Population molecular analysis among the narrow-clawed crayfish (*Astacus leptodactylus*) based on microsatellite DNA loci

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The genetic variation of narrow-clawed crayfish (*Astacus leptodactylus*, Eschscholtz, 1823) from Caspian Sea including two sites (Astara and Kiashahr), Aras region, Anzali lagoon and three rivers (Chafrood, Masuleh, and Siah Darvishan) was evaluated and their genetic variation on the basis of DNA microsatellite loci was estimated. DNA from pleopods of 194 specimens extracted and was examined with six microsatellite markers. Genetic differences between the populations were discerned by pairwise comparison based on allelic distribution. The average numbers of alleles per locus ranged from 2 to 10, while the average observed heterozygosity (*Ho*) at various loci varied between 0.222 to 0.732, implying that a midway level of genetic variation. Among seven populations Siah Darvishan population displayed the highest level of variability in terms of heterozygosity. Tests of Hardy-Weinberg showed that the microsatellite loci deviated significantly in most populations indicating deficit of heterozygote. The results indicate that some of the above populations are significantly differentiated from one another based on pairwise F_{ST} estimates. Genetic distance based measures supported the clustering of Siah Darvishan, Chafrood and Astara may be genetically discrete from other narrow-clawed crayfish populations. The neighbor-joining dendrogram topology and multidimensional scaling approach (MDS) constructed on the basis of genetic distances among populations supported observed division between the populations. The non-significant differentiation between crayfish samples from the Anzali lagoon, Kiashahr and Masuleh can be explained by a relatively recent disconnection of these three populations and/or small amounts of gene flow. These results could give applicable information for cultivation of stocks and also for future genetic improvement of this commercial species by selective breeding program.

Key words: *Astacus leptodactylus, Microsatellite DNA, Stock structure, Caspian Sea*

INTRODUCTION

Freshwater crayfishes are a highly varied group of decapod crustaceans (Holdich, 1993; Crandall, 2006). They inhabit a diversity of streams, estuary, reservoirs and lakes. The family Astacidae (freshwater crayfish), from the viewpoint of taxonomy, be part of Decapoda (Holdich, 2002; Machino & Holdich, 2006; Souty-Grosset *et al.*, 2006) and are allotted amid three families, two (Cambaridae along with Astacidae) in the northern side of Hemisphere and one (Parastacidae) in the

southern side of Hemisphere, specifically in Madagascar, southern South America and Australia (Köksal, 1988; Holdich, 1993; Crandall & Buhay, 2007).

According to Karimpour *et al.* (2004) the freshwater crayfish in Iran are represented by two native crayfish species; the noble-crayfish, *Astacus astacus* and the narrow-clawed crayfish, *Astacus leptodactylus*. The narrow-clawed crayfish *A. leptodactylus* belong to the genus *Astacus* is scattered in streams, rivers, lagoons and lakes throughout of Iran and many of natural groups have been lost or are in serious decline (Souty-Grosset *et al.*, 2006; Gherardi *et al.*, 2013; Harlioğlu and Harlioğlu, 2009). Karimpour *et al.* (2004) suggested that *A. leptodactylus* occurred naturally in Caspian Sea basin such as Anzali lagoon with its rivers, Aras as well as in the Siah Darvishan, Chafrood and Masuleh rivers.

Based on their distribution *A. leptodactylus* is a cold water species; that is, they will tolerate cold winter conditions and range of its reproduction cycle differs habitat climate in which it inhabits (Holdich, 2002; Karimpour *et al.*, 2004). On the southern part of the Caspian Sea the spawning time commonly happens between winter and spring/early summer (Karimpour *et al.*, 2011). In Astara region, for instance, the spawning initiates on November 1 and ends on June 20. At the time of spawning season, crayfish catchment was prohibited by the Iranian administration in the Caspian Sea (Karimpour *et al.*, 2011). Narrow-clawed crayfish grade among the most economical crustaceans and ranked as a delicious food, which are of large industrial significance (Karimpour *et al.*, 2011). Due to their industrial prominence, large investigations have been assigned to the research of their life history, natural science, fisheries (Harlioğlu & Holdich, 2001; Harlioğlu & Harlioğlu, 2005; Gherardi, 2007) and population genetics (Gouin *et al.*, 2000; Edsman *et al.*, 2002; Gouin *et al.*, 2011; Gross *et al.*, 2013).

