Warning of Reducing Genetic Diversity of *Abramis brama* (Berg, 1905) in Gilan Coast Using SSR Markers

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Abstract

Genetic diversity is one of the three levels of biodiversity. The aim of present study was to compare levels of genetic polymorphism between wild Bream populations using seven microsatellite loci. Genetic diversity was investigated by studying samples collected from two regions, the coast of Chamkhale and Bandaranzali of Gilan province. A total of seven microsatellite loci (MFW_7, MFW_{26}, Mcs1EH, Rser10, Bl_1-153, Bl_2-114 and IC_{654}) were used. The average number of alleles in Chamkhale and Bandaranzali coast were 10 and 10.71 alleles, respectively. The numbers of effective alleles were 7.05 and 7.74 alleles in each population. Allele frequency was declined in wild fish due to inbreeding and genetic drift. The mean of observed heterozygosity values were 0.66 and 0.70 in Chamkhale and Bandaranzali coast, respectively. Approximately, all of loci showed deviation from Hardy-Weinberg equilibrium. The genetic similarity and distance between the two populations were 0.316 and 0.684, respectively. The results of Molecular Variance Analysis revealed that genetic diversity within locations was 97 percent, while among them was 3 percent. The F_{st} value was 0.024 that indicates the low genetic differentiation between the two locations which could be explained by the low number of alleles in two populations. Furthermore, the Natural Migration (Nm) between two stations was obtained 16.30. According to the analysis, it seems that *Abramis brama* has not a desirable genetic diversity in the investigated regions.

Introduction

There are several species of fishes from Agnatha to Teleostomi in the Caspian Sea, the largest lake in the world .The *Abramis brama orientalis* (Berg, 1906) from order cypriniformes is one of important endemic fish in the southern Caspian Sea. Furthermore, it habitat in the Anzali wetland and the neighboring Caspian coast (Kiabi, Abdoli and Naderi 1999). Considerable differences have been seen among the Caspian coast based on ecological conditions such as temperature, salinity and morphology. These factors can influence the populations of fishes including *A. brama.* Nevertheless, evidence indicate that there is loss genetic diversity among them (Ghasemi et al, 2007; Keivanshokooh and Ghasemi, 2009). Species ability to survive in the nature is determined by genetic variation that affects their ability to adapt environmental changes. Thus, genetic variation is necessary for the species survival and resistance (Bataillon et al., 1996). Microsatellite DNA markers or simple sequence repeats (SSRs) are tan-dem repeated motif of 1-6 bases found in all prokaryotic and eukaryotic genomes that have been utilized in the assessment of genetic variation and population differentiation studies for a variety of vertebrates (O'Connell and Wright, 1997; Neff and Gross, 2001).This is mainly due to high frequency in genome, Mendelian inheritance, being semi-dominant, small loci size, ease to determine genotype by polymerase chain reaction (PCR) and great polymorphism (Chen et al., 2008; Dewoody and Avise 2000). Recently, microsatellites have been extensively used to evaluate genetic diversity and structure of wild fish species, such as *Liza aurata* (Ghodsi et al., 2011), *Rutilus rutilus caspicus* (Rezae et al., 2009), *Paraschistura bampurensis* (Askari and Shabani., 2013) and *Alosa braschnicowi* (jafari et al., 2014). Since there is no specific SSR primers for these species from gene banks, we used 7 SSR primer pairs from specific species belongs to Cyprinidae. Nowadays, overfishing and degradation of natural environment in Iran resulted in a sharp decline in population of *A.brama.*

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Hence, they need to be protected via a restocking program (Coad, 2014).

Restocking programs are a managerial strategy, in which mature individuals are caught from wild and propagated under controlled conditions. Then, fries are released to their natural habitats and this process is repeated next years (Fiumera et al., 1999). Therefore, Iranian Fisheries Organization (Shilat) tried to increase bream populations through the release of artificially bred fry. In 1986, this artificial reproduction performed using only a single pair of spawned for giving eggs and milt. Bream population increased annually using the generations descended from the same pair. For the preventation of loss genetic heterozygosis through inbreeding, Ghasemi et al (2007) and Keivanshokooh and Ghasemi (2009) offer Shilat to import bream stocks from Azerbaijan.

