





Using Inter Simple Sequence Repeat Multi-Loci Markers for Studying Genetic Diversity in Guppy Fish

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Abstract

The aquarium fish industry has become a capacity for job creation and income generation. Aquarium fish were used for decoration and are creating tranquility in the environment and beautification in public places. This study investigated genetic variation between different populations of various varieties of guppy ornamental aquarium fish using inter simple sequence repeat (ISSR) markers in Iran for the first time. DNA was extracted from twelve strains. The polymerase chain reaction was performed using seven ISSR primers. GenAlex 6.501 and XLSTAT software were used to analyze data. Results of analysis of molecular variance showed that 54% of observed polymorphism belongs to variance among populations, and 46% corresponds to diversity between populations. The highest and the lowest polymorphism percentage belonged to (GAG)5GC (90%) and (AG)8C (41%) markers, respectively. The percentage of polymorphic loci ranges from 32.31% to 86.15%. The results of the phylogenetic tree showed that this tree was divided into two major clusters. Cluster I included only two populations and cluster II contained the other ten studied populations. It is suggested that this marker system was efficient in discriminating each genotype at the molecular level and can be used for genetic diversity analysis for aquarium guppy fish because a greater understanding of the species' potential is necessary for supporting long-term genetic improvement.

Introduction

With the increasing use of aquarium fish for decoration and creating tranquility in the environment, and beautification in public places, the aquarium industry has become an essential source for job creation and income generation. Annually, about 200 million pieces of aquarium fish are bred and reproduced in Iran. Among the ornamental aquarium fish, guppies (*Poecilia*

reticulata) are one of the most important and popular fish (Kucuk, 2009). Researchers have created various varieties in terms of body shape and color by performing different crosses between guppies (Khoo et al., 2003; Kucuk, 2009). The guppy is a live-bearing fish used as a model creature for population genetics studies (Suk and Neff, 2009), growth, aging, inbreeding depression, and heterosis (Shen et al., 2007).

The agglomeration of inbreeding and the detriment of genetic diversity is anxiety in animal conservation (Barazandeh et al., 2012; Shamsalddini et al., 2016). Inbreeding enhances the danger of surrogating a rare recessive genetic disease and diminishes the population's fitness (Nassiry et al., 2005; Vajed Ebrahimi et al., 2017). In addition to that, plenary exploration of the breed specifications is necessary for impressive management of genetic resources in animals (Kharrati Koopaei et al., 2012; Moghadaszadeh et al., 2015). The utilization of molecular genetics has very significant benefits (Mohammadi et al., 2009; Gholamhoseini et al., 2018). One of the most important advantages is the genotyping of individuals for particular genetic loci (Mohammadabadi et al., 2010; Pasandideh et al., 2015).

Inter Simple Sequence Repeat (ISSR) is the genome region between microsatellite loci (Mohammadabadi 2017). The ISSR is a molecular marker method, which does not need the genome sequence information and leads to multi-loci and highly polymorphic patterns (Zamani et al., 2011). Each ISSR band corresponds to a DNA sequence delimited by two inverted microsatellites. The ISSR loci are dominant markers with the assumption of only two alleles per locus. It has been shown that the ISSR markers are universal, quick, easy to apply, highly reproducible, and polymorphous (Zamani et al., 2011). Their utility has been confirmed in various studies, such as detection of somaclonal variation (Leroy et al., 2000; Sarwat, 2012), genetic stability (Yuan et al., 2009; Lata et al., 2010; Zhang et al., 2010), gene tagging (Ammiraju et al., 2001; Marczewski et al., 2002), cultivar identification (Nagaraju et al., 2002), hybrid identification (Lin et al., 2010) and phylogenetic studies (Paris et al., 2003; Han et al., 2010), genetic relatedness, (Rajwade et al., 2010), and also to determine production quality (Tamhankar et al., 2009; Wu et al., 2010; Sarwat, 2012). Although ISSR markers have many advantages, it should be noted that these markers are dominant, so heterozygotes cannot be distinguished from homozygotes. Therefore, compared to codominant markers, this is a defect for ISSR markers.

Carvalho et al. (1991) used allozymes to investigate genetic diversity between populations of the guppy fish. In another study, Fajen and Breden (1992) investigated genetic diversity for different populations of guppy fish using mitochondrial DNA. Many researchers (Becher et

al., 2002; Watanabe et al., 2003; Paterson et al., 2005) have used microsatellite markers for studying genetic variations in the guppy fish. In the earliest, Suk and Neff (2009) used microsatellite markers to study genetic diversity in 15 populations, including 373 fishes of Trinidadian guppies (*Poecilia reticulata*). They showed a high level of genetic diversity between studied populations. RADP markers have also been used for determining genetic diversity between guppy fishes (Kucuk, 2009).

