are synonymous terms to describe the movement of one taxon from one location to another (Minckley 1995). On the other hand, captive breeding is conservation of the fish by breeding them in captivity (Vrijenhoek 1998). Though both approaches address the problem of population decline, they can also lead to reduced genetic variation if a small number of individuals is used to establish new populations or if parental fishes used in breeding are closely related. Conservation of genetic diversity is very important since it is the raw material for adaptive capacities and therefore the survival of populations and species (Sinha and Heaney 2006). Hence, true and long-term management of fish resources must include the conservation of genetic diversity that would equip a species to adapt to changing environments, now and in the future. Periodic survey of genetic variation or molecular genetic studies is thus imperative to find appropriate conservation management strategies (Stockwell et al. 1996).

Microsatellites or simple sequence repeats (SSRs) are tandem arrays of one to six nucleotide bases. They are neutral markers that are densely and randomly distributed along prokaryotic and eukaryotic genomes. Their high mutation rates, co-dominant nature and ease of amplification make microsatellites the marker of choice in genetic diversity analysis, assessment of population genetic structure, individual and parentage identification, genetic mapping and marker-assisted breeding (Ellegren 2004; Sekar et al. 2009). In spite of their usefulness, however, microsatellite markers have not been developed for most Philippine freshwater fishes, including *L. plumbeus*. This is largely due to the need for known genomic sequences before SSR primers can be designed. Fortunately, numerous strategies have already been developed to facilitate the isolation of microsatellite loci in non-model organisms. Given the ecological and economic importance of silver perch and its dwindling population, thorough assessment of its genetic diversity and structure are important in finding appropriate measures for its long-term conservation.

This study thus aimed to isolate and characterise microsatellites from *L. plumbeus*, design microsatellite primers and use the primers for the assessment of genetic diversity among populations of *L. plumbeus* from three Philippine lakes—Sampaloc Lake, Taal Lake and Laguna de Bay.

Materials and Methods

Sample collection and DNA isolation

Silver perch were collected from each of the three lakes in the Philippines namely: Calamba area of Laguna de Bay (LDB), Talisay area of Taal Lake (TL) and Sampaloc Lake (SL). To ensure extraction of high quality DNA for library construction, pooled DNA from ten individuals of silver perch was isolated using a commercially available DNA Isolation Kit (Qiagen, USA) following the manufacturer's protocol. For the genetic diversity study, muscle tissues from at least 30 individuals from each lake were used to isolate genomic DNAs using routine phenol-chloroform isolation.

Microsatellite isolation and primer design

Genomic DNA successfully digested using *Rsa* 1 was purified using Qiaquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. Prior to ligation, the Rsa21-Rsa25 adapter (Edwards et al. 1996) was prepared by mixing equal volumes of 10 μM Linker1 (Rsa21:5' CTCTTGCTTACGCGTGGACTA) and Linker 2 (Rsa25:3'ACACGAGAACGAATGCGCACCTG AT-p, where p indicates phosphorylation). Ligation of the adapter to the digested gDNA fragments was performed in a total volume of 40μl. For every one microgram digested DNA, 12 pmol adapter, 2U *Rsa*1 (Vivantis), 4U *Bst*PAI, 1x ligation buffer and 5U T4 Ligase (Promega USA) were used. Successful ligation was checked through polymerase chain reaction (PCR) using Rsa21 as primer. The linker-ligated DNA was used for subtractive hybridisation following a modified protocol of Glenn and Schable (2005). In brief, linker-ligated DNA was hybridised to biotinylated oligonucleotides [$(CA)_{10}$, $(TC)_{10}$ and $(TG)_{10}$], captured on Dynabeads M-280 streptavidin magnetic particles (Invitrogen, USA) and recovered through re-precipitation of the stringently washed DNA.

Successful enrichment was checked through PCR and the product was purified using the Qiaquick Purification Kit (Qiagen). The repeat-enriched PCR product was ligated to pGEM-T Easy Vector (Promega) and the plasmid was used to transform *E. coli* JM109 competent cells (Promega) through the heat-shock method. White colonies were checked for insert size using *Eco*RI digestion and several clones of different sizes were sent for sequencing to Macrogen Inc., South Korea. The quality of the sequences was checked for clarity and absence of overlaps between peaks using the programme ChromasPro 1.32 (Technelysium, Australia). Trimmed of any portion of vector and adapter sequences, unique sequences were analysed using NCBI-BLAST (Altschul 1990) to check for similarity to any fish microsatellite sequences.