Currently, stock rehabilitation program of *Abramis brama* is conducted by releasing artificially propagated fries with Azerbaijan stocks to the Caspian Sea.

Now, after several years of implementation of conservation program, the aim of this study is to compare the levels of genetic polymorphism of Bream between the coast of Chamkhale and Bandaranzali of Gilan province. The results of this study will have implications for the conservation of genetic resources of this species. Such information is essential for effective culturing, management and conservation of fish populations. Based on this goal and to make decisions on the brood stock to be used for hatcheries, this work could be used as a guideline of the Iranian Fisheries Organization for a successful repopulation.

Materials and Methods

Samples Collection and DNA Extraction

In Autumn 2012, A total of 60 specimens were collected from two station, Chamkhale (37° 11′N , 30° 10′E) and Bandaranzali (37° 28′ N, 49° 28′E) costal of Gilan province (30 specimens from each station). Total genomic DNA was extracted from fin pectoral tissue by using the traditional proteinase-K digestion and standard phenol/chloroform techniques (Hillis et al., 1996). Approximately, 100 mg tissue was treated with 25µl proteinase K (10 mg/ml) and 50µl sodium dodecyl sulfate (SDS) (10%) in a 500 µl Sodium Chloride-Tris- Ethylenediamine tetra acetic acid (STE) buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M Na_2EDTA, pH: 8.0) overnight at 37°C. After incubation, DNA was isolated by two phenol-chloroform (25 phenol: 24 chloroform: 1 isoamyl

alcohol) steps followed by precipitation with cold absolute ethanol. The quality and concentration of DNA were assessed by 1.0% agarose gel electrophoresis and stored at -20 °C until use (King et al., 2001).

Molecular Analysis

In this study, seven microsatellite markers were amplified by polymerase chain reactions (PCR) using the following primers (Hosseinnia et al., 2014): (Rser10, Ic654, Bl_2-114, Bl_1 -153, Mcs1EH, MFW_7 and MFW_{26}) (Table 1). PCR amplification were carried out in 0.2 ml PCR tubes with an Eppendorf thermal cycler (BIO-RAD, MJ Mini Thermal Cycler). Initial denaturation was achieved at 94°C for 3 min followed by 30 denaturation cycles for 30 s at 94°C, 30 s at the respective annealing temperatures extending to 72°C for 1 min. The final step was extended to 3 min at 72°C. PCR products were separated using 8% polyacrylamide gels stained with silver nitrate (Rajora et al., 2000).

Statistical Analysis

The presence of null alleles was tested using Microchecker version 2.2.3 (Van Oosterhout et al., 2004). Allelic and genotypic frequencies, observed (Ho), expected heterozygosity (He), test for deviations from Hardy-Weinberg Equilibrium (HWE), Fst values and number of migrant (Nm) were calculated by Genealex ver. 6.5 Software (Peakall and Smouse, 2012). Deviation distribution, genetic distance, genetic identity (Nei, 1978), Difference in Ho, He and allelic variation was determined by Wilcoxon's test using statistical software, SPSS v. 16.

Results

In this study, structure and genetic diversity of *A. Brama* was studied at seven microsatellite loci for two stations in Iran. Micro-checker showed no evidence for large allele dropout or stutter-band scoring at any of the five loci but null alleles can exist in all loci. Allele sizes ranged from 100 to 276 bp (Table 1). The Bl_2-114, MFW26 and Rser10 primer showed the maximum allele number (13) compared to other primers. No statistically significant difference was observed between the numbers of alleles per each locus (Table 2). The average number of effective alleles per locus (Ne) in Chamkhale and Bandaranzali were 7.05 and 7.74, respectively, showing no significant difference (*P*>0.05) between two populations (Wilcoxon-Mann-Whitney test).