In comparison with mentioned used markers, ISSR markers are helpful to investigate population genetic studies, gene mapping, germplasm identification, and characterize gene bank accessions and identify closely related populations. ISSRs are also simpler, faster, cheaper (Sarwat, 2012), unnecessary to know the genetic background of the fish studied to investigate genetic diversity based on ISSR markers (Zamani et al., 2011). However, no attempt has been made to characterize the genetic diversity of guppy ornamental aquarium fish through ISSRs. Hence, this study aimed to investigate genetic variation between different populations of various guppy ornamental aquarium fish using ISSR markers in Iran for the first time.

Materials and Methods

Fish Varieties

Twelve strains of aquarium guppy fish (*Green Red Dragon, German Platinum Crown Tail, Blue Moscow, Blue Moscow Albino, Black Moscow, Mozaic, Red Mozaic, Super Red Singapore, Japan Blue, Full of Black Thai, Metal Lace Thailand, and Cobra*) were bought from various aquarium fish shops. 30 fish were selected from each strain. A total of 360 fish were used in our study.

Sampling and DNA Extraction

Due to the small size of the fish, part of the muscle tissue in the fish's abdomen was removed for DNA extraction. The removed part of muscle tissue was added to 500 ml of TES buffer including proteinase K (200 mg/ml), 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 25 mM EDTA (pH 8.0) via the Phenol: Chloroform: Isoamyl Alcohol procedure (Sambrook and Russell 2001) to extract total DNA. Both spectrophotometry and agarose gel (1%) were applied to determine the quality of extracted DNA.

Selection of Primers

From the various ISSR primers used to study the genetic diversity of fish, (AG)8G, (GAG)5GC, (CA)8AG, (GTG)5GC, (AG)8C, (GACA)4, and (CA)8AC primers (Table 1) which had higher polymorphisms and were better amplified than other primers were selected (Labastida et al., 2015).

Polymerase Chain Reaction

Amplification of DNA was carried out in a 25 μ L PCR reaction. Each reaction consisted of 20 ng/ μ L DNA, 2.5 μ L PCR buffer, 200 μ M of each dNTP, 3 mM MgCl₂, 0.3 μ L Taq DNA polymerase, sterile water was added until the reaction volume reached 25 μ L. The PCR condition was 94°C for 5 min (initial denaturation); 94°C for 45s (denaturation), annealing temperature which differed for each primer and ranged from 56 to 66 °C (Table 1) and 72°C for 2 min (extension) for 40 cycles; 72°C for 7 min (final extension). Negative controls were also used. The PCR products were electrophoresed on 1% agarose gel with 1 \times TAE buffer at 80V for 2h along with 0.1 kb ladder (CinnaGen Co., Iran). The gels were stained with ethidium bromide and visualized under UV light

(BTS-20.M, UVItect Ltd., UK). ONE-Dscan software (Scanalytics, Inc., Fairfax, VA) was applied to define the amplified fragments' size. Based on the presence or absence of the bands, the ISSR profiles were scored as 1 or 0, respectively. It is assumed that in any locus, every created ISSR band is a dominant allele.

Data Analysis

A non-parametric analysis of molecular variance (AMOVA) (Excoffier et al., 1992) and Nei's genetic distance analysis were carried out using GenAlex 6.501 (Peakall & Smouse, 2006) to characterize the genetic structure and variability between populations. XLSTAT (2017) was used to draw a phylogenetic tree for studied populations based on 7 ISSR markers. The principal component analysis (PCA) was performed using GenAlex 6.501 (Peakall & Smouse, 2006).

Population structure was determined using STRUCTURE software version 2.3.4 and performed with a burn-in of 10 000 iterations followed by 100 000 Markov chain Monte Carlo (MCMC) iterations under the admixture model. The analysis was run for the range of genetic clusters (K) from K= 2 to K= 10 with five repetitions for each K. The optimum K

Table 1. Characteristics of used ISSR primers for studied guppy fish strains

Primer	Primer sequence (5'-3')	Annealing Temperature (°C)
(AG)8G	5'-AGA GAG AGA GAG AGA GG-3'	56
(GAG)5GC	5'-GAG GAG GAG GAG GAG GC-3'	63
(CA)8AG	5'-CAC ACA CAC ACA CAC AAG-3'	60
(GTG)5GC	5'-GTG GTG GTG GTG GTG GC-3'	66
(AG)8C	5'-AGA GAG AGA GAG AGA GC-3'	57
(GACA)4	5'-GAC AGA CAG ACA GAC A -3'	56
(CA)8AC	5'-CAC ACA CAC ACA CAC AAC-3'	60