Contrary to inland finfish species, the genetic structure of crayfish groups in the Caspian Sea basin has scarcely been addressed (Rezvani, 2000; Qasemi *et al.*, 2004; Norouzi, *et al.* 2009; Khoshkholgh *et al.*, 2011, 2013; Porkazemi *et al.*, 1999, 2012; Nazari *et al.*, 2013). Many studies have been done to describe the genetic variation within and among populations of freshwater crayfish using various markers, which is the basic goal of population genetics (Buhay & Crandall, 2005; Trontelj *et al.*, 2005; Fetzner & Crandall, 2003; Khoshkholgh & Nazari, 2015). Previous study examining population structure in *A. leptodactylus* has primarily utilised mitochondrial DNA (mtDNA) markers. This study found levels of genetic variation to be relatively high in populations across geographic range (Khoshkholgh & Nazari, 2015).

To better clarify the population genetics of this important species, identification of *A. leptodactylus* genetic stock structure in Iran is essential. Conservation genetic and optimum lasting authority rely on knowing the distribution, characteristics of all stock segments and maintaining their diversity (Shaklee & Currens, 2003), therefore in the present work, microsatellite DNA loci in *A. leptodactylus* were utilised to delineate the level of genetic diversity among collections of crayfish.

MATERIAL AND METHODS
Sample collections
A total of 194 crayfish samples from seven sampling areas (Fig. 1, Table 1) were obtained. Sample collection of adult *A. leptodactylus* carried out from October through late November of the 2010 commercial fishing seasons. Immediately after the pleopods of every individual were obtained, crayfish specimens were delivered at the place where they were caught.
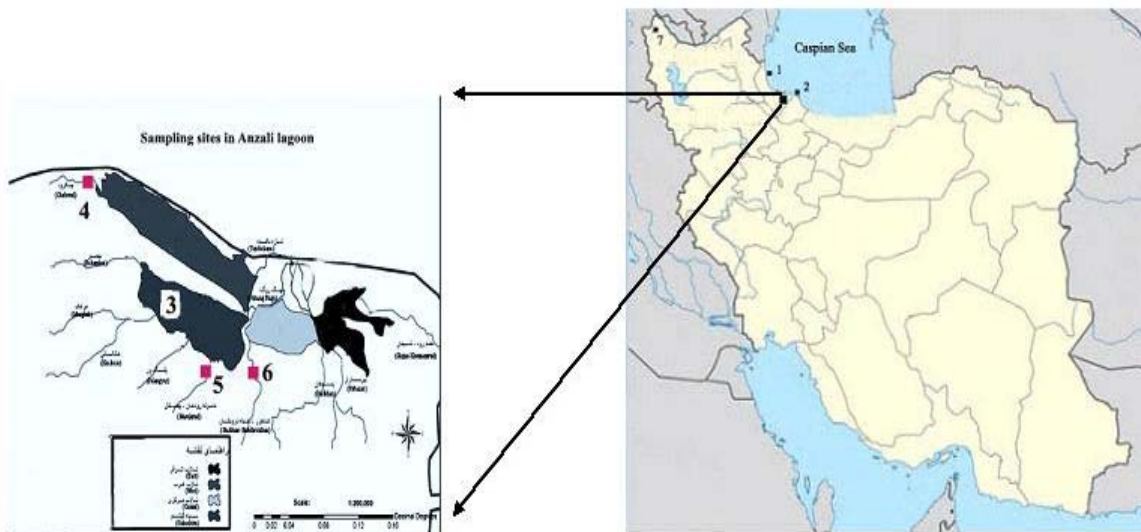
FIGURE 1. Sampling sites of the narrow-clawed crayfish, *A. leptodactylus*. The localities are labeled with numbers. Detailed information about the sites can be found in Table 1.