Sequences were also analysed using the BioPHP Microsatellite Finder tool now available at http://insilico.ehu.es/mini_tools/microsatellites/ (Bikandi et al. 2004). From the sequences of the inserts, ten primer pairs to amplify microsatellite regions were designed using the Primer3 Software Package (Untergasser et al. 2012) in NCBI. The designed primers were sent for synthesis to Invitrogen (USA) and the functionality of each was tested on *L. plumbeus* individuals. The ten primer sequences and corresponding characteristics were deposited at the NCBI Probe database (Table 1).

Genetic diversity analysis

Nine functional SSR primer pairs were used to amplify microsatellites from *L. plumbeus.* The standard PCR condition used was as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at corresponding T_a for 45 sec and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. For touchdown PCR, the following profile was used: initial denaturation at 94 °C for 5 min; 20 cycles of denaturation at 94 °C for 1 min, annealing for 45 sec at ±5 °C of T_a with 0.5 °C decrement every cycle and extension at 72 °C at 1 min; standard 20 cycle of 94 °C for 1 min, annealing at corresponding T_a for 45 sec and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min.

The presence of PCR products was checked in 8 % polyacrylamide gels and visualised using the Bio-Rad Gel Documentation System. The resulting bands were scored by estimating the fragment length of a band/allele. For microsatellite markers that showed shadow bands, the strongest band was consistently scored. The Polymorphic Information Content (PIC) of each marker was computed using PowerMarker 3.25 (Liu and Muse 2005). Micro-checker 2.2.3 software (Van Oosterhout et al. 2005) was used to check for the presence of null alleles, large allele drop-out and scoring errors. For all locus with evidence of null alleles, the adjusted genotypes were estimated using the Brookfield 1 (Brookfield 1996) equation and these corrected genotypes were used for the subsequent genetic diversity analyses. The levels of genetic diversity and pairwise genetic distances were estimated using the GenAlex software version 6.5 (Peakall and Smouse 2012) with the following statistics: number of effective allele (A_e), observed heterozygosity (H_o), unbiased expected heterozygosity (uH_e), pairwise F_{st} values, and Nei's genetic distances. GENEPOP (Rousset 2008) was used to perform the Hardy Weinberg (H-W) equilibrium exact test (Guo and Thompson 1992) and to compute for the inbreeding coefficient (F_{IS}) estimates by Weir and Cockerham (1984). NeEstimator v2.01 (Do et al. 2014) was used to estimate the effective population sizes (N_e) based molecular co-ancestry (Nomura 2008) at 95 % confidence intervals (CI) obtained using Jackknife on loci method. Unadjusted genotypes were also used to examine genetic relationships among the three populations via unweighted pair-group method using arithmetic means (UPGMA) cluster analysis carried out with PowerMarker. Nei's (1973) similarity coefficient was used and the reliability of the resulting tree was tested by generating 1000 bootstrapped trees which were analysed in the Consense package of the Phylip 3.695 (Felsentein 2009). The dendrogram was drawn by TreeView 1.6.6 (page 1996).

Table 1. Characteristics of ten primer pairs developed from *Leiopotherapon plumbeus*.