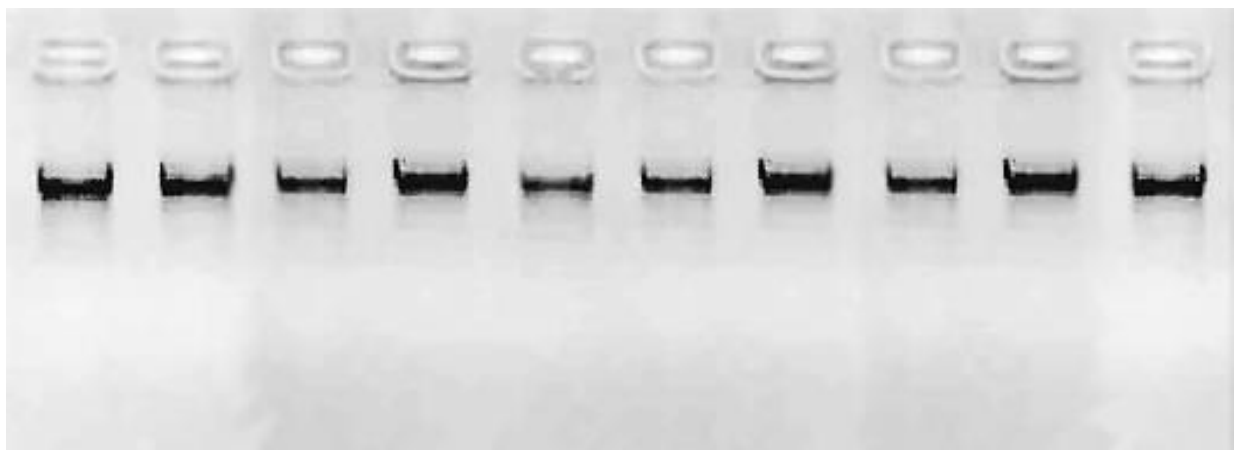


Figure 1. Quality of extracted DNA from studied guppy fish on 1% agarose gel

was determined based on ΔK calculated by the following equation:

$$\Delta K = m|L''(K)|/s[L(K)]$$

which is based on the rate of change in the log probability of data between successive K values via the ΔK method of Evanno et al. (2005), using STRUCTURE HARVESTER (Earl and VonHoldt, 2012).

Results

The quality of the extracted DNA was suitable for further research (Figure 1). Using seven specific primers, PCR products were amplified as well (Figure 2). In all studied samples, the size of amplified PCR fragments varied from 100 bp to 1800 bp (Table 2). The AMOVA analysis detected that 54% of observed polymorphism belongs to

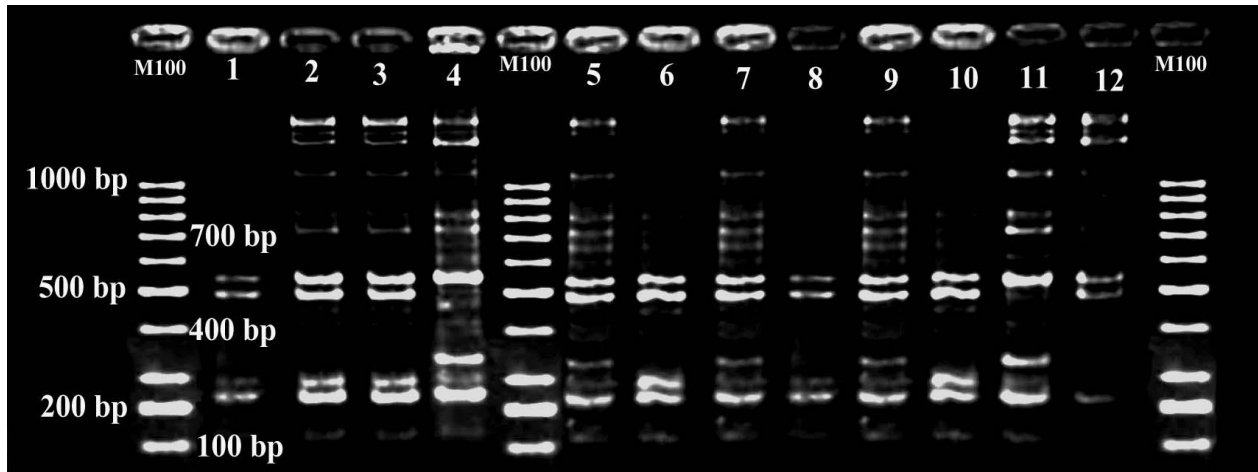


Figure 2. Profiles of two ISSR markers; (AG)8G and (GAG)5GC for twelve studied guppy fish on 2 % agarose gel. Lines 2, 3, 4, 5, 7, 9, 11 and 12 correspond to (AG)8G marker and 1, 6, 8 and 10 correspond to (GAG)5GC marker. M100 is DNA ladder