DNA extraction and microsatellite genotyping

Tissue samples, obtained from one of the pleopods, were immediately maintained either in 96% ethanol, and then stored at -20˚C freezer, until being processed for DNA extraction. Muscle tissues of ethanol-preserved crayfishes were incubated in lysis buffer with proteinase K at 37°C overnight for 16 hours. Total genomic DNA was extracted from muscle of the legs (about 50–100 mg) according to the standardized procedure described by Hillis & Moritz (1990) as modified by Nazari *et al.* 2016, and then stored at -20°C. DNA Extraction was examined for concentration using spectrophotometer (Nanodrop ND1000) and standardized to a specific concentration (for example, 50 ng/μl for Polymerase Chain Reaction (PCR)). The quality DNA specimens were checked optically on a 0.8% agarose gel.

TABLE 1. Narrow-clawed crayfish samples collected for population genetic analysis. Sampling localities, numbers (see Fig. 1), and number of individuals sampled (*n*)

Region	Map no.	Sampling site	*n*
Caspian Sea	1	Astara	34
	2	Kiashahr	31
Anzali lagoon	3	Central part	27
	4	Chafrood	28
River	5	Masuleh	14
	6	Siah Darvishan	23
Reservoir	7	Aras	37
Total			194

Crayfish-specific dinucleotide microsatellite loci were exploited in this study (Gross *et al.*, 2011). Each primer set was tested by varying the PCR conditions and evaluating the PCR products on 1.5% agarose gels. Amplifications were performed in 20μl reaction volume with template DNA (50 ng) using an Eppendorf 5331 thermocycler (Eppendorf, Germany). Experimental condition tested included, MgCl2 concentration (1-2.2 mM), deoxyribonucleoside triphosphate (dNTP) concentration (150-190 μM), 0.75 -1.2U of Taq DNA polymerase (Vio Taq^{Tm} VT1001, Fermentase)

and 1μl each of forward and reverse primers (10-20 pmol) . The PCR reaction comprised leading denaturing for 4 minutes at 94°C, followed by 25 cycles of 30 second at 94°C, 30 second at best annealing temperature (Table 2), and 10 minutes for ending extension at 72°C, followed by 4°C hold. Products of PCR were separated by 6% non-denaturing polyacrylamide gels electrophoresis in 0.5× TBE buffer for 2 to 3 hours at 250 V and subsequently checked by silver staining method. The pictures acquired were examined testing BioCapt software (version 2.0) (Table 2).

Statistical analysis
Number of Alleles, expected heterozygosity (H_E) and observed heterozygosity (H_O) (Nei, 1972) were analysed for each locus by the Excel Microsatellite Toolkit (Park, 2001). The Hardy–Weinberg equilibrium (HWE) tests for each locus were estimated by GENEPOP version 3.2 software (Raymond and Rousset, 1995) with the Markov chain parameters. The frequencies of null allele were assessed using the software MICRO-CHECKER (Van *et al.*, 2004).

TABLE 2. PCR condition and reaction, locus, product sizes and repeat motifs on narrow-clawed crayfish.

Locus	Product size (bp)	components	Cycling condition	Repeat motif	Number of allels
Aas2	197-224	1.6mM MgCl2, 160μ MdNTPs, 10pmol each primer and 1.1UTaq DNApolymeraz	95/3min[94/30sec, 57/25sec and 72/30sec]³⁰,72/5min	GT(23)	13
Aas7	225-322	1.5mM MgCl2, 180 μMdNTPs, 20pmol each primer and 1.2UTaq DNApolymerase	95/3min[94/30sec, 62/25sec and 72/30sec]³⁰,72/5min	CT(24)	20
Aas766	186-216	1 mM MgCl2, 175μMdNTPs, 10pmol each primer and 0.85 UTaq DNApolymerase	95/3min[94/30sec, 60/25sec and 72/30sec]³⁰,72/5min	GT(29)	6
Aas1198	191-268	1.2mM MgCl2, 190 μMdNTPs, 20pmol each primer and 1.1UTaq DNApolymerase	95/3min[94/30sec, 58/25sec and 72/30sec]³⁰,72/5min	CT(24)	4
Aas3666	100-142	1.3mM MgCl2, 175 MdNTPs, 10pmol each primer and 1.1UTaq DNApolymerase	95/3min[94/30sec, 59/25sec and 72/30sec]³⁰,72/5min	GT(27)	7
Aas3040	105-128	2.2mM MgCl2, 150 μMdNTPs, 10pmol each primer and 0.75UTaq DNApolymerase	95/3min[94/30sec, 61/25sec and 72/30sec]³⁰,72/5min	TA(20)	6