Table 1. Characteristics of *Abramis brama* microsatellite loci used in the present study

Microsatellite locus	Primer sequence (5→3)	N	Size (bp)	Annealing (°C)	Gene bank ID number
Bl2-114	F:ATCACTGCCATTTTATTA R:CTGCTCCGCTCTGTTCCA	13	168-272	52	FJ468352
Bl1-153	F:GCACAGCTCTAATCGGTCACT R:TATGGTCAAACACGGGTCAA	8	200-276	53	FJ468350
Mcs1EH	F:ACCGGGCTTTAGGCTGTTGGTCA R:TGAGACACATCCCATCACTGCCTACG	9	100-200	59	AY770926
Ic654	F:TGAGCCGACACTAGAAACAGAGC R:GACAAAGTGCAGGCACAGAATG	9	128-172	52	EU252096
MFW7	F:TACTTTGCTCAGGACGGATGC R:ATCCCTGCACATGGCCACTC	7	160-200	61	-
MFW26	F:CCCTGAGATAGAAACCACTG R:CACCATGCTTGGATGCAAAAG	13	100-148	48	-
Rser10	F:TGCGTAATCGTGAAGCGGTG R:GCCACTAAAGCGCAGAAGCC	13	160-232	57	AJ312850

Table 2. Genetic diversity parameters for seven microsatellite loci in *A. Brama*

Location		Bl2-114	BL1-153	MFW7	MFW26	Rser10	IC654	Mcs1EH
Chamkhale	N_a	12	13	8	7	13	8	9
	N_e	9.412	10.127	5.674	4.520	8.333	4.678	6.667
	H_o	0.70	1.00	0.55	0.30	0.65	0.70	0.75
	H_e	0.894	0.901	0.824	0.701	0.880	0.786	0.850
	Fis	0.217	-0.110	0.332	0.615	0.261	0.110	0.118
	P_{Hw}	Ns	Ns	Ns	**	***	Ns	Ns
Bandaranzali	N_a	14	13	10	7	13	9	9
	N_e	10.667	9.639	7.080	5.063	10.127	5.479	6.154
	H_o	0.85	0.85	0.90	0.60	0.85	0.55	0.65
	H_e	0.906	0.896	0.859	0.803	0.901	0.818	0.838
	Fis	0.062	0.052	-0.048	0.252	0.057	0.327	0.224
	P_{Hw}	Ns	Ns	***	***	Ns	***	***

The number of observed allele (Na), Ne, observed heterozygosity (Ho), expected heterozygosity(He) and fixation index (F_{is}) are shown in Table 2. H_o and expected H_e means for all samples were 0.30-1.00 and 0.701-0.906 respectively. In the Chamkhale sample, the mean for heterozygosity and expected heterozygosity values were 0.664 and 0.833, respectively. In the Bandaranzali samples, these values were 0.75 and 0.860, respectively, but there was no significant difference in the average expected and observed heterozygosity between the populations (Wilcoxon-Mann-Whitney test). All seven loci were tested for deviation from the HWE (Table 2). There were significant deviations from

Hardy -Weinberg equili-brium at most of the loci in the two populations. After sequential Bonferroni correction (Rice, 1989), among the 14 population-locus tests (2 populations × 7 loci) six out of 14 possible HWE tests were statistically significant. Fixation index (Rst , Fst) were calculated 0.010 and 0.024 between two wild fish populations; respectively. These values were calculated according to the formula [Nm = [(1 / F_{st}) - 1] / 4] between populations. The mean Nm was obtained as 15.509, between Chamkhale and Bandaranzali station, and the minimum and maximum amount calculated for loci MFW_7 and loci Bl_1-153 were 0.138 and 15.978, respectively (Table 3).

Table 3. FST and Nm for tested

	Bl2-114	Bl1-153	MFW7	MFW26	Rser10	Ic654	Mcs1EH
F_{ST}	0.016	0.015	0.038	0.007	0.015	0.016	0.039
Nm	15.319	15.978	6.410	33.289	16.570	14.919	6.081

The average content of F_{is} index was 0.223 across all loci. Furthermore, the analysis of molecular variance and index F_{st} in 99% showed the high genetic diversity (97%) within populations and the low genetic variation among populations (3%) (figure 1).
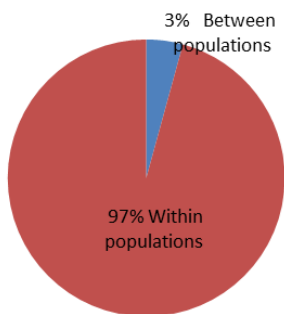
percentage of genetic diversity

Figure 1. The distribution of genetic diversity on F_{st} standard

Genetic distances and similarities (Nei et al, 1975) computed between the Chamkhale and Bandaranzali fish populations were 0.316 and 0.684, respectively (table 4).

