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Genetic diversity of mosquito fish(*Gambusia affinis*) in hatchery and wild populations

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Abstract. The invasive mosquito fish *Gambusia affinis*, affected hatchery populations and abundant in different wetlands of Jashore regions which carry ectoparasites. This study evaluated genetic diversity between hatchery and wild populations of *G. affinis* using polymorphic SSR DNA markers, which have not yet been clarified. A total of 50 polymorphic SSR and microsatellite alleles were identified by electrophoresis with a mean value of polymorphic information content (PIC) of 0.94, highest PIC values (0.97) were found for CytBand Gaaf15 marker, and lowest values (0.92) for Gaaf14, Gaaf22, and Gaaf23.A total of 50 (ranges 165-452bp) DNA fragments (alleles) were scored, of which 307 fragments were highly polymorphic with cytBSSRmarker. A UPGM Adendrogram constructed based on Nei's coefficient, generated by SSR DNA marker showed very limited genetic variation. Genomic sequences of DNA fragments for each population were found genetically closest with good adaptability in changing environmental scenario. The SSR marker techniques are found to be the rapid and effective tool for diversity assessment, this result might be helpful to the development of sterile *G. affinis* to control further reproduction.
Keywords: Mosquito fish (*Gambusia affinis*), PCR amplification, DNA sequencing, Genetic diversity

Introduction

The western (*Gambusia affinis*) is a species of freshwater fishcommonly known as mosquitofish. The worldwide distribution of *G. affinis* and *G. holbrooki* had been largely unclear until a few years ago, mainly because of taxonomic confusion (Pyke 2008, Vidal *et al*. 2010). No taxonomic record is available from Bangladesh literature about the mosquito fish (Shafi and Quddus 1982, IUCN 2000, Rahman 2004, Akhter 2007). Because of their reputation as mosquito-control agents, *G. affinis* has been stocked routinely and indiscriminately in temperate and tropical areas around the world. Mosquitofish were introduced directly into ecosystems in many parts of the world as a biocontrol to lower mosquito populations which in turn had negatively affected many other species in each distinct bioregion. Mosquitofish is distributed all over Bangladesh but especially highly abundant in Jashore region. It is found in different habitat including the natural ditches, carp rearing ponds, drains, and nursery ponds. It carries parasites such as *Lernaea* sp. which is a major threat for the hatchery population. To overcome the issue, a broad study on genetic diversity in hatchery and wild population is obligatory to control the species of *G. affinis*.

The evolutionary forces determine local divergence as well as the temporal components of the genetic diversity of mosquito fish. Such genetic information could assist in controlling invasive success and prevent further expansion of current populations. The genetic diversity and

their comparison between hatchery and wild population of *G. affinis* will facilitate the way to understanding the population structure of the species. Using microsatellite DNA markers, and CytB gene sequence markers we assessed the genetic diversity of mosquitofish in hatchery and wild populations.

Materials and Methods

***Sample collection*:** A total of 180 individuals from six different populations (30 each) of *G. affinis* were collected from Jashore region. DNA. The samples were taken in an icebox full of crushed ice and brought to the Laboratory of Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA) and stored at -20°C. Detail information about the sampling location and habitat is listed in Table I.

Table I. Relevant information on sampling sites of six different populations of *G. affinis*

Category	Habitat	Geographical position	Water depth (feet)	Sample size (inch)	Number of samples
Hatchery	Carp rearing pond (semi-intensive)	23°10′05″N 89°12′15″E	5-6	1-1.5	30
	Brood culture pond	23°10′05″N 89°12′15″E	5-6	1-1.5	30
	Nursery pond (High density)	23°10′05″N 89°12′15″E	3-4	1-2	30
	Drain (outlet of the pond)	23°10′05″N 89°12′15″E	1-2	1-1.5	30
Wild	*Beel*	23°10′05″N 89°12′15″E	5-11	1-2	30
	Drain (outlet of city)	23°10′05″N 89°12′15″E	2-3	1-2	30

