Research Article

Analysis of parentage assignment and parental contribution of silver carp (*Hypophthalmichthys molitrix*) in a semi-natural system of propagation using microsatellites

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Abstract

Study on pedigree information and genetic diversity is essential for effective management of hatcheries. In this study we used six microsatellite loci for analysis of offspring parentage from two sets of brooders, to evaluate the genetic diversity and parental contribution in the production of progeny of *Hypophthalmichthis molitrix*. The effective number of alleles and the heterozygosity for parents and offspring was (3.63 and 4.38) and (0.943 and 0.960) for two groups, respectively. The contribution of the females and males to the offspring for the two groups was 61% and 91%, respectively. Females mated with 2–6 males, and males fertilized 2–5 females, revealing multiple paternity in this species. Our results revealed that the ratio of males to females plays an important role in parental contribution to offspring production. Despite a decrease in the heterozygosity among larvae towards their parents, the values obtained were still acceptable, which supports the hypothesis of genetic factors involves in mating patterns selected by parents to maintain the proper levels of heterozygosity in their progenies.

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Introduction

One of the main strategies for sustainable aquaculture is the planning of basic breeding of hatcheries. Several studies have been focused on breeding programs in recent decades due to the occurrence of various problems (Hulak *et al.,* 2010, Wedekind *et al.,* 2007, Fessehaye *et al.,* 2006).

Keeping genetic diversity safe and monitoring its changes in hatcheries is one of the main plans of genetic management involved

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in aquatic breeding programs (Hedrick, 2004). Genetic diversity, particularly in mammals, birds, and fish is related to the effective population size (Horreo *et al.,* 2008). A sharp decline in genetic diversity would impair population, and even lead to the extinction of species (Machado-Schiaffino *et al.,* 2007).

There are many determinative factors which can influence the actual participation of broodstocks during mating in generations. These factors include the size of the broodstock in the hatcheries, contribution of males and females to reproduction (due to unequal sex ratios or differences in reproductive success between them), the criteria of broodstock selection, and gamete collection and the way of encountering them (Tiou, 2010; Gall, 1988). Executive protocols in hatcheries play an effective role in reducing effective reproduction size of aquatic organisms and the genetic diversity of their offspring.

Genetic diversity in a population is dependent on the diversity of alleles at different genetic loci. Genetic diversity can be assessed through the evaluation of the number and the distribution pattern of alleles among individuals and populations (Abdul-Muneer, 2014). So far, several molecular techniques have been used to examine genetic changes in natural populations, in breeding stocks and in their interactions (Subasinghe *et al.,* 2003; Yudha *et al.,* 2012; Ruzzante *et al.,* 2016).

Microsatellites are noncoding, highly polymorphic, codominant DNA markers with short nucleotide repetitions (1–6 bp) that are powerful tools to evaluate genetic diversion (Kantrartzi, 2013). Microsatellites have several advantages, such as good frequency and distribution at the genomic level (O'Connell & Wright 1997), high diversity and polymorphism, codominant inheritance, compliance with Mendelian laws (DeWoody & Avise 2000), lack of functional features in the genome (in most cases), need for smalls amounts of DNA samples, and the applicability of microsatellite primers for closely-related species (Briñez *et al.,* 2011).

Simple sequence repeats (SSRs) have been used in various population genetics studies, for instance; study on the effects of domestication on the genetic structure of populations of Atlantic salmon (*Salmo salar*) (Koljonen *et al.,* 2005), turbot (*Scophthalmus maximus*) (Coughlan *et al.,* 1998), brown trout (*Salmo trutta*) (Hansen, 2002, Was & Wenne 2002), common carp (*Cyprinus carpio*) (Bartfai *et al.,* 2003) and black tiger Shrimp (*Penaeus monodon*) (Xu *et al.,* 2001). Majority of them revealed weak genetic diversity of hatchery stocks.