Table 2. Genetic diversity parameters over loci for each population using seven ISSR markers

Population		N	Na	Ne	I	He	uHe	P%
Green Red Dragon	Mean	30.000	1.662	1.291	0.300	0.186	0.189	83.08%
	SE	0.000	0.094	0.039	0.029	0.021	0.021	
German Platinum Crown Tail	Mean	30.000	1.477	1.191	0.197	0.119	0.121	73.85%
	SE	0.000	0.110	0.038	0.028	0.020	0.021	
Blue Moscow	Mean	30.000	0.892	1.315	0.258	0.177	0.180	44.62%
	SE	0.000	0.124	0.050	0.038	0.026	0.027	
Blue Moscow Albino	Mean	30.000	0.646	1.255	0.199	0.139	0.141	32.31%
	SE	0.000	0.117	0.049	0.037	0.026	0.026	
Black Moscow	Mean	30.000	0.954	1.225	0.209	0.135	0.137	47.69%
	SE	0.000	0.125	0.042	0.032	0.023	0.023	
Mozaic	Mean	30.000	1.677	1.575	0.461	0.315	0.320	83.08%
	SE	0.000	0.091	0.051	0.033	0.024	0.025	
Red Mozaic	Mean	30.000	1.692	1.346	0.344	0.218	0.222	84.62%
	SE	0.000	0.090	0.040	0.029	0.021	0.021	
Super Red Singapore	Mean	30.000	1.692	1.189	0.225	0.129	0.131	84.62%
	SE	0.000	0.090	0.033	0.024	0.017	0.018	
Japan Blue	Mean	30.000	1.077	1.308	0.269	0.179	0.182	53.85%
	SE	0.000	0.125	0.047	0.035	0.025	0.025	
Full of Black Thai	Mean	30.000	1.569	1.349	0.323	0.208	0.211	78.46%
	SE	0.000	0.103	0.046	0.032	0.023	0.024	
Metal Lace Thailand	Mean	30.000	1.231	1.260	0.250	0.159	0.162	61.54%
	SE	0.000	0.122	0.041	0.032	0.023	0.023	
Cobra	Mean	30.000	1.754	1.563	0.456	0.309	0.315	86.15%
	SE	0.000	0.079	0.051	0.032	0.024	0.025	
All populations	Mean	30.000	1.360	1.322	0.291	0.189	0.193	67.82%
	SE	0.000	0.033	0.013	0.010	0.007	0.007	

*Na = No. of Different Alleles, Ne = No. of Effective Alleles, I = Shannon's Information Index, He = Expected Heterozygosity, uHe = Unbiased Expected Heterozygosity, P% = Percentage of Polymorphic Loci

Table 3. The AMOVA (Analysis of Molecular Variance) calculated by the GeneAlex 6.41 (Peakall and Smouse, 2006) for twelve strains of aquarium guppy fish using seven ISSR markers

Source	df	SS	MS	Est. Var.	%
Among populations	11	2470.122	224.557	7.280	54%
Within populations	348	2141.267	6.153	6.153	46%
Total	359	4611.389		13.433	100%