***Genomic DNA isolation and quantification*:** From each fish sample, approximately 30mg of flesh was cut into slight pieces with the help of scissors and forceps and taken in 1.5ml micro-centrifuge tube. Genomic DNA was extracted from the fish using Proteinase-K digestion, phenol-chloroform-isoamyl alcohol purification and ethanol precipitation method (Islam and Alam., 2004).The DNA quality was checked by on 1% agarose gel electrophoresis using a high-performance ultraviolet trans-illuminator of a gel documentation system (BioDoc-It™Imaging system), the purity and concentration of eluted DNA were measured using a Nanodrop 2000-Spectrophotometer (Nanodrop Calibri, Titertek Berthold, Germany).

PCR amplification of SSR markers and gel-electrophoresis of PCR products: The amplification of DNA was performed following the method(Bala et al., 2017)with some modification. We used five sets of SSR primers isolated from *G. affinis* for PCR (Purcell et al., 2011) (Table II). The amplified PCR products of the SSR primers were separated electrophoretically on 1% agarose gel containing ethidium bromide in a 1×TAE buffer at 120 V for 90 min. TrackIt™ 1kb PlusDNA Ladder was used as a molecular weight marker. DNA bands were observed on a high-performance, ultraviolet transilluminator and photographed with a gel documentation system (BioDoc-It™Imaging system, Cambridge, UK).

Table II. The total microsatellite SSR markers used to resolve various reagents of the genome sequence included gene-specific marker with repeat motif

Name of primer	Base sequence	No of Base	Product size (bp)	Repeat motif	Annealing Temp. °C
Gaaf10_F	GAACTGAACCACCCAAAGGC	20	252-386	(ATCC)	59.4
Gaaf10_R	TCCATCTGGAGACAGGTGTG	20			59.4
Gaaf14_F	ATCCTTGCCAGATAGAACGTC	21	253-339	(GGAT)	57.9
Gaaf14_R	TGGATCCTAACACAACCTGGG	21			59.8
Gaaf15_F	TGCATGTGTGTTTGGTAAGG	20	148-166	(AATG)	55.3
Gaaf15_R	GATCCCTGTTACACTGCTGG	20			59.4
Gaaf22_F	ATGCGACCTGAAACTTCTGC	20	249-300	(ATC)	57.3
Gaaf22_R	CCGAGGTCCTTGAGGTTTATAG	22			60.3
Gaaf23_F	TCCTCTTGGCATTGCTGAAAC	21	170-220	(GAT)	57.9
Gaaf23_R	GGCTGCTCCATCCAATGTG	19			58.8
CytBF1	ATG GCC AAC CTA CGA AAA AC	20	307	-	55.3
CytBR1	GGG TAG RAC ATA ACC TAC GAA G	22			59.3

***Scoring of the SSR genotypes*:** The gene-scan files of the electrophoresis gel were analyzed with the PyElph GUI software (PyElph is its interactive Graphical User Interface (GUI), © 2012 Pavel and Vasile; licensee BioMed Central Ltd) to reveal the genotypes from the PCR amplified DNA fragments for gel images analysis and phylogenetics. The polymorphism information content (PIC) values were computed using the formula of (Milbourne *et al*. 1997) and (Liu *et al*. 2011).

$$PIC = 1 - \sum_{J=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of a unique genotype of j_{th} allele assuming that each SSR marker represents a single locus with n of SSR genotypes.

***PCR amplification of CytB gene and sequencing*:** An approximately 307 bp fragment of the CytB gene was amplified from the *G. affinis* DNA samples by using the universal primer pairs CytBF1/CytBR1 (Table II). The fragment was purified and sequenced using Big Dye Terminator v3.1 (Life Technologies, Thermo Fisher, Mulgrave, VIC) and resolved using a 3500xL genetic analyzer (Life Technologies). The resolved regions were incorporated into the genome sequence using Sequencer v5.1 (Gene Codes, Ann Arbor).