The application of SSRs for determining the relationship between brooders and progenies for parentage assignment has emerged simultaneously with a software revolution in the life sciences and the development of statistical approaches (Liu and Cordes 2004). Application of microsatellites includes assessment genetic parameters for desirable traits during breeding (Herbinger *et al.,* 1995; Vandeputte *et al.,* 2004), evaluation of inbreeding in hatcheries (Letcher & King, 2001), determining the exact number of parents

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(Frost *et al.*, 2006), and estimating differences in individuals (Norris *et al.*, 2000.; Jerry *et al.*, 2004.; Liu *et al.*, 2012).

Silver carp (*Hypophthalmichthys molitrix* from Cyprinidae family), is one of the most important species in aquaculture from China. Because of the achievement in captive reproduction through hormone injections, Silver carp introduced to many parts of the world (Kolar *et al.*, 2005) and its Commercially reproduction and farming spread rapidly. Production of silver carp has been reached over 85–170 thousand tons recently, the first place of production of freshwater fish in Iran (FIGIS 2016).

Almost 80% of fingerlings are supplied by private hatcheries, while the rest comes from governmental hatcheries that belong to Iran's Fishery Organization.

Reduction in genetic variability as a consequence of inbreeding in the populations of *H. moliterix* led to a low production of fish, to an increase in offspring mortality and deformity, as well as to imbalanced ratio of males and females in hatcheries. Despite the overall growth of cyprinid farming in Iran, there is still a relatively low production rate of fish, in comparison to other Asian countries (Motallebi & Sharif-rohani, 2011). Possibly due to genetic variability reduction, in Iran death and deformities of juveniles of silver carp (Mortezaei, 2006), and imbalanced ratio of males and females in hatcheries (five females per male), have become a major concern in cyprinid production in captivity. Evaluating the reproductive success rate of each parent provides a good picture of the distribution of genetic diversity between two generations. Thus, in this study we examined genetic diversity indicators between silver carp progeny and their parents to evaluate the effectiveness of semi-natural breeding method as a common type of reproduction of this species in Iran. We used a number of pre-introduced microsatellites for parentage assignment, and then calculated the success rate of each parent to contribute to the breeding process.

Materials and methods

Breeding and sampling

The experiments were performed in a private hatchery, named Abzigostaran, in Khouzestan (32˚ 01′ 03″ N, 48˚ 51′ 16″ E) province of Iran. Two independent groups with different sex ratios were set. In the first group (named Semi1), 8 females and seven males with an average weight of 5320 ± 438.16 g, and 4 ± 192.72 g, respectively, were used. In the second group (Semi2) 8 females and 10 males with an average weight of 5313.75 ± 250.81 g and 6220 ± 496.3 g, respectively, were used. Parents were prepared for spawning using the standard method in warm-water fishes, which involves two rounds of hormone injections of pituitary gland extract (Horvath *et al.*, 2015). Females were injected intramuscularly with 2 mg/kg PG, 10% of which (0.2 mg/kg) was injected 12 h before the second injection (1.8 mg/kg). Simultaneously males were injected with 0.5 mg/kg PG. Then, they were transferred to 50–60 m^3 circular tanks. Running water (0.2–0.3m/s) toward the center of the pool where an outlet pipe leading to the egg collection chamber, was regularly checked and transferred

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in to the incubators. Spawning started ~8 h after the second injection, and lasted for 6–7 h. Fin clips of all brooders were taken and preserved in 95% alcohol. Fertilized eggs were incubated at 25–26 °C. Approximately 24–36 h later, the larvae were hatched and manual feeding was performed from the third day after fertilization. At days 5–6 of the incubation period, larvae (about 100 larvae/incubator) were sampled; they were preserved in 95% alcohol and keep in a fridge until DNA.