Genotype distributions between populations were inspected with the software GENEPOP 3.2 (Raymond & Rousset, 1995). All populations were estimated by the genetic differentiation index (F_{ST}, Weir & Cockerham, 1984) using Arlequin software (Excoffier *et al.*, 2005). Genetic relationships arrangement between collections was assessed by multidimensional scaling method (MDS) with the software SYSTAT (Wilkinson *et al.*, 1992). Optical evaluations of the genetic connections between populations were created over the structure of a neighbour-joining tree according to the Cavalli-Sforza & Edwards (1967) chord distance implemented in PHYLIP software (Felsenstein, 2005) and the bootstrap amount was estimated depend on 1000 repeats.

RESULTS
The average of allele numbers inspected at each locus varied between 2 to 10 (Table 3). Mean expected heterozygosity of each population varied between 0.346 (Masuleh River) to 0.575 (Siah Darvishan River), and the total mean was 0.318. Initially, 17 of 42 exact tests significantly departure from HWE at 0.05. Most of overall similarities were significant subsequent sequential Bonferroni adjustment, after pooling rare alleles (Table 4). Entire loci had one significant deviation from HWE

at least, with no locus out of HWE for more than two groups significantly and three populations including Siah Darvishan and Chafrood River and Astara out of HWE for more than four loci and statistically significant. All departures from HWE were steady with heterozygote surplus. Examinations of genetic distinctiveness indicated that the narrow-clawed crayfish collections did not show one panmictic population. Pairwise F_{ST} values varied between 0.0115 to 0.087 and exposed that most adult populations were genetically noticeable from each and every one (Table 5).

For each population and locus: Number of samples used (n), Number of alleles per loci (Na), Effective number of alleles per loci (A_e), Number of rare alleles [A_r], Frequency of the most common allele [Fmax (S= size in bp)], Frequency of the less common allele [Fmin (S= size in bp)], Expected heterozygosity (Nei, 1978), and Observed heterozygosity.

Population correspondings with non-significant F_{ST} measurements included (1) Kiashahr versus Anzali, Masuleh; (2) Aras versus Anzali, Masulah, Kiashahr. Mean F_{ST} for all seven populations was 0.023. Populations from the Siah Darvishan and Chafrood River were highly distinct from populations of the others (Table 5). The overall trial had a chi-square measure of endlessness and Differences between allele frequencies among all populations were significant at all loci ($p < 0.0001$).

TABLE 3. Allelic variability at 6 loci in the survey *A. leptodactylus* populations.

	Variable	Khazar-Astara	Khazar-Kiashar	Anzali wetland	Chafrud	Masuleh	Siah-Darvishan	Aras
Locus								
	N	31	30	26	25	12	23	35
Aas2	**N_a**	5	4	2	4	2	5	3
	A_r	2.82	2.43	1.3	2.18	1.3	2.54	1.66
	F max	0.346	0.256	0.285	0.369	0.214	0.398	0.325
	F min	0.048	0.012	0.019	0.065	0.007	0.026	0.042
	He	0.644	0.536	0.482	0.539	0.494	0.681	0.570
	Ho	0.505	0.542	0.506	0.465	0.517	0.498	0.591
Aas7	**N**	30	28	25	26	12	21	33
	N_a	7	5	4	8	4	10	6
	A_r	3.94	2.46	2.13	4.19	1.98	4.96	3.14
	F max	0.368	0.226	0.218	0.446	0.368	0.498	0.317
	F min	0.034	0.011	0.024	0.071	0.006	0.041	0.029
	He	0.615	0.546	0.414	0.521	0.436	0.583	0.315
	Ho	0.732	0.521	0.382	0.420	0.378	0.469	0.357
Aas766	**N**	32	30	27	26	13	22	36
	N_a	6	4	4	8	4	8	5
	A_r	3.68	2.45	2.62	3.58	2.11	3.89	2.15
	F max	0.469	0.385	0.228	0.376	0.469	0.525	0.189
	F min	0.019	0.010	0.014	0.023	0.008	0.086	0.013
	He	0.572	0.390	0.434	0.492	0.384	0.540	0.476
	Ho	0.635	0.402	0.459	0.516	0.372	0.559	0.425