***Data Analysis*:** Allelic frequency, gene diversity and polymorphism at five SSR loci in six different populations of *G. affinis* were analyzed using PyElph GUI software. Clustering of different population of *G. affinis* using the unweighted Pair Group Method of Arithmetic Mean (UPGMA) algorithm, which was constructed on the basis of (Nei 1972)genetic distance summarizing differentiation according to microsatellite profiles (Excel to NTedit 1.1, checked coefficient SM (Similarity) by SimQual method and clustered (SAHN) UPGMA method using NTSYSpc 2.2 software).The mitochondrial CytB gene sequences were analyzed using BLAST, computers a pair-wise significant alignment between a query and the database sequences based on partial DNA nucleotides sequences in NCBI.

Results

***Allelic and loci variation within the genotypes*:** A total of 44 alleles were identified using five sets of SSR primers from the DNA of 36 genotypes of *G. affinis* using agarosegel electrophoresis detection system (Fig. 1). The polymorphism information content (PIC) values of those markers varied from 0.92 to 0.97 with an average of 0.94 (Table III). Generally, highest PIC value (0.97) were found for cytBandGaaf15 marker and lowest PIC value 0.92 (Gaaf14, Gaaf22, and Gaaf23)tested to *G. affinis*in six populations.

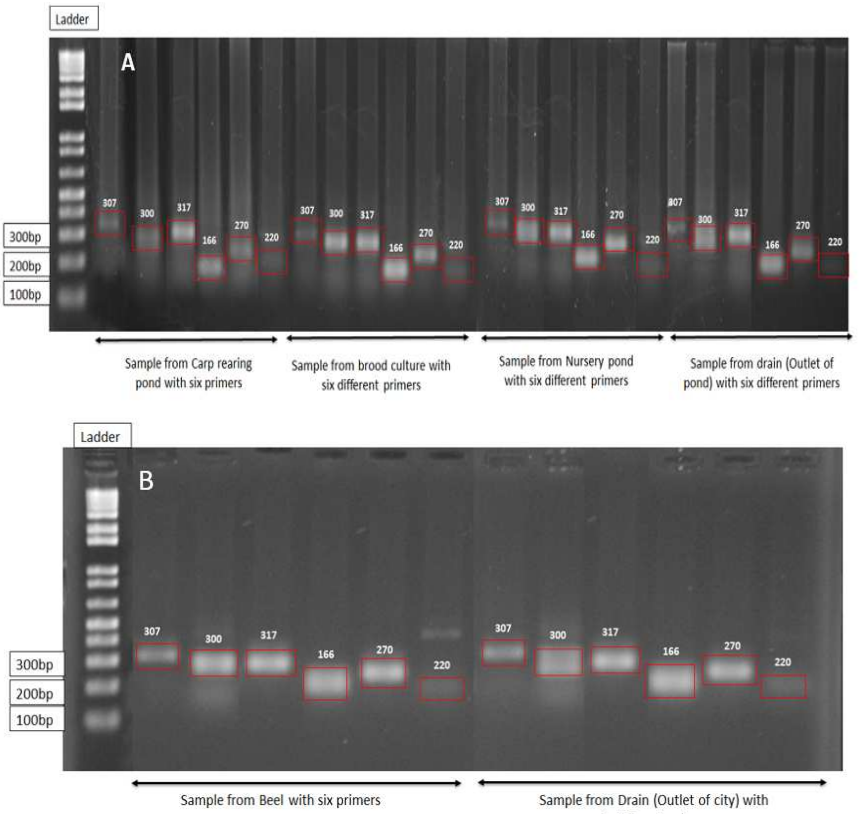

Fig. 1. Electropherogram of five microsatellite loci (Gaaf10, Gaaf14, Gaaf15, Gaaf22 and Gaaf23) amplified from *G. affinis* samples collected from six locations in Jashore region. A (hatchery populations: lane 2-25): lane 1: ladder; lane 2-7: carp rearing pond; lane 8-13: brood culture pond; lane 14-19: nursery pond; lane 20-25: drain of pond; B (wild populations: lane 2-13): lane 2-7: Beel; lane 8-13: municipal drain.