Analysis of the sperm quality

Male brooders were sampled for evaluating sperm motility and concentration according to Rahman, Rahman & Hasan (2011), using a hemocytometer (Depth 0.1 mm). Sperm was diluted 1000 times in a 0.3% NaCl solution. After 10 min (to allow for sperm sedimentation), the number of spermatozoa were counted in 16 cells and calculated as follows:

Sperm density (ml) = 1000 × number of counted sperm / [area (mm^2) × chamber depth (mm) × dilution ratio]

To evaluate sperm motility, the total period of sperm motility was measured, until 95% of the spermatozoa were immotile. For this purpose a light microscope (Nikon Eclipse 50i) was used at 400 × magnification. Sperm was diluted 1:10 in 0.3% NaCl. Measurements were performed twice for each sample.

DNA extraction

DNA was extracted from 160 larvae (80 larvae from each group, Semi1 and Semi2), and from fin clips of 33 males and females using Chelex (Chelex® 100, Sigma-Aldrich) (Estoup *et al.*, 1996) and the phenol-chloroform method (Hillis, 1996). The concentration of total DNA was estimated from absorbance readings at 260 nm (Nanodrop2000). DNA quality was verified by measuring the A260 nm/A280 nm ratio (> 1.8) and the A230 nm/A260 nm ratio (> 2), and by gel electrophoresis. DNA samples were stored at −20 °C until analysis.

Microsatellite analysis

Ten primers were selected for this study from a set of microsatellite markers previously introduced for silver carp (Gheyas *et al.*, 2006) (Table 1). All PCRs were performed using a Bio-Rad (T100) thermal cycler. PCR amplification was performed in 10 µl with 1× PCR Buffer (MasterMix, Ampliqon) containing 1.5 mM MgCl2, 0.4 mM dNTP, 1unit Taq DNA polymerase, 0.2 µM of each primer and 20 ng of extracted DNA template. The following program was used: 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 50 s, annealing at 60–64 °C (depending on the primer) for 50 s, and extension at 72 °C for 50 s, followed by a final step of 72 °C for 10 min. PCR products were electrophoresed using 6% polyacrylamide gels for 4 h at 180–200 V, followed by silver staining (Benbouza *et al.*, 2006).

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Table 1- Microsatellite primers for the 10 loci used in parentage assignment. The first two columns indicate the loci name and their accession numbers, respectively. Sequences correspond to forward (F) and reverse (R) primers used for microsatellite genotyping of *H. molitrix*. Tm, annealing temperature (°C)

Locus name	Accession no.	Sequence (5′-3′)	Tm (°C)
Hmo11	AM086451	F: CTG CTT GAT CAC AGG GTT TG R:CCT TAC AGA TAG ACA GAT ATT CAG	60
Hmo13	AM086452	F:AAA CCT GGA AGA TGT TCA CTG AAT R:GCG CGA GTG TTT GAA GTC TG	60
Hmo25	AM086454	F:TGT GCT GCA TTT TCA CTT CA R:TTC TTA CTA TCC ACA TTT GTT GTA TG	60
Hmo26	AM086455	F:GAT TTC AGG CAC ATT GCT TAT CT R:GAG CGT TTC TCA TTT GTA CTT ATT TT	60
Hmo33	AM086458	F:GTG CAG CAG TAT GTG AAT CAG GAC AC R:GTG CTT CGG GAT ACC ACA CTC TTG	60
Hmo34	AM086459	F:GTT CCC TGA GGC TTT ACA A R:GGG TCA TTA TCC TCT CAC TTT	60
Hmo36	AM086460	F:ATC GGA GGA GTG CTG TTC AGT CTG GA R:ACG ATT GTT GCC GAA CGG GTT GAT	64
Hmo37	AM086461	F:CAC AGC GGA GGG GCA AAG GTC R:GGA CGC CGT GTG ACT GGA GAT TTT	64
Hmo39	AM086462	F:ACA GTT ATG AGC TAG CAG CAG TTT CT R:TAC GTC GTA ATA CCA GTG TAA TAC CC	60
Hmo40	AM086463	F:CAG GCA GGC ATC CAC ATA GAG AAT C R:AGA AGA AAT CTG ATC GTC ACC TAT GA	64