TABLE 3. Continued

|	Variable	Khazar-Astara	Khazar-Kiashar	Anzali wetland	Chafrud	Masuleh	Siah-Darvishan	Aras
Locus								
	N	31	29	25	25	12	21	32
Aas1198	**N_a**	5	3	2	4	2	6	5
	A_r	2.66	1.69	1.14	2.13	1.3	2.86	2.93
	F max	0.246	0.154	0.322	0.476	0.171	0.299	0.184
	F min	0.012	0.102	0.008	0.019	0.005	0.027	0.021
	He	0.544	0.468	0.396	0.456	0.288	0.641	0.434
	Ho	0.405	0.543	0.427	0.512	0.326	0.523	0.383
	N	30	29	23	25	11	21	31
	N_a	4	2	2	3	2	5	3
	A_r	2.14	1.46	1.12	1.39	1.4	2.28	1.48
Aas3666	**F max**	0.329	0.256	0.325	0.297	0.139	0.289	0.228
	F min	0.014	0.010	0.004	0.013	0.009	0.022	0.008
	He	0.445	0.431	0.318	0.387	0.368	0.428	0.346
	Ho	0.331	0.362	0.222	0.315	0.276	0.313	0.264
Aas3040	**N**	31	30	25	24	12	21	32
	N_a	4	3	3	5	3	6	3
	A_r	2.68	1.45	1.23	2.22	1.4	2.79	1.5
	F max	0.469	0.285	0.378	0.426	0.271	0.386	0.250
	F min	0.018	0.010	0.029	0.025	0.009	0.034	0.012
	He	0.579	0.490	0.432	0.498	0.397	0.585	0.568
	Ho	0.425	0.422	0.465	0.371	0.325	0.422	0.426

TABLE 4. Exact *P* value for Hardy Weinberg Equilibrium (HWE) estimation for the 7 different crayfish samples after sequential Bonferroni adjustments.

	Khazar-Astara	Khazar-Kiashar	Anzali	Chafrud	Masuleh	Siah Darvishan	Aras
Aas2	<0.001*	(0.005) 0.248	(0.005) 0.174	<0.001*	(0.003) 0.42	<0.001*	(0.005) 0.356
Aas7	<0.001*	(0.01) 0.25	<0.001*	<0.001*	(0.006) 0.319	<0.001*	(0.004) 0.234
Aas766	<0.001*	(0.003) 0.217	(0.002) 0.159	(0.000) 0.113	(0.005) 0.456	(0.004) 0.017	(0.005) 0.165
Aas1198	(0.003) 0.217	(0.004) 0.414	(0.002) 0.288	(0.002) 0.241	(0.004) 0.514	<0.001*	<0.001*
Aas3666	<0.001*	(0.005) 0.16	(0.002) 0.327	<0.001*	(0.01) 0.148	<0.001*	<0.01*
Aas3040	<0.001*	<0.001*	(0.02) 0.486	<0.001*	<0.01*	<0.001*	<0.001*

TABLE 5. Pairwise estimates of F_{ST} between populations of *A. leptodactylus* (above diagonal) and values of D_A for each population pair (below diagonal)