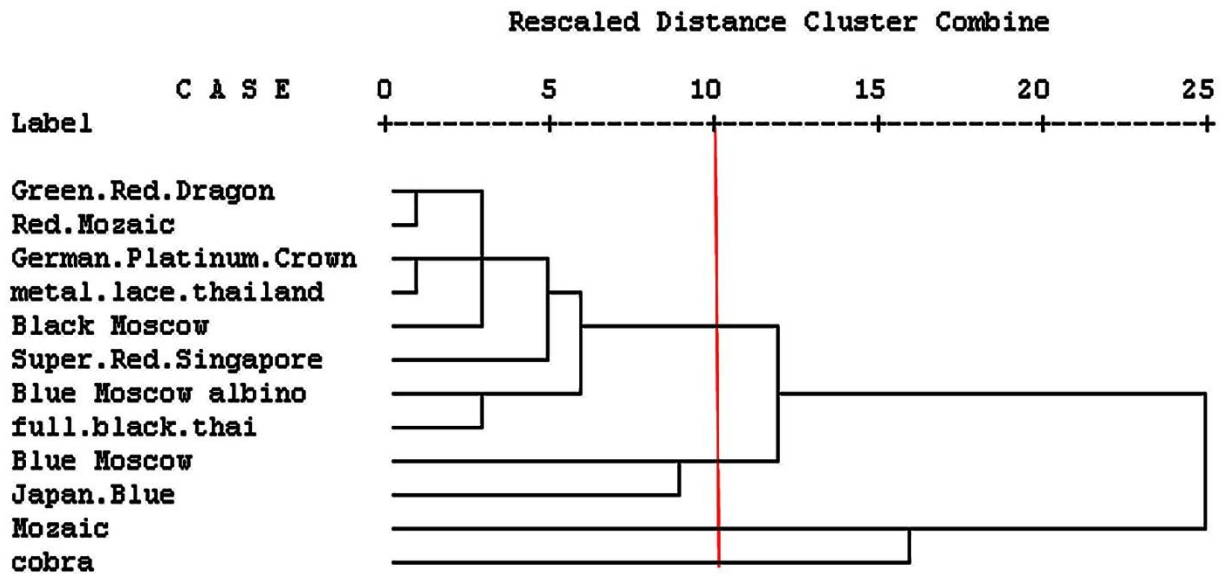


Figure 3. Dendrogram represent similarity coefficients of twelve strains of aquarium guppy fish in assessing the phylogenetic relationships using ISSR markers

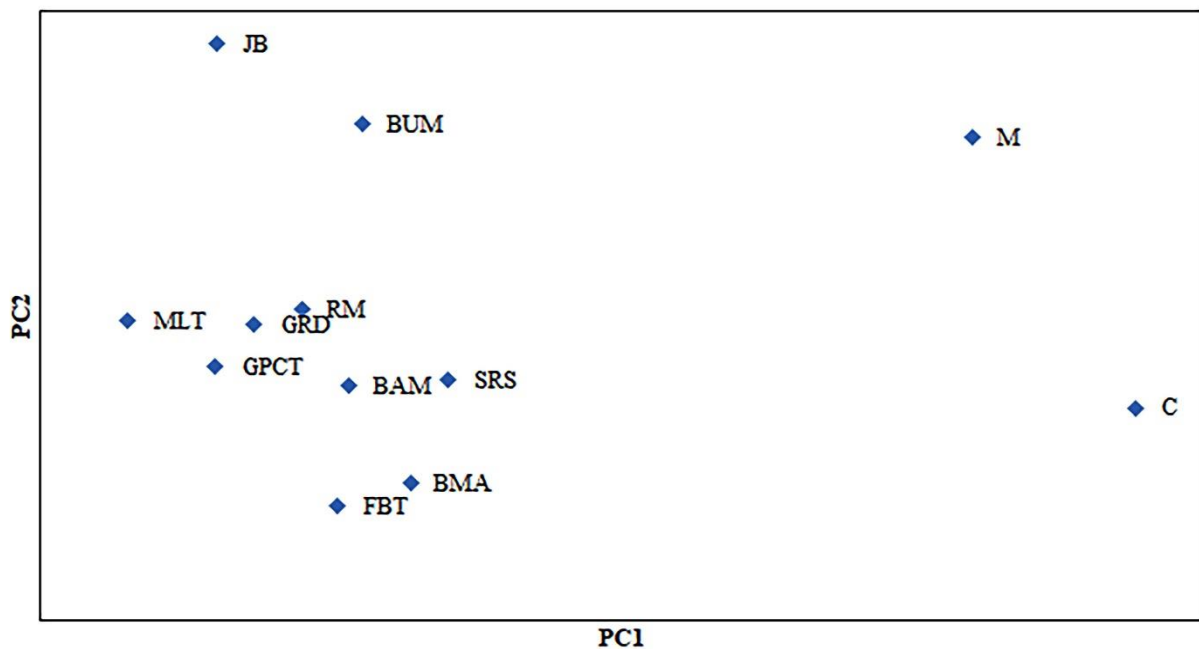


Figure 4. The populations clustering base on principal components analysis for twelve strains of aquarium guppy fish using seven ISSR markers. The proportion of total variation explained by first and second principal components, were 29.60 and 23.58 %, respectively. GRD=Green Red Dragon, GPCT=German Platinum Crown Tail, BUM= Blue Moscow, BMA= Blue Moscow Albino, BAM=Black Moscow, M=Mozaic, RM=Red Mosaic, SRS=Super Red Singapore, JB=Japan Blue, FBT=Full of Black Thai, MLT=Metal Lace Thailand and C=Cobra