Data analysis

The size of the alleles obtained for each sample was calculated using Gel Scanner program (ver. 1.3). The allele number, allele frequency, observed heterozygosity (Ho) and expected heterozygosity (He), were calculated using GenAlEx (Peakall & Smouse, 2006, 2012). The Cervus Ver. 3.0.7 program (Marshall, 2014) was used to estimate polymorphic information content, null alleles frequency, and exclusionary power indicators, including Excl-1P (mean power assignment for a parent), Excl-2P (mean power assignment for one parent and the other parent of the opposite sex with a given genotype), and Excl-PP (mean power assignment for a pair parent), which are required to determine the assignment power of parents. According to relations A and B, the calculated values for the power exclusion (PE) of loci were used to determine the combined exclusion power (CPE) of parents (Vandeputte, Kocour, Mauger *et al*., 2004): relation A, PE = 1-Excl; relation B; CPE = 1-Multiplication of PEs for all loci.

Parentage assignment analysis was performed in Cervus Ver. 3.0.7 (Marshall, 2014), using the maximum likelihood method with the most potential exclusion power. The natural logarithm likelihood ratio is called LOD. Through 10,000 simulations in the Cervus program, the distinctive limit was generated in the form of a LOD score between the first and second possible parents with confidence levels greater than 95% and lower than 90%. From

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this analysis the number of larvae produced by both male and female parents, and consequently the contribution rate of each parent to the process of reproduction, was determined. Chi-square analysis was used to determine significant differences in contribution between brooders ($p < 0.05$).

The effective genetic size of the population (Ne) was calculated to examine the effects of family sizes according to this formula (Chevassus 1989): Ne = [4(n -2)] / [(Ks + Vs / Ks) + (Kd + Vd / Kd)]-2; where n is the sample size of the larvae, Ks, and Kd are the mean number of larvae produced per sire and dam, respectively, and Vs and Vd are the variances of family sizes produced by sire and dam (Falconer, 1989). Assuming random family samples within equally sized families, the effective population size of brooders is calculated by the following formula (Falconer 1989): Ne = 4(Nm × Nf) / (Nm + Nf); where Ne is the effective population size of parents, and Nm, and Nf are the number of male and female parents participating in a mating group, respectively.

Results

Genetic diversity

Analysis of genetic diversity in parents using eight microsatellite loci showed the lowest number of alleles in loci Hmo11, Hmo39 and Hmo40 (4 alleles), and the highest number of alleles in loci Hmo37 locus (10 alleles) (Table 2). The mean number of alleles per locus was 6.25. The highest rates of expected heterozygosity were obtained at loci Hmo37 (0.860) and Hmo34 (0.796), while the lowest rate was seen at Hmo40 locus (0.414). The maximum and minimum rates of polymorphism information content were obtained at loci Hmo37 (0.845) and Hmo40 (0.378), respectively. The null allele frequency was negative in all loci except in the Hmo40 locus (0.418) (Table 2). After computing the loci exclusion power, six loci were selected to assign the parents (Hmo13, Hmo25, Hmo25, Hmo26, Hmo37, and Hmo39). The CPE of parents for all studied loci were Excl-1P = 0.959, Excl-2P = −0.996, and Excl-PP = 0.999. For selected loci in this study, the factors estimated as 0.943, 0.992 and 0.999, respectively.

Table 2. N.A., Number and size of alleles; Ho, observed heterozygosity; He, expected heterozygosity; PIC, polymorphic information content; N.U., null alleles; Excl-1P, Excl-2P, and Excl-PP, average exclusion probabilities