UPGMA dendrogram: The unweighted pair group method of averages (UPGMA) dendrogram based on genetic distance computed by(Nei 1972)resulted in two major clusters: the four hatchery populations alone were in one cluster and the remaining two wild populations in the other cluster (Fig. 2).

Table III: The number of alleles, allele sizes, and polymorphism information content (PIC) at five SSR loci of *G. affinis* collected from six locations

No.	Marker	Size range (bp)	No of bands	Alleles (bp); automatically computed using PyElph GUI software*	PIC
1	Gaaf10	291-452	8	291, 298, 302, 302, 322, 336, 452, 452	0.95
2	Gaaf14	165-295	10	165, 171, 171, 172, 239, 257, 270, 270, 272, 295	0.92
3	Gaaf15	171-213	6	171, 175, 183, 193, 193, 213	0.97
4	Gaaf22	170-452	10	170, 176, 215, 225, 237, 237, 255, 302, 368, 425	0.92
5	Gaaf23	170-239	10	170, 174, 175, 175, 177, 220, 228, 233, 233, 239	0.92
	Total		44		0.936

*The molecular weights are fitted after a linear electrophoresis migration model:
in (Weight) = $\alpha *$ Migration Distance + β, $\alpha < 0$, $\beta > 0$(Pavel and Vasile, 2012)

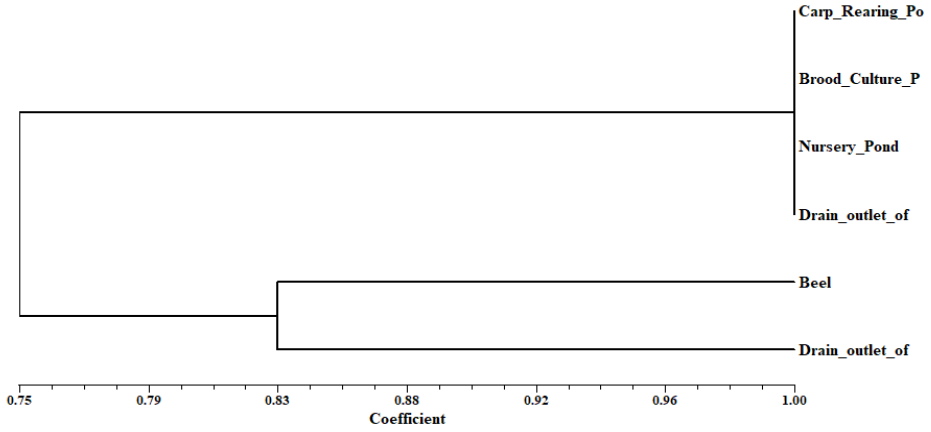

Fig. 2. Clustering of different population of *Gambusia affinis* using the unweighted pair group method of arithmetic mean (UPGMA) algorithm, which was constructed on the basis of (Nei 1972)genetic distance summarizing differentiation according to microsatellite profiles.

***CytB gene sequence*:** The DNA sequences of partial fragment of the CytB gene of *G. affinis* has been checked in NCBI using standard nucleotides BLAST. The CytBsequences of *G. affinis* produces significant alignment with query cover (96%) and identities (98%) between *G. affinis* sequences. Comparisons between genome sequences are summarized in Table IV.