Location	Astara	Kiashahr	Anzali	Chafrood	Masuleh	Siah Darvishan	Aras
Astara	-	0.0115	.0.179	0.0427	0.0328	0.0713	0.0245
Kiashahr	0.0072	-	0.0128	0.0339	0.0104	0.0682	0.0167
Anzali	0.0096	0.0023	-	0.0246	0.0116	0.0741	0.0198
Chafrood	0.0518	0.0081	0.0036	-	0.0418	0.0867	0.0469
Masuleh	0.0123	0.009	0.0094	0.0778	-	0.0635	0.0213
Siah Darvishan	0.0285	0.0162	0.0865	0.0875	0.0172	-	0.0395
Aras	0.0063	0.0036	0.0039	0.0765	0.0091	0.0145	-

The neighbour-joining tree and MDS approach corroborated significant pairwise F_{ST} values by spawning the narrow-clawed crayfish from the Siah Darvishan, Astara and Chafrood River with high bootstrap support and the Siah Darvishan River also had the higher branch lengths. The tree indicated that the Astara and Kiashahr region (south coast of Caspian Sea) regularly clustered composed, with more support (Fig. 2, 3). In general, longer arm lengths isolated the crayfish populations of Siah Darvishan River from those of the other location, and populations of Astara and Chafrood River did not group with that of in the south shore, indicating substantial population genetic structure in this portion of Caspian Sea. In addition the genetic distinctiveness observed in south part of Caspian Sea enhanced, spawning locations that were more geographically remote to the rest of the Caspian Sea basin (Fig. 2).
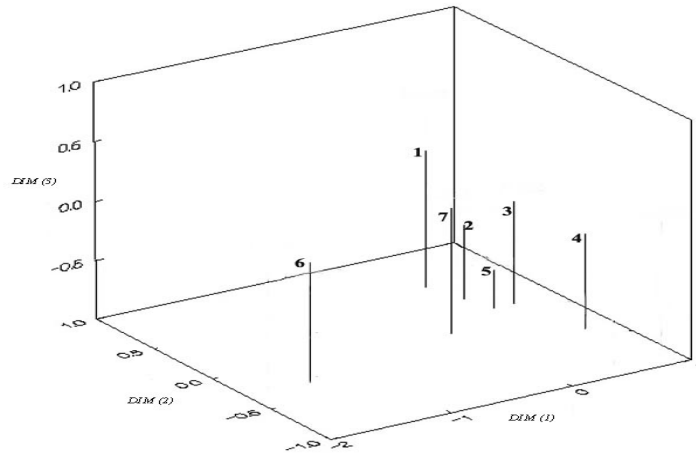
FIGURE 2. A multidimensional (MDS) plot based on D_A distances showing genetic relationships among seven populations of *A. leptodactylus*. 1- Astara, 2- Kiashahr, 3- Anzali, 4- Chafrood, 5- Masuleh, 6- Siah Darvishan and 7- Aras
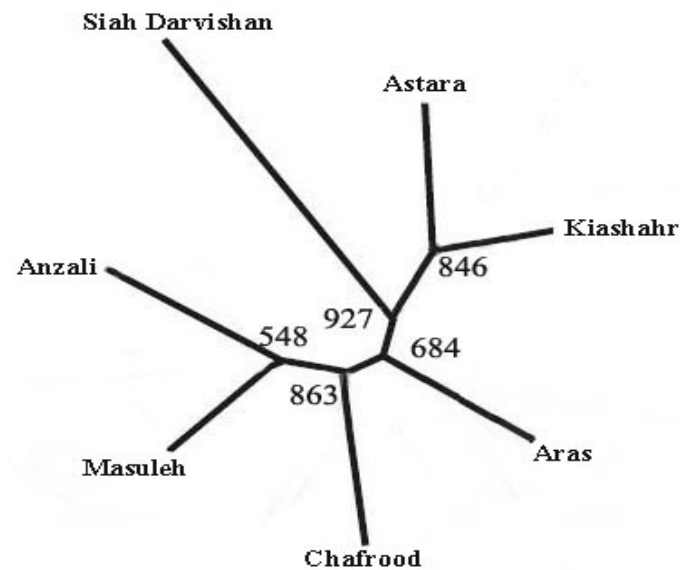
FIGURE 3. Unrooted neighbour-joining cluster analysis diagrams based on Cavalli-Sforza and Edwards' (1967) chord distance for the gene locus and microsatellite markers. The data were bootstrapped over loci, with replacement, for 1000 replicates; the numbers represent the percent support of the branch.