Locus	N.A.	H_o	H_e	PIC	N.U.	Excl-1P	Excl-2P	Excl-PP	Size of alleles (bp)
Hmo11	4	1	0.631	0.567	-0.268	0.533	0.366	0.213	142-148
*Hmo13	6	0.805	0.696	0.653	-0.068	0.652	0.463	0.287	136-162
*Hmo25	6	1	0.686	0.643	-0.230	0.643	0.454	0.279	134-144
*Hmo26	9	1	0.767	0.739	-0.154	0.767	0.573	0.391	146-220
*Hmo34	7	1	0.796	0.768	-0.122	0.791	0.606	0.428	114-128
*Hmo37	10	0.976	0.860	0.845	-0.068	0.887	0.723	0.564	148-194
*Hmo39	4	1	0.707	0.653	-0.174	0.621	0.449	0.28	128-140
Hmo40	4	0.366	0.414	0.378	0.041	0.359	0.22	0.087	208-238
Means (all studied loci)	6.25	0.893	0.695	0.655	-0.131	0.999	0.996	0.959	-
Means (Selected - loci)	7	0.963	0.752	0.716	-0.137	0.999	0.992	0.943	-

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Comparison of genetic diversity of parents and offspring

Genetic diversity indices obtained for the larvae in groups Semi1 and Semi2, and for the contributing parents in each group, are presented in Table 3. The numbers of alleles were identical in all loci of parents and larvae, except for Hmo25, where one allele less is observed in the larvae (Semi2).

The mean values for He, Ho, and polymorphic information content in the parents of group Semi1 were 0.714, 0.962, and 0.673, respectively, while the equivalent values in their larvae were 0.693 ± 0.034, 0.925 ± 0.039, and 0.646 ± 0.096, respectively. Also, in parents of larvae in the group Semi2, the mentioned indicators were 0.755, 0.969, and 0.718, respectively, while the estimation in the offspring of this group was 0.745 ± 0.036, 0.925 ± 0.032, and 0.705 ± 0.107, respectively. Chi-square test showed no significant differences between parents and their larvae for both of the groups. The mean number of effective alleles in the parents and larvae of group Semi1 was 3.70, and 3.56, respectively. The mean number of effective alleles in the parents and larvae of group Semi2 was 4.40 and 4.37, respectively.

Table 3. Genetic diversity in parents and offspring in two group semi-natural propagation of *Hypophthalmichtys molitrix*. N.A., Number and size of alleles; Ho, observed heterozygosity; He, expected heterozygosity; Ne, Number of effective alleles

Loci	Groups	N.A.		He		Ho		Ne	
	Sample	Semi1	Semi2	Semi1	Semi2	Semi1	Semi2	Semi1	Semi2
Hmo13	Parents	4	4	0.618	0.689	0.769	0.813	2.62	3.22
	Larvae	4	4	0.651	0.656	0.825	0.8	2.86	2.9
Hmo25	Parents	6	6	0.686	0.699	1	1	3.18	3.32
	Larvae	6	5	0.665	0.714	1	0.988	2.98	3.5
Hmo26	Parents	7	7	0.738	0.811	1	1	3.44	5.270
	Larvae	7	7	0.617	0.799	0.788	0.938	2.61	4.960
Hmo34	Parents	7	7	0.809	0.752	1	1	4.5	4.030
	Larvae	7	7	0.728	0.8	0.938	1	3.67	5.000
Hmo37	Parents	8	10	0.843	0.857	1	1	5.28	7.010
	Larvae	8	10	0.843	0.859	1	0.988	6.38	7.080
Hmo39	Parents	4	4	0.711	0.719	1	1	3.15	3.550
	Larvae	4	4	0.648	0.64	1	1	2.88	2.770
Mean	Parents	6	6.33	0.714	0.755	0.962	0.969	3.7	4.400
	SE	0.683	0.843	0.028	0.027	0.038	0.031	0.993	1.480
	Larvae	6	6.167	0.693	0.745	0.925	0.952	3.56	4.370
	SE	0.683	0.946	0.034	0.036	0.039	0.032	1.42	1.640

Parent's contribution to offspring

The parentage analysis, using six selected loci, in two groups of Semi1 and Semi2 assigned 100% and 95% of offspring to their parents, respectively. Individual parents, and the number