NCBI ProbeDatabse Accession No.	Code	Target clone repeat motif		Sequence (5'->3')	Tm	T_a used (°C)	Expected Product size (bp)	Approximate Size Range (bp)	No. of alleles	PIC
Pr032825044	Lp3	(AC)10	F	GAAAACAGCAGCAATAGTC	52.2	49	240	218-266	8	0.75
			R	TGGTCCATAGGTATGAGTA	51					
Pr032825050	Lp5	(AC)4 gcacgc	F	TGTCTACACCTCTCCCAG	54.4	52	180	180	1	0
		(AC)4	R	CAGATTTGTGTTCGGTGTG	54.7					
Pr032825051	Lp8	(GA)6 ggg (GA)12	F	TGCGAGGTAAAGCGGAGCAG	57.1	52[td]	±200	192-234	4	0.54
			R*	CAGAGCTGCAGGGACGATTCC	57.3					
Pr032825042	Lp11[n]	(TG)8 cacgtgttttattg	F	CATGCTGGTCTGTTGTGGAC	53.4	N	132	n	n	N
		(GA)19	R	ACATCAAACATCACAGCACCA	53.6					
Pr032825043	Lp13	(CA)6	F	TGGAAGGCGGATCATTTCATT	52.1	50	101	<100-101	2	0.12
			R	CTGTAAATGTATTTTTAAGGCAAGG	49.4					
Pr032825045	Lp30	(TG)5 tttgac (TG)9	F	GAGCGCACCCGAAACACAG	56.4	52[td]	131	126-184	7	0.75
			R	CACCGTGTGTTTGAACGGCC	57					
Pr032825046	Lp31	(TG)10 (AG)12 aa	F	CAGTTGGGAAGTGTCAGGGC	55.1	51.6	202	200	1	0
		(AG)12	R	GTGGCGCTCAGCCTTCAC	55.8					
Pr032825047	Lp34	(TG)9	F	GTACGCTTACCAGAGTACAGTG	52.5	48[td]	127	125-137	6	0.72
			R	TGAACTGTGGGTTCTGGTCC	53.6					
Pr032825048	Lp43	(TG)14	F	GCATGAACATGCTTTCCCTCTAGCC	58.2	54[td]	101	<100-110	3	0.18
			R	ACACAGCACTTCCACCTCCAGTG	58.8					
Pr032825049	Lp48	(CA)21 cg (CA)9 ct	F	ACCGGGGACAAAGTGACGTG	56.8	52[td]	164	164-194	3	0.26
		(CA)13	R	GGAGCGTGTATGGGGGTGTTC	57.2					

Tm- melting temperature; Ta-annealing temperature; n- non-functional; *Reverse sequence from from gi|306478369|gb|HQ163783.1| *Chaetodon ornatissimus* microsatellite Cor28 sequence (NCBI); td-touchdown PCR; s-not optimised

Results

Microsatellite marker development

Twenty six clones were sequenced from the microsatellite-enriched library. Twenty five sequences had good quality reads with linkers and vector sequences located and subsequently removed. However, multiple copies of the same clone (i.e. samples/clones with the same sequences) were observed and these were considered as one clone. Thus, only 18 unique sequences were considered in the BLASTN and Repeat Finder analyses. This redundancy in the sequenced fragments could be due to the amplification step prior to cloning. With query coverage and maximum identity of 50–99 % and 85–96 %, respectively, the sequences showed similarity to sequences from fishes such as *Danio rerio* Hamilton 1822 (zebra fish), *Sepiella maindroni* de Rochebrune 1884 (Japanese spineless cuttlefish) and *Salmo salar* Linnaeus 1758 (Atlantic salmon). BLAST hits also showed similarity to fragments containing microsatellites. This shows the successful isolation of microsatellite loci from *L. plumbeus* and suggests the conservation of some sequences in fishes.

Fifteen out of 18 unique sequences (83.33 %) contained microsatellite repeats with the number of repeats ranging from 4 to 21. This is comparable to the results of Nunome et al. (2006), Segelbacher et al. (2008) and Hill et al. (2008) with enrichment efficiency equal to 74 %, 65 % and 81 %, respectively. The latter two studies also used the Glenn and Schable (2005) microsatellite enrichment protocol. Fourteen of the clones contain $(CA/GT)_n$ while three clones contain $(AG)_n$ repeat motif. This is consistent with previous results that showed the prevalence of the $(CA/GT)_n$ type in fishes (Gao et al. 2009) and in vertebrates in general (Ellegren 2004; Sekar et al. 2009). It should be noted however that the library constructed in this study may not represent the overall true microsatellite distribution in *L. plumbeus* due to bias in the biotinylated oligos used in subtractive hybridisation. This is also the reason why all of the microsatellite loci detected were dinucleotides. From the 15 unique clones carrying microsatellite loci, ten were amenable to primer design. Some of the sequences were not suitable for primer design due to short available flanking sequences.