variance among populations, and 46% corresponds to diversity within populations (Table 3). The highest and the lowest polymorphism percentage belonged to (GAG)5GC (90%) and (AG)8C (41%) markers, respectively. Our results showed that the highest percentage of polymorphic loci belongs to Cobra population (86.15%), and the lowest corresponds to Blue Moscow Albino population (32.31%). Other polymorphic indices, such as Shannon's information index (I), for these two populations also confirm estimated diversity (Table 2). Finally, we observed 489 bands for used ISSR markers on studied populations, *Green Red Dragon*: 48 bands, *German Platinum Crown Tail*: 29 bands, *Blue Moscow*: 29 bands, *Blue Moscow Albino*: 21 bands, *Black Moscow*: 31 bands, *Mozaic*: 56 bands, *Red Mozaic*: 49 bands, *Super Red Singapore*: 47 bands, *Japan Blue*: 35 bands, *Full of Black Thai*: 47 bands, *Metal Lace Thailand*: 39, and *Cobra*: 58 bands.

The dendrogram was drawn using coefficients of twelve strains of aquarium guppy fish in assessing the phylogenetic relationships using ISSR markers (Figure 3). The phylogenetic tree results showed that this tree was divided into two major clusters (Figure 3). Cluster I included only two populations *Mozaic* and *Cobra*, and cluster II contained the other ten studied populations. Cluster II is divided into two sub-clusters. The first sub-cluster included two populations; *Blue Moscow* and *Japan Blue*, and the second sub-cluster contained eight populations; *Green Red Dragon*, *German Platinum Crown Tail*, *Blue Moscow Albino*, *Red Mozaic*, *Super Red Singapore*, *Full of Black Thai*, *Metal Lace Thailand*, and *Black Moscow*.

The populations clustering base on principal components analysis for twelve strains of aquarium guppy fish using seven ISSR markers is shown in Figure 4. The proportion of total variation explained by the first and second principal components were 29.60 and 23.58 %, respectively. As this figure shows, two populations *Mozaic* and *Cobra*, are located far from the other ten populations. Between these ten populations, two populations *Blue Moscow* and *Japan Blue*, are separated from the other eight populations. These results were in agreement with the phylogenetic tree (Figure 3).

The number of clusters (K) present in twelve strains of aquarium guppy fish was determined by structure analysis based on the method adopted by Evanno et al. (2005). According to the results

obtained by HARVESTER STRUCTURE, the highest level of ΔK corresponded to $K = 3$ (Figure 5 and Figure 6). Based on the population structure analysis, strains were separated into three groups (Red, green and blue) with different genetic structures (Figure 6). As seen in this figure, two populations, *Mozaic* and *Cobra* (populations 6 and 12) with blue color, have high genetic similarity, and the other two populations *Blue Moscow* and *Japan Blue* (populations 3 and 9) with green color, have high genetic similarity with each other. These results were in agreement with the phylogenetic tree and PCA analysis.

Discussion

We studied the genetic diversity between different populations of 12 various guppy ornamental aquarium fish species using ISSR markers, which are useful for investigating genetic polymorphism in different fishes (Tong et al., 2005; Saad et al., 2012). The observed bands in this study are considered sufficient for species determination and studies of population genetics. Moreover, other molecular investigations for studying population genetics in fish species reported a similar number of bands and have confirmed our results. Labastida et al. (2015) studied lionfish in Cuba using ISSR markers and observed 113 bands ((GACA)4WB: 18 bands; (CA)8AC: 17 bands; (CA)8AG: 23 bands; (AG)8YC: 24 bands and (GAG)5GC: 31 bands) on 34 individuals. Casu et al. (2009) studied *Dentex dentex* L. 1758 (*Perciformes*, *Sparidae*) using 8 ISSR primers and reported 97 fragments. Liu et al. (2008) investigated *Cynoglossus semilaevis* Günther, 1873 (*Pleuronectiformes*, *Cynoglossidae*) applying 19 ISSR markers and identified 137 bands. A study on *cyprinodontiform* fish using 9 ISSR primers showed 101 bands (Maltagliati et al. 2006). Foo et al. (1995) studied the inheritance of RAPD markers in the Guppy fish, *Poecilia reticulata*. They used three oligonucleotide primers and their paired combinations (14 RAPD markers) for two guppy varieties, Green Snakeskin and Black, and showed that of these markers, 60% of them were polymorphic. In another investigation, Khoo et al. (2002) studied genetic diversity within and among feral populations and cultured strains of the guppy (*Poecilia reticulata*) by RAPD fingerprinting. They collected Feral guppies from 6 isolated populations (*Bukit Timah*, *Nee Soon*, *Tuas*, *Mount Faber*, *Kranji*, *Laboratory-inbred feral lines*) and sampled *Tuxedo*