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of larvae produced by each parent, are presented in Table 4, according to parentage assignment in Semi1 and 2. Assuming a balanced participation, Chi-square tests in the Semi1 group showed significant differences on the number of larvae produced by the sires (and dams), regardless of type of crossing with dams (and sires) ($p<0.05$). In Semi1, 30% and 32% of the progeny (more than half of the larvae) belonged to females F1 and F2, respectively. The female F8 had the lowest percentage of the progeny (5%). Among males, M4, M5 and M6 produced more than 60% of larvae (Figure 1), revealing a disproportionate contribution to the progeny. None of the larvae in Semi1 group were attributed to females F3 or F4. Therefore, females F3 or F4 were eliminated from the participating brooders of the group. The mean larvae production in Semi1 by males and females was 16.32 and 18.7, respectively (Table 5). The variance of larvae production in this group by males and females was 44.34 and 69.78, respectively (Table 5).

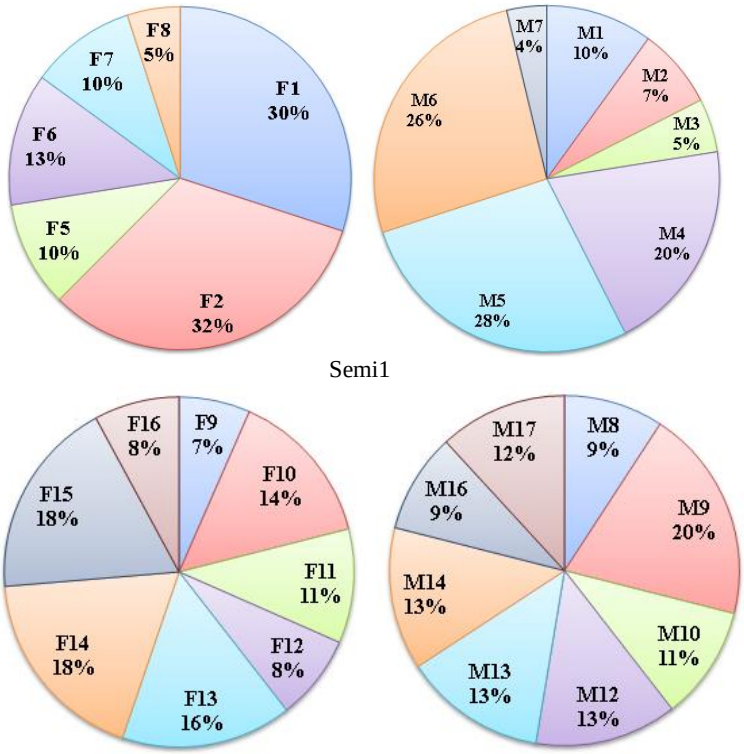


Figure 1. Parental contribution to offspring production of *Hypophthalmichtys molitrix* in two groups of semi-natural reproduction system (Semi1 and Semi2).

In the group Semi2, the Chi-square test showed no significant differences between contributing males and females to produce larvae ($p > 0.05$). except for the male M9, there was a roughly balanced male participation. although among the females (Figure 1). Most of the larvae (>50%) were produced by females F13, F14, and F15. In Semi2 group no progeny was attributed to males M11 and M15. Thus, they were eliminated from the participating males of the group. The mean and variance of larvae reproduction in this group by males and females were calculated respectively as (10.10–0.71) and (7.21–9.96) (Table 4).

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Comparing the numbers presented in Table 5 in relation to the effective population size of parents in the both groups, a significant reduction of Ne can be clearly seen in the group Semi1. Assuming full participation of all males and females in Semi1, the expected effective population size would be 12.9, however this number was actually 7.9, which is equivalent to a 39% reduction compared to equally sized families. The decline in the population of Semi2 group was 9.5% (Table 5).

The females were in participant with 2–6 males, while the males fertilized 2–5 females, which can be indicative of multiple paternity in this species (Table 4). Qualitative sperm analysis in males of Semi1 and Semi2 showed that the mean concentrations were 421.66 ± 6.37, and 398.5 ± 12.74, respectively. Average sperm motility in Semi1 and Semi2 was 65.23 ± 0.45, and 63.16 ± 0.35, respectively (Table 6). Comparing to previous results in other studies of this species, all of the males were able to fertilize the females.