Following the definition used by Varshney et al. (2009), functional primer pairs are those that amplified the predicted allele size while non-functional primers amplify nonspecific alleles or nothing at all. The ten species-specific primer pairs were used on 90 individuals of *L. plumbeus.* Results showed that nine primer pairs were functional and one (Lp11) was non-functional. The allele sizes ranged from less than a hundred to 266 bp. Among the functional primers, all but two (Lp5 and Lp31) detected polymorphism across the *L. plumbeus* individuals. The seven pairs revealed a total of 33 alleles at an average of 4.7 alleles per locus. The markers varied in their degree of polymorphism and PIC values ranged from 0.12 (Lp13) to 0.75 (Lp3 and Lp30) with a mean of 0.47. Polymorphism Information Content (PIC) is a measure of the polymorphism of a marker for a certain locus. Based on Botstein et al. (1980), four primers

were highly informative (PIC > 0.50), one was reasonably informative (PIC = 0.5-0.25) and two were slightly informative (PIC < 0.25) (Table 1).

Genetic diversity analyses

Table 2 shows the different diversity parameters of the three populations of *L. plumbeus* computed using the adjusted genotypes. The number of effective alleles (A_e) is an estimate of the number of equally frequent alleles in the population and is corollary to expected heterozygosity (H_e) (Racchi et al. 2014). In the Laguna de Bay (LDB) population, A_e ranged from 1.265 to 3.315 and Lp34 showed the highest gene diversity (uH_e) at 0.732. In the Taal Lake (TL) population, A_e ranged from 1.153 to 3.492 with Lp3 locus showing the highest gene diversity at 0.727. Among the three populations, Sampaloc Lake (SL) showed the widest range of A_e from 1.039 to 5.788 and the highest gene diversity (0.855) in the Lp3 locus.

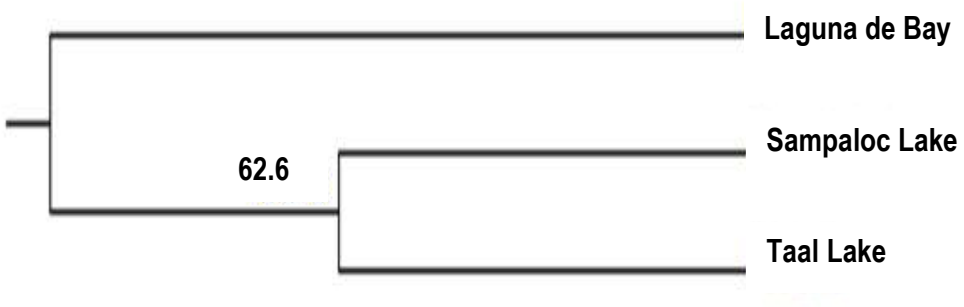

Fig. 2. UPGMA dendogram analysis of three *Leiopotherapon plumbeus* populations constructed using Nei's (1973) genetic distance. Bootstrapped value (626/1000) indicated at the node.

The overall mean H_o and H_e values over all the seven loci and across the three populations of *L. plumbeus* were 0.246 and 0.494, respectively. Moderate level of genetic diversity was found in all three populations (mean uH_e for LDB: 0.488; TL: 0.509; SL: 0.484) and the computed means uH_e and H_o for each population were not significantly different from each other indicating the same level of genetic diversity within each population. Hardy-Weinberg Equilibrium (HWE) exact test showed significant heterozygote deficit in six, four and two loci in the LDB, TL and SL populations, respectively. The highest mean inbreeding coefficient (F_{IS}) was obtained by LDB population at 0.639, followed by TL at 0.486 and SL obtained the least at 0.298. In terms of the effective population size (N_e), the LDB population had the smallest at 2.3 (95 % CI = 1.2-3.8), followed by TL ay 2.5 (95 % CI = 1.7-3.5) and lastly, SL at 8.2 (95 % CI = 0.0-41.2).

Table 2. Estimates of different diversity parameters, Hardy-Weinberg exact test and F_{IS} values for the three populations of *Leiopotherapon plumbeus* using the adjusted genotypes.