Table 4. Genetic identity (regular font) and genetic distance (bolded font) of *Abramis brama* originated from Chamkhale and Bandaranzali coast

Area	Chamkhale coast	Bandaranzali coast
Chamkhale coast		0.68
Bandaranzali coast	**0.31**	

Discussion

Genetic diversity is important for ecological and evolutionary processes ranging from individual fitness to ecosystem function. Heterozygocity and allele number are important parameters in population genetic variation which determine the ability of organism to compete and survive in natural habitats (Reed, 2009; Hakansson and Jensen, 2005; Frankharn, 2008; Diz and Persa, 2009). In genetic variation studies, allelic richness is more worthy than heterozygocity. In fact, higher allelic richness shows higher effective population size and use of allelic richness is suitable for populations which are treated by selection or conservation programs (Diz and Persa, 2009). In the case of allele number, Ho and He, the present study is in line with the previous report on *Abramis brama* with the different primers (Keivanshokooh

and Ghasemi, 2009). The results of this study indicated that the average number of alleles per locus and observed heterozygosity were comparable, but there was no significanct difference between two populations (p>0.05). In our result, observed heterozygosity were higher than the average for freshwater fish (Ho = 0.54 ± 0.25, Dewoody and Avise, 2000). The mean number of alleles are little more than that noted for fresh water fish (Na = 9.1 ± 6.1, Dewoody and Avise, 2000) and anadromous fish (Na = 10.8 ± 7.2, Dewoody and Avise, 2000). The allelic diversity and heterozygosity are both indicative of genetic variation, but allele number is dependent on the effective population size much more than that of heterozygosity (Nei et al., 1975). The Iranian Fisheries Organization produces and releases up to 15 million bream fry into the Anzali wetland annually. Because the hatchery population used for restocking has been founded with a small effective number of parents (Ne), it is likely that these populations have lost some alleles during restocking. Loss of allelic variation has also been reported with (Ghasemi et al, 2007; Keivanshokooh and Ghasemi, 2009). In the genetic population studies allele number is used to determine diversity and is preferred to heterozygosity, because allele number may be lost faster than genetic heterozygosity loss, and low frequency alleles contribute little to overall heterozygosity (Lind et al., 2009). It seems that artificial interbreeding of the Iranian and Azeri broodfish in hatcheries slightly improve the genetic heterozygosity of the Iranian stocks. Unfortunately, number of allels is not satisfying. As showed in table 2, Ho was less than He (Ho= 0.70, He= 0.83). A high gene flow and a low number of specimens are some reasons for a low Ho (Li et al., 2009, Skalla et al., 2004). Eight of 14 tests showed a significant deviation from HWE. Beyond the hypothesis of null alleles, genetic drift and inbreeding are likely to be the causes for deviation from the H–W disequilibrium. In addition, the values obtained from inbreeding index (F_{is}) showed significant defference (p< 0.05), indicating increased inbreeding and nonrandom mating (Xu et al., 2001).

Analysis of molecular variance (AMOVA) is a suitable criterion to assess population structure, and to determine the differentiation and genetic similarity between populations (Grassi et al, 2004). According to our obtained F_{st} index, the genetic diversity between the two populations was 3%. The F_{st} index mean was about 0.024, which represents the low differentiation between two populations. According to Wright (1987), F_{st} value of less than

0.05 indicates low differentiation among communities.

In this study, number of migrant's average was reported as 15.50, that was in line with previous study on *Abramis brama* populations which had Nm = 16.30 (Keivanshokooh and Ghasemi, 2009). Li et al. (2007) reported that when Nm> 1, it can be an agent factor for number of migrant and low genetic diversity between populations.

In present study, the genetic identity was 0.68. Shaklee et al, (1982) and Thorpe and Sol-Cave (1994) showed that genetic distance values (Nei, 1972) for cenospecies populations averaged 0.05 (range: 0.002- 0.07), averaged 0.30 for congeneric species (range: 0.03-0.61), and ranged from 0.58 to 1.21 for confamilial genera. The genetic distance between the Chamkhale and Bandaranzali populations falls within the range of congeneric species, suggesting their genetic convergence.