Table 4. Parentage assignment for two groups of *Hypophthalmichtys molitrix*

Semi1	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**Total**			
F1	1	0	0	2	18	3	0	24			
F2	1	5	1	7	1	9	2	26			
F3	0	0	0	0	0	0	0	0			
F4	0	0	0	0	0	0	0	0			
F5	5	0	1	0	0	2	0	8			
F6	0	0	0	5	2	3	0	10			
F7	1	1	2	0	0	4	0	8			
F8	0	0	0	2	1	0	1	4			
Total	8	6	4	16	22	21	3	80			
Semi2	**M8**	**M9**	**M10**	**M11**	**M12**	**M13**	**M14**	**M15**	**M16**	**M17**	**Total**
F9	4	0	0	0	0	0	1	0	0	0	5
F10	1	1	2	0	3	4	1	0	0	0	12
F11	1	1	0	0	2	0	0	0	3	1	8
F12	0	0	1	0	0	0	4	0	0	1	6
F13	0	0	2	0	0	4	3	0	0	3	12
F14	1	1	0	0	5	2	0	0	2	2	13
F15	0	10	3	0	0	0	0	0	1	0	14
F16	0	2	0	0	0	0	1	0	1	2	6
Total	7	15	8	0	10	10	10	0	7	9	

Table 5. Number of broodstock in each group

Group	**Number of broodstock**	**Mean contribution of parents**		**Variance of produced larvae**		$N_e(1)$	$N_e(2)$	$N_e(2)/N_e(1)$
		K_s (Male)	**K_d (Females)**	**V_s (Male)**	**V_d (Females)**			
Semi1	15	16.32	18.7	44.34	69.78	12.9	7.9	0.61
Semi2	18	10.10	10.71	7.21	9.96	16	14.48	0.91

Kd, mean contribution of males parents to produced larvae, Ks, mean contribution of female parents to produced larvae; Vd, variance of produced larvae for males; Vs, variance of produced larvae for females; effective population sizes using 4 (Nm×Nf)/(Nm+Nf), (Ne (1)), effective population sizes using Ne (2)=4(N 2)/[(Ks+Vs/Ks)+(Kd+Vd/Kd)-2] (Chevassus, 1989) (Ne (2)) and the ratio of Ne (1)/Ne(2).

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Table 6. Sperm characteristics of *Hypophthalmichtys molitrix*. Compared to other studies in the same species

Species	Mean concentration of sperm(10^7/ml)	Mean motility of sperm (s)	Reference
H. molitrix	315±2	96.1 ± 1(early season)	Khara, Baradaran, Dadras, Rahbar, Ahmadnejad & Khodadoost (2012)
H. molitrix	-	36 ± 9	Rahman, Rahman & Hasan (2011 (2011)
H. molitrix	421.66 ± 6.37	65.23 ± 0.45	This study, group Semi1
H. molitrix	398.5 ± 12.74	63.16 ± 0.35	This study, group Semi2

Discussion

In this study the parentage assignment was successful for about 97% of all larvae studied. It seems that factors such as the absence of null alleles and using loci with appropriate diversity (proper amount of effective allele size) have led to the efficient performance of the technique on the larvae analyzed. In numerous reports, an appropriate success rate in detecting parents and their contribution to offspring production have been reported using SSRs: Weinman *et al*. 2014, 95%sucess; Gheyas *et al.* 2009, 96.3%; and Liu *et al*. 2012 99.6%. In a microsatellite study on Caspian Salmon (*Salmo trutta caspius*) discrimination of more than 98% of brooders was successfully performed (Sourinejad *et al.*, 2011).