Pop	Laguna de Bay					Taal lake					Sampaloc Lake				
Locus	A_e	H_o	uH_e	H-W test p-value	F_{IS}	A_e	H_o	uH_e	H-W test p-value	F_{IS}	A_e	H_o	uH_e	H-W test p-value	F_{IS}
lp3	2.149	0.211	0.549	0.0000*	0.623	3.492	0.607	0.727	0.0005*	0.167	5.633	0.692	0.855	0.0066*	0.197
lp8	3.000	0.000	0.727	0.0014*	1.000	2.571	0.000	0.667	0.0043*	1.000	1.960	0.000	0.527	0.0177	1.000
lp13	1.265	0.143	0.215	0.2345	0.341	1.153	0.143	0.135	1.0000	-0.059	1.039	0.038	0.038	-	-
lp30	2.951	0.552	0.673	0.0008*	0.183	3.113	0.550	0.696	0.2431	0.214	5.788	0.733	0.841	0.1163	0.130
lp34	3.315	0.273	0.732	0.0058*	0.639	2.522	0.154	0.628	0.0008*	0.762	5.143	0.500	0.841	0.0029*	0.416
lp43	1.286	0.036	0.227	0.0001*	0.845	1.400	0.056	0.294	0.0004*	0.815	1.069	0.067	0.066	1.0000	-0.018
lp48	1.398	0.048	0.292	0.0003*	0.840	1.682	0.214	0.421	0.0261	0.500	1.276	0.143	0.222	0.0957	0.362
Mean	2.195	0.180	0.488		0.639	2.276	0.246	0.509		0.486	3.130	0.311	0.484		0.298
SE	0.338	0.072	0.089			0.335	0.090	0.087			0.856	0.121	0.141		

A_e- number of effective alleles; H_o- observed heterozygosity; uH_e- unbiased expected heterozygosity; F_{IS}-inbreeding coefficient; HWE-Hardy Weinberg Equilibrium;*-significantly deviate from HWE at 1 % level of significance

The overall F_{ST} value across the three populations (0.137) suggests moderate genetic differentiation among the populations (Wright 1978). Pairwise comparison among the populations showed significant moderate differentiation between all population pairs (Table 3) wherein SL and TL were the least differentiated at 0.040. On the other hand, pairwise population comparison based on Nei's genetic distance ranged from 0.089 between TL and SL to 0.432 between LDB and TL. The dendrogram constructed using the unadjusted genotypes revealed two main branches with TL and SL clustering together (Fig 2) and this corroborates with the closer relationship between TL and SL populations using the adjusted genotypes as seen in the pairwise F_{ST} and Nei's genetic distances.

Table 3. Pairwise F_{ST} estimates* and genetic distance** among the three populations of *Leiopotherapon plumbeus.*

Population	Laguna de Bay	Taal Lake	Sampaloc Lake
Laguna de Bay	-	0.432	0.394
Taal Lake	0.132	-	0.089
Sampaloc	0.138	0.040	-

*F_{ST} estimates- below diagonal **Nei's genetic distance - above diagonal

Discussion

A previous report on the genetic diversity of *L. plumbeus* populations using allozyme analysis obtained very low H_o and H_e values ranging from 0.0014–0.0099 and 0.0041–0.0140, respectively (Quilang et al. 2008). The higher heterozygosity values obtained from this study could be attributed to the recognised higher level of polymorphism in microsatellite loci than allozymes (Lehmann et al. 1996; Estoup et al. 1998). It is noteworthy that the H_o values in the three populations were less than expected. This is in agreement with the low *L. plumbeus* intrapopulation genetic diversity detected by Quilang et al. (2008) which was hypothesised to be due to the significant decline in the population of silver perch in LDB. In the early 1960s, *L. plumbeus* together with *Glossogobius giurus* Hamilton 1822 ('biyang puti'), and *Arius manilensis* Valenciennes 1822 ('kanduli') comprised 95 % of the annual harvest (83,000 mt) in the lake. Overfishing and snail-dredging caused the collapse of the whole fishery of the lake around 1970 and, although the production of the lake increased again after the collapse, milkfish and tilapia had become the primary choice for aquaculture instead of silver perch (Bagarinao 2001). Several studies showed that decline in the population and overfishing could lead to a decrease in genetic variation (Smith et al. 2013; Pinsky and Palumbi 2014). Yap (2012) reported a minimal but consistent decline in the population of silver perch in Laguna de Bay which poses a potential threat to the species' long-term survival since reduction in genetic variability as population size declines can result in decreased fitness and reduced adaptability (Gregory et al. 2012). H_e and H_o in the TL and SL populations are highly similar. The species was only translocated from Laguna de