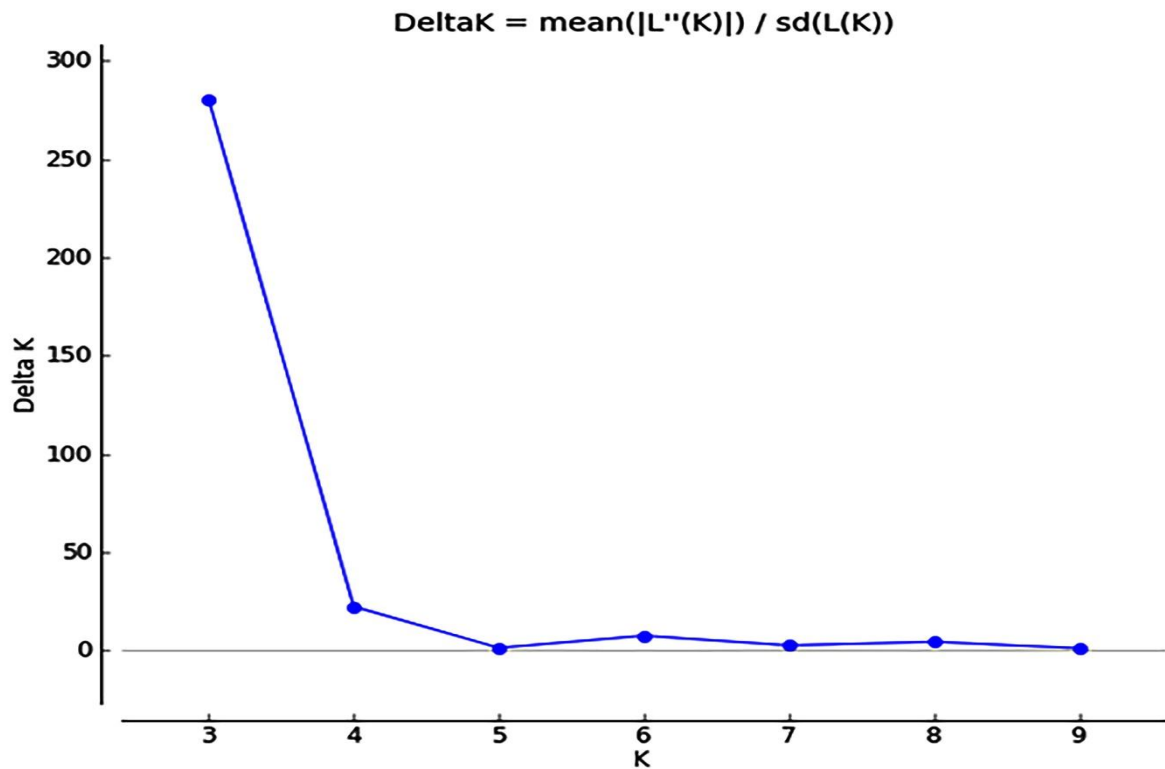


Figure 5. The graph (Delta K, K) of Evanno's method to determine the best K values for twelve strains of aquarium guppy fish using seven ISSR markers

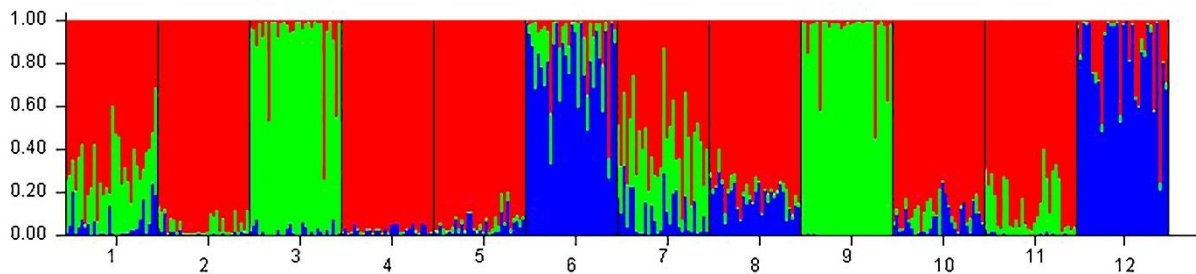


Figure 6. Biplot based on data obtained from seven ISSR primer combinations using the structure software for twelve strains of aquarium guppy fish. Red, green and blue indicate aquarium guppy fish divided into three subpopulations. 1=Green Red Dragon, 2=German Platinum Crown Tail, 3= Blue Moscow, 4= Blue Moscow Albino, 5=Black Moscow, 6=Mozaic, 7=Red Mozaic, 8=Super Red Singapore, 9=Japan Blue, 10=Full of Black Thai, 11=Metal Lace Thailand and 12=Cobra

and Green Variegated strains from 2 guppy farms in Singapore. They reported that the percentage of polymorphic loci ranged from 54.96% to 68.70%. In contrast, the results of our current study showed that the percentage of polymorphic loci ranges from 32.31% to 86.15%. This comparison demonstrates that genetic variations and polymorphism in studied Iranian guppies are higher than the guppy (*Poecilia reticulata*) in Singapore.