The use of a minimum number of microsatellite loci to achieve a high level of parentage assignment power in parental groups is one of the main goals of this technique (Castro *et al.,* 2007). However different factors, such as null alleles, mutations, and genetic linkage between the examined loci, can affect the exclusionary power and consequently the success rate of parentage assignment (Vandeputte *et al.,* 2011). In previous studies, 6–8 microsatellite loci were considered a reliable number for parentage assignment (Fessehaye *et al.,* 2006). In this study, we tried to minimize the number of loci required for analysis on silver carp (Gheyas *et al.,* 2006), using SSRs markers and preliminary evaluation and selection of the most appropriate loci (in terms of discrimination power; CPE calculation).

In the animal kingdom, fish have the most complex mating systems (Neff 2001). Multiple paternity has also been observed in other species, such as Perches, Gastropods and Paco fish (*Piaractus mesoptamicus*) (Xue, 2014; Fessehaye *et al.,* 2006; Povh *et al.,* 2010). Mating with a greater number of females increases the chance for male reproductive success, which highlights the role of males as the determinant of the fertilization event. However, the ongoing effort of females for mating with a greater number of males should not be ignored. Besides fish, polyandrous events associated with adaptive behaviors have been reported in a variety of animals (Gowaty, 1994). Females try to maximize adaptation, survival, and fitness through mating with multiple males. which include successful fertilization despite poor reproductive success

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in males (poor quality sperm), achieving better genes for their larvae, reproducing offspring with greater genetic diversity (and as a result higher adaptability), and preventing inbreeding (Avise *et al.,* 2002). This event is possible through multiple spawning of females at different intervals (DeWoody & Avise, 2001). In this study, evaluation of the mating patterns between parents of Semi1 and Semi2 groups revealed that females mated with two or more males, which supports the polyandrous participation hypothesis in silver carp, and this could have occurred in several stages.

According to the results of parent contribution, two males of the second group (M13 and M15) did not participate in reproduction. Comparing the sperm quality in this study with previous studies (Khara *et al.*, 2012, Rahman *et al.*, 2011), the sperm of fish in our study had appropriate motility and concentration values (Table 6). So it does not seem to be related to sperm quality factors, since other parents with similar sperm features participated in reproduction and produced larvae. On the other hand, all of the females produced larvae so it seems no problem with them. However, the incidence of some differences in the values obtained may be attributed to various reasons such as differences in the maturity of parents, in the hormonal stimulation methods, contamination during sperm collecting, differences in concentration estimation methods, different time periods during a reproductive season, and the conditions of parents in terms of nutrition, age, etc. (Khara *et al.*, 2012, Alavi *et al.*, 2010).

In this study, the difference in the participation rate of parents in reproduction (especially for males) and a high variance in family size in Semi1 limited Ne (7.9), when compared to the expected Ne value (12.9) (Table 5). However, in the group Semi2, which had an equal contribution of parents to reproduction, the estimated Ne (14.4) was closer to the expected value (16). Evaluating the genetic diversity in parents and their progeny showed that despite the decrease in the effective size of broodstock in both groups, the heterozygosity in offspring was maintained at acceptable levels that were similar to the parents' (0.962–0.952 in group Semi1, and 0.969–0.952 in group Semi2). This issue, would strengthen the hypothesis of reproductive behaviors, such as dissimilar mating to profit the progenies from its effects as an increase in the amount of heterozygosity (Tregenza & Wedell, 2000), when considering the negative F values reveals the selection in favor of heterozygous individuals. According to heterozygosity-fitness correlations at noncoding genetic markers, heterozygosity is a sign of fitness effects, which is the result of genes distributed through the genome (Hansson *et al.*, 2004).

In general, the microsatellite markers used in this study were useful for assigning parentage with a high degree, and made it possible to estimate Ne during a semi-natural process of propagation. The analysis of parentage assignment suggested an unequal contribution of parents in an unbalanced sex ratio. However, in the group with a balanced sex ratio, a balanced participation of parents in

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reproduction was observed, which highlights the importance of sex ratio among males. The use of equal sex ratios of males and females in silver carp, and ensuring the sexual maturity of the brooders and their health status, can benefit the proportional distribution of genetic diversity from parents to the offspring.

Conflict of interest

The authors have no conflict of interest in this work.

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