Bay to Sampaloc Lake in the late 1950's and in Taal Lake in the early 1970's (Santos et al. 2010). The reported low H_o in this study is in agreement with the hypothesis that the low genetic diversity in translocated populations (SL and TL) indicates the already low genetic diversity in the founding population from LDB (Quilang et al. 2008).

Surveys of both natural and semi-natural populations of fishes at the biochemical genetic level often reveal lower frequencies of heterozygotes than expected under HWE (Waldman and McKinnon 1993). The lower H_o than H_e values and the result of the HWE exact test in this study are suggestive of non-random mating and heterozygote deficit in some loci. Heterozygote deficit could arise due to several mechanisms including inbreeding, population sub-structuring (Wahlund effect), null alleles, natural selection, non-random sampling (Castric et al. 2002; Ruzafaa et al. 2006), variation in effective population size, unequal number of sexes and contemporary decline in spawning population or bottleneck (Brown et al. 2009). The computed positive values for the inbreeding coefficient within individuals or F_{IS} in the three populations seem to suggest the tendency of inbreeding in *L. plumbeus*. The mean F_{IS} values in each population significantly differ from each other with the LDB population having the highest value (0.639) that may indicate a greater degree of heterozygote deficiency or inbreeding in the LDB population. The low effective population size (N_e) obtained in the current study, especially for the LDB population (N_e=2.3), may indicate the occurrence of inbreeding.

Inbreeding occurs when there is mating within relatives. It increases the proportion of homozygotes at all loci and, in effect, decreases the levels of individual genetic diversity. Furthermore, it reduces the over-all diversity of a population when measured as H_o without immediately affecting H_e (Freeland et al. 2011). Inbreeding has been known to occur in natural fish populations especially in freshwater fishes (Waldman and McKinnon 1993; Langen et al. 2011; O'Leary et al. 2013). Small population size, limited dispersal, population sub-structuring and mating preference are some of the demographic factors that could cause inbreeding (Shields 1993). Several studies on freshwater fishes such as the mouth brooding tilapia (*Sarotherodon melanotheron* Rüppell 1852) (Pouyaud et al.1999), Eurasian or English perch (*Perca fluviatilis* Linnaeus 1758.) (Gerlach et al. 2001; Godel et al. 2006) and cichlid *(Pelvicachromis taeniatus* Boulenger 1901*)* (Langen et al. 2011) showed inbreeding due to population sub-structuring and/or behavioural kin preference and recognition. Heterozygote deficiency due to inbreeding was also reported in the overexploited flatfish, plaice (*Pleuronectes platessa* Linnaeus 1758). Interestingly, the emergence of inbreeding in this species was found to coincide with increased fishing mortality that directly reduced the size of the spawning population and/or indirectly disrupted the courtship behaviour of the fish population (Hoarau et al. 2005). It is thus possible that the mentioned collapse in the *L. plumbeus* population due to overfishing and habitat degradation contributed to the lower observed heterozygosity and higher inbreeding coefficient. There is still no report on the biological aspect of inbreeding, kin preference or assortative mating in *L. plumbeus.* This area of research could thus be explored to ascertain

the possibility of inbreeding occurring in the wild population, especially in the LDB population.