Table IV. Identity of CytB sequence of *G. affinis* samples collected from Jashore region with standard nucleotides FASTA sequences in NCBI

Accession	Species	Max Score	Total score	Query cover	Identity
EF017514.1	*Gambusia affinis*	605	605	96%	98%
DQ075686.1	*Gambusia affinis*	605	605	96%	98%
AP004422.1	*Gambusia affinis*	605	605	96%	98%
HM443906.1	*Gambusia affinis*	601	601	96%	97%
DQ075683.1	*Gambusia affinis*	601	601	96%	97%
AF412123.1	*Gambusia affinis*	601	601	96%	97%

Discussion

Mosquito fish *G. affinis* is one of the carriers of parasites such as anchor worm *Lernaea* sp. which spread the disease lernaeasis all over the freshwater system especially in the culture system (Hossain *et al*. 2018). The history of the introduction of mosquitofish still remains unknown and analysis of genetic diversity of mosquitofishhas not been done yet in Bangladesh. Environmental factors and genetic modification are evolved to change the genetic characteristics of fish. One of the major objectives of the present study was to assess genetic variation among different stocks of *G. affinis*. All studied specimens from Jashore region sampling sites yielded the exact same sequences of the CytBfragment. Based on the low degree of genetic variability, all mosquito fish in six Upzilla of Jashore district may have been introduced from the same source population.

The mosquitofish introductions continue to impose a massive threat not only to the conservation of indigenous biodiversity but also to fisheries (Arthington and Marshall 1999, Rowe *et al*. 2007). Where mosquitofish became invasive, they tend to cause decreases in populations of native aquatic species, including different detritivorous, zooplankters (rotifers, crustaceans, backswimmers etc.), amphibians and fishes, all of which have the potential to affect vital components of ecosystem functioning (Margaritora *et al*. 2001,Rincón *et al*. 2002,Blanco *et al*. 2004, Mills *et al*. 2004). Even the introduction of only a few individuals bears to the risk of local population establishment and further range expansion, as mosquitofish have a high reproductive potential (Vargas and De Sostoa 1996).

In this study, we have investigated genetic diversity in *G. affinis* samples collected from hatchery and wild sources of Jashore region by using microsatellite and mitochondrial CytB DNA markers. The 5 SSR primer pairs amplified a total of 44 polymorphic alleles detectable by the gel electropherogram platform, of which no alleles have never been reported before in Bangladesh. Every primer pair was able to amplify varying numbers of SSR alleles from all accessions tested, regardless of their geographical origins. The DNA sequence of 6 fish generated by a universal primer pair for the CytB produced were identical. Therefore, this cytB SSR primer pairs could not detect any variations among the hatchery and wild samples of *G. affinis*. However, the CytB gene sequence correctly identified the species as *G. affinis* though BLAST analysis (Table IV).

The sizes of alleles obtained at the 5 SSR loci varied from 165-452bp with an average PIC content of 0.936 which is very high and effective for studying genetic diversity. Therefore, for genetic diversity analysis of *G. affinis*, the nuclear microsatellite markers are more effective than the mitochondrial CytB gene sequence. The overall genetic variation values from this study were higher than those reported by (Purcell and Stockwell, 2015). We hypothesize that this phenomenon was due to the utilization of a larger number of SSR and microsatellite primer pairs and the large number of accessions from diverse *Gambusia* species in our study. A UPGMA dendrogram was constructed based on the genetic distance calculated from the polymorphic allele frequencies of five microsatellite loci resulted in two clusters: The fish originated from hatchery and fish farms formed one cluster and the fish originated from *beel* and out-let of the city drain formed another cluster. The information about genetic diversity might be utilized in future control treatment programs for eradication of *G. affinis*. We used combined molecular information (based on phylogenetic analyses of sequence variation of the mitochondrial

cytochrome b gene) to confirm the presence of only one species, *G. affinis*, across six sampling sites in greater Jashore district of Bangladesh. The finding from the current study will help broaden our understanding of the population genetic structure and genetic diversity of *G. affinis*. The information on the genetic identification of the present study may be used as a starting point information regarding the control program to eradicate the invasive fish species in our native fish culture region.

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