Conclusion

The results of this study suggested that genetic diversity of *A. brama* was rather high in Gilan coasts of the Caspian Sea, but genetic variation between two populations was very low (3%). It seems that mode of reproduction and no appropriate release of larvae are main reasons for genetic differentiation and also, genetic bottleneck in the Bream populations. Restoration of rivers where this species spawned there and providing natural reproduction of brood stocks are best ways for preventing this process.

Acknowledgment

References

1. Askari Gh. and Shabany A. (2013) Microsatellite loci to determine population structure of *Garra rufa* (Heckel, 1843) in the Khuzestan Province (Iran). International Journal of Aquatic Biology 1:4: 188-194.

2. Avise J. C. (1994) Molecular Markers, Natural History and Evolution. Chapman and Hall, New York. 551 pp.

3. Bataillon T. M., David J. L. and Schoen D. J. (1996) Neutral genetic markers and conservation: Simulated germplasm collections. Genetics 144: 409-417.

4. Chen L., Li Q. and Yang J. (2008) Microsatellite genetic variation in wild and hatchery populations of the sea cucumber (*Apostichopus japonicus Selenka*) from northern China. Aquaculture 39: 1541-1549.

5. Coad B. (2014) Fresh water fishes of Iran. Retrieved from http:// www. briancoad.com/ contents.htm.

6. Crooijmans R. P. M. A., Bierbooms V. A. F., Komen J., Van der poal J. J. and Groenen M. A. M. (1997) Microsatellite markers in common carp (*Cyprinus carpio L.*). Animal Genetics 28: 129-134.

7. Dewoody J. A. and Avise J. C. (2000) Microsatellite variation in Marin, freshwater and anadromous fishes compare with other animal. Journal of Fish Biology 56 : 61-473.

8. Diz P. A. and Presa P. (2009) The genetic diversity pattern of *Mytilus alloprovincialis* in Galician Rías (NW Iberian estuaries). Aquaculture 287: 278-285.

9. Ferguson A., Taggart J. B., Prodohl P. A., McMeel O., Thompson C., Stone C., McGinnity P. and Hynes R. A. (1995) The application of molecular markers to the study and conservation of fish populations with special reference to Salmo. Journal of Fish Biology 47: 103-126.

10. Fiumera A. C., Wu L., Parker P. G. and Fuerst P. A. (1999) Effective population size in the captive breeding program of the Lake Victoria cichlid *Paralabidochromis chilotes*. Zoo Biology 18: 215-222.

11. Frankham R. (2008) Genetic adaptation to captivity in species conservation programs. Molecular Ecology 17: 325-333.

12. Ghasemi A., Keivanshokooh S., Shahriari-Moghadam M., Khara H. and Sourinejad I. (2007) Genetic comparison of Iranian and Azeri populations of the oriental bream *Abramis brama orientalis* (Berg) using microsatellites. Aquaculture Research 38: 1742-1746.

13. Ghosi Z., Shabani A. and Shaabanpour B. (2011) Genetic diversity of *Liza aurata* (Risso, 1810) in the coastal regions of Golstan province, using microsatellite marker. Taxonomy and Biosystematics 6: 35-46.

14. Grassi F., Imazio S., Gomarasca S., Citterio S., Aina R., Sgorbati S., Sala F., Patrignani

G. and Labra M. (2004) Population structure and genetic variation within *Valeriana wallrothii* Kreyer in relation to different ecological locations. Plant Science 166: 1437-1441.

15. Hakansson J. and Jensen P. (2005) Behavioural and morphological variation between captive populations of red jungle fowl (Gallus gallus) – possible implications for conservation. Biological Conservation 122: 431-439.

16. Hillis D. M., Mortiz C. and Mable B. K. (1996) Molecular systematic. Sinauer Association Inc. Sunderland, MA. 655 pp.

17. Hosseinnia Z., Shabany A., Kolangi miandare H. (2014) Comparison of genetic variation of wild and farmed Bream (*Abramis brama orientalis; berg, 1905*) using microsatellite markers. Molecular Biology Research Communications 3: 187-195.

18. Jafari O., Shabany A. and Kolangi Miandare H. (2014) A study of genetic population of *Alosa braschnicowi* (Borodin, 1904) in Sari and Mahmodabad coasts in the Caspian Sea, using Microsatellite loci. International Journal of Aquatic Biology 2:1, 20-26.