Population structuring is another common reason for heterozygote deficit. It is defined as any population subdivision due to deviations from random mating (Avise and Felley 1979). Several studies have shown that for a certain species within a lake or tributary, genetic sub-structuring could occur such that individuals from different parts of the lake form different local populations (Gerlach et al. 2001; Taylor et al. 2001; Northrup et al. 2010). There had been reports on the significant morphological variation between *L. plumbeus* populations from two distant areas (Binangonan and Tanay) of Laguna de Bay (Quilang et al. 2007; Santos et al. 2010). This may indicate sub-structuring of *L. plumbeus* populations within the lake. There had been no report, however, on population substructuring within Taal and Sampaloc Lakes probably due to the smaller sizes of these two lakes compared to Laguna de Bay, the largest lake in the Philippines. Since this study focused only on one area of Laguna de Bay, the occurrence of population sub- structuring cannot be ascertained. With several possible intercalated reasons to account for the observed heterozygote deficit, including the use of loci suggestive of stutter bands, the microsatellite data in this study cannot confidently confirm nor discount any explanation.

In terms of the diversity among the three populations, the present data agree with the greater degree of differentiation between LDB and TL observed using allozyme analysis that compared *L. plumbeus* populations from two areas of LDB (Tanay and Binangonan), TL and SL (Quilang et al. 2008). However, the allozyme data revealed only minimal genetic differentiation among the populations with very low pairwise F_{ST} estimates ranging from 0.0043 between Binangonan, LDB and SL to 0.0386 between Tanay, LDB and TL.

Using landmark-based morphometric analysis, distinct morphological variations among native and translocated populations of *L. plumbeus* had been reported by Santos et al. in 2010. The study showed high similarity in shape and gill raker counts of TL and SL populations. Appreciable similarity was also found between SL and LDB populations but such close similarity was not observed between TL and LDB specimens. The microsatellite data in this study seems to concur with the reportedly higher phenotypic similarity between TL and SL populations. In addition, the morphological dissimilarity of TL and LDB specimens was congruent with the high degree of differentiation between TL and LDB populations.

Congruent genetic and morphological differentiation of populations had been reported in the ray-finned fish *Labeo victorianus* Boulenger 1901(Rutaisire et al. 2005) and shrimp-feeding cichlid fish, *Altolamprologus compressiceps* Boulenger 1898 (Spreitzer et al. 2012). It is important to note that SL and TL populations have the same genetic background because both were from the same source population—Laguna De Bay. Sampaloc Lake and Taal Lake are also deeper compared to the shallower Laguna de Bay (Santos et al. 2010). These factors may have contributed to the observed closer morphological and genetic relationship between TL and SL populations.

Correlation of genetic and environmental variations to morphometric variations had been observed in the Baltic Sea herring (*Clupea harengus* Linnaeus 1758) (Jorgensen et al. 2008) and the Mediterranean goby (*Pomatoschistus tortonesei* Miller 1969) (Mejri et al. 2012). The previously discussed possibility of population sub- structuring within the LDB population may have also contributed to its farther genetic distance from TL and SL populations.

Conclusion

This study reports the successful isolation of ten microsatellite loci from the Philippine-endemic silver perch, *Leiopotherapon plumbeus*. Seventy percent of the designed species-specific primers were able to detect polymorphism across *L. plumbeus* individuals. The study also demonstrated the use of the designed primers in assessing the genetic diversity and relationships among silver perch populations from Laguna de Bay (LDB), Taal Lake (TL) and Sampaloc Lake (SL). The three populations exhibited low intrapopulation diversity with observed heterozygosity (H_o) and expected heterozygosity (uH_e) values ranging from 0.180 to 0.311 and 0.484 to 0.509, respectively. Low but significant interpopulation differentiation was observed with Sampaloc Lake and Taal Lake populations being the most similar obtaining an F_{ST} value at 0.040 and Nei's genetic distance at 0.089. Inferences from microsatellite data obtained in this study can be used in developing breeding programmes and conservation strategies to save the dwindling population of silver perch.

The current microsatellite data may serve as baseline information on the genetic diversity of each population and provide insight on relevant issues (e.g. low genetic diversity, inbreeding) that should be addressed and considered in the conservation management of *L. plumbeus*. Conservation of this species through captive breeding programmes must ensure that there is high genetic diversity in the parental population to maximise the diversity in the stocked population which is vital in the long-term survival of the species. In addition, maintenance of within and among population genetic diversity is an important factor in the survival of a species. The significant but low variation among the three *L. plumbeus* populations observed in this study highlights the need to maintain or, better yet, improve the current diversity of *L. plumbeus* in the wild.

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