Shen et al. (2007) studied guppy (*Poecilia reticulata*) using fifty-one microsatellite DNA markers. They demonstrated that all of the

markers show moderate allelic variation. The number of alleles for each locus ranged from two to 10. Observed and expected heterozygosities varied from 0.10 to 0.63 and from 0.23 to 0.77, respectively. Suk and Neff (2009) studied genetic diversity in 15 populations, including 373 fishes of Trinidadian guppies (*Poecilia reticulata*) located in three drainages (northern coast, Caroni, and Oropouche) using seven microsatellite markers. They showed that all seven microsatellite loci are polymorphic and the observed number of alleles at a locus ranged from 5 to 44, and heterozygosity ranged from 0.037 to 0.855. Kucuk (2009)

estimated genetic diversity among 64 guppies, *Poecilia reticulata* using nine RAPD markers. They showed that seven of nine RAPD markers are suitable for the investigation of diversity in the guppy. The genetic difference was low (ranged from 0.052 to 0.330). KuĖnstner et al. (2016) studied the genome of the Trinidadian guppy, *Poecilia reticulata*, and variation in the *Guanapo* population. They sequenced ten wild-caught male individuals and showed that the identified 5 million SNPs correspond to an average nucleotide diversity (π) of 0.0025. They concluded that the genome assembly and SNP map provide a rich resource for investigating adaptation to different predation regimes. The results of the study of guppy populations with different markers show that the diversity observed in the strains studied in our study with ISSR markers is higher than in other studies. It can be concluded that Iranian populations of guppies are less affected by selection pressure and are a good genetic resource for breeding programs.

The ISSR markers were used to study genetic diversity between three *Paralichthys olivaceus* populations by Liu et al. (2006), and it was shown that these markers are delicate and reproducible tools for population genetic analysis of fish. Moreover, Yun et al. (2006) demonstrated that determination of genetic diversity for domestic hatchery populations implicating the conservation of natural *Paralichthys olivaceus* resources. AMOVA analysis in this study showed that 54% genetic variance among populations is a high level of variance in twelve strains of aquarium guppy fish. Genetic diversity could be originated from various production systems and ecological conditions in which aquarium guppy fish in Iran have been historically preserved.

Since the adaptability of the aquarium guppy fish in Iran with their environments is the product of long-term selection pressures and cannot be attributed to recent times, admixed aquarium guppy fish should also be considered in the conservation strategies. An appropriate conservation strategy should maintain maximum genetic diversity in the global gene pool while maintaining breed diversity to reduce inbreeding and preserve genetically differentiated groups (Talle et al., 2005). In general, close genetic composition and similar environments indicate that similar and probably related conservation programs can be applied for these populations. However, control of crossbreeding, breeding

centers development and improvement of recording systems conserve these populations under in situ conservation situations.

Conclusion

In recent years, with the wide application of DNA markers, the research of molecular marker-assisted breeding has made great progress. The ISSR loci are highly polymorphic and could be used for genetic diversity studies. Further studies should be performed with more ISSRs to obtain higher accurate results. Genetic relationship among populations is a priority for managing genetic diversity. ISSR markers are efficient in discriminating each genotype at the molecular level. They can be used for genetic diversity analysis for aquarium guppy fish because a greater understanding of the species' potential is necessary for supporting long-term genetic improvement. Since ISSRs are simpler, faster, cheaper, and more effective than other markers, we suggest that this technique is an excellent alternative for low-cost genetic monitoring focused on improving control programs of species in regions with insufficient financial resources.

Ethical Statement

Not applicable.

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Author Contribution

Mohammadreza Mohammadabadi, Valentyna Oleshko and Alevtina Bazaeva conducted the experiments; Olexandr Oleshko and Leonid Heiko analyzed the data and wrote the manuscript; Iryna Starostenko, Zahra Roudbari and Jurii Kunovskii contributed to data analysis and revising the manuscript; Mohammadreza Mohammadabadi supervised the whole project.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal

relationships that could have appeared to influence the work reported in this paper.

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