19. Keivanshokouh S. and Ghasemi A. (2009) Genetic diversity of *Abramis brama* using microsatellite marker. Modern Genetic 5: 47-52.

20. Kiabi B. H., Abdoli A. and Naderi M. (1999) Status of the fish fauna in the south caspian basin of Iran. Zoology in the Middle East 18: 57-65.

21. King T. L., Kalinowski S. T., Schill W. B., Spidle A. P. and Lubinski B. A. (2001) Population structure of Atlantic salmon (*Salmo salar L.*): a range-wide perspective from microsatellite DNA variation. Molecular Ecology Notes 10: 807-821.

22. Li J., Wang G. and Bai Z. (2009) Genetic variability in four wild and two farmed stocks of the Chinese freshwater pearl mussel (*Hyriopsis cumingii*) estimated by microsatellite DNA markers. Aquaculture 287: 286-291.

23. Lind C. U., Evans B. S., Knauer J., Taylor J. J. U. and Jerry D. R. (2009) Decreased genetic diversity and a reduced effective population size in cultured silver-lipped pearl oysters (Pinctada maxima). Aquaculture 286: 12-19.

24. Liu Z. J. and Cordes J. F. (2004) DNA marker technologies and their applications in aquaculture genetics. Aquaculture 238: 1–37.

25. Lundrigan T. A., Reist J. D. and Ferguson M. M. (2005) Microsatellite genetic variation within and among Arctic charr (*Salvelinus alpinus*) from aquaculture and natural populations in North America. Aquaculture 244: 63-75.

26. Neff B. D. and Gross M. R. (2001) Microsatellite evolution in vertebrates: inference from AC dinucleotide repeats. Evolution 55: 1717–1733.

27. Nei M. (1978) Estimation of average heterozygosity and genetic distance from small number of individuals. Genetics 89: 583-590.

28. Nei M., Maruyama T. and Chakraborty R. (1975) The bottleneck effect and genetic variability in populations. Evolution 29: 1-10.

29. O'Connell M. and Wright J. M. (1997) Microsatellite DNA in fishes. Fish Biology 7: 331-363.

30. Oosterhout C. V., Hutchinson W. F., Wills D. P. M. and Shipley P. (2004) MICRO CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535-538.

31. Park L. K. and Moran P. (1995) Developments in molecular genetic techniques in fisheries. Molecular Genetics in Fisheries. London.

32. Rajora O. P., Rahman M. H., Buchert G. P. and Dancik B. P. (2000) Microsatellite DNA analysis of genetic effects of harvesting in old-growth eastern white pine (*Pinus strobus*) in Ontario, Canada. Molecular Ecology 9: 339-348.

33. Reed D. H. (2009) when it comes to inbreeding: slower is better. Molecular Ecology 18: 4521-4522.

34. Rezae M., Shabany A., Shaabanpoor B. and Kashiri H. (2010). Study of genetic structure of *Rutilus frisii kutum* in Golestan province coastal watersusing microsatellite markers. Iranian Scientific Fisheries Journal 19: 151-156.

35. Shaklee J. B., Tamaru C. S. and Waples R. S. (1982) Speciationand evolution of marine fishes studied by electrophoretic analysis of proteins. Pacific Science 36: 141-157.

36. Skalla A., Hbyheim B., Glover K. and Dahle D. (2004) Microsatteletie analysis in domesticated and wild Atlantic salmon. Aquaculture 240: 131-143.

37. Thorpe J. P. (1982) The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematic. Annual Review of Ecology and Systematics 13: 139-168.

38. Weber J. L. and May P. E. (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain-reaction. Am. J. Hum. Genet 44: 388–396.

39. Wright S. (1987) Evolution and the genetics of populations, vol. 4: Variability within and among natural populations. University of Chicago Press, Chicago. 590 pp.

40. Xu Z. h., Primavera J. H., Dela Pena L. D., Pettit P., Belak J., Alcivar-Warren A. (2001) Genetic diversity of wild and cultured Black Tiger Shrimp Penaeus monodon in the Philippines using microsatellites. Aquaculture 199: 13-40.

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