DISSCUSSION

In the present study, specific amplifications of 6 microsatellite loci in the narrow-clawed crayfish were obtained after optimizing the experimental conditions. Identical result was attained by Gross *et al.* (2013), who observed a cross-amplification of 12 *Astacus stacus* (Linnaeus, 1758). The results of the genetic population identification showed that narrow-clawed crayfish adult accumulates did not establish one panmictic population and structuring continued. In the natural condition, if migration between populations is low, it means association with high balanced of genetic distinction (Bohonak 1999).

Our results indicate that there is a genetic structure in the narrow-clawed crayfish populations and these findings agree with the patterns of cytochrome oxidase subunit I (*COI*) variation reported by Khoshkholgh & Nazari (2015), who observed significant differences in mtDNA *COI* segment among seven populations using polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis of the mitochondrial DNA. Significant variance in microsatellite allele frequency (mean F_{ST} = 0.023) provide evidence that narrow-clawed crayfish populations are spatially genetically structured. Genetic affinities among populations revealed in the neighbour-joining tree and multidimensional scaling showed high bootstrap and genetic distance support for three distinct population segments, generally corresponding to location of origin (Siah Darvishan River, Chafrood River and Astara (Fig. 2). The majority of the pairwise estimates of inter-population variance in allele frequencies (F_{ST}) were also found to be statistically significant.

Our results, therefore, do not support the null hypothesis of a homogeneous gene pool for *A. leptodactylus* inhabiting the in the Caspian Sea basin. However little level of genetic distinctiveness in *A. leptodactylus* from Anzali, Kiashahr, Aras and Masuleh River could be related high migration rate of this species. The species' life history also plays a role in influencing contemporary levels of spatial population structure. No barriers exist in the south coast of Caspian Sea that prevents narrow-clawed crayfish from dispersing over long distances.

Recent studies have suggested that despite extensive marine migrations, crayfish exhibit remarkable figure of stock-specific migration and accumulation (Fetzner *et al.*, 1999; Gouin *et al.*, 2002, 2011; Grandjean *et al.*, 2001; Gross *et al.*, 2013). Hence, it is imaginable that for *A. leptodactylus* movement models are recognizable between sites and the connection of these migratory models stays unexplored. Identification of population complicatedness at different locations is crucial for efficient species management and rehabilitation programme (Largiader *et al.*, 2000; Schulz, 2000).

The mean F_{ST} value also displayed genetic distinction between populations and suggests these populations are separate. In the examination of genetic structure of *A. leptodactylus* populations in the Caspian Sea basin, Khoshkholgh & Nazari (2015) reported significant genetic differentiation between some collections. Our data and the research by Khoshkholgh & Nazari (2015) approve the idea that there is gene flow between adjacent populations and that topographical separation of genetic stock structure occurs above long intervals (Bohonak, 1999; Miller, 2003). Possible reasons for the discrepancies in genetic stock structure of *A. leptodactylus* in Siah Darvishan River, Chafrood River and Astara include habitat characteristics.

The genetic variability detected in the present study for *A. leptodactylus* is almost identical to that observed in other finfish species in the Caspian Sea. For example, Norouzi *et al.* (2009) discovered significant discrepancies among the adjacent neighbours of some river districts for Stellate sturgeon *A. stellatus* (Pallas, 1771) using microsatellites, resulting in the identification of three management units and also pronounced genetic differentiation has been found between south populations and Ural River populations of Ship sturgeon *A. nudiventris* (Lovetsky, 1828) using mtDNA variation (Qasemi *et al.*, 2004). These studies and limited information on *A. leptodactylus* suggest that *A. leptodactylus* spawning habitat is distinctive within the river systems they use for spawning.

The results showed a moderate genetic structure between populations. However, supplementary microsatellite loci should be applied to complete a genetic population identification programme and quantify the potential subscription of distinct stock segments to mixed stocks found in the regions. This would donate managers with information on migratory patterns and give them a more comprehensive understanding of the stock complexity of *A. leptodactylus*. Development of further microsatellite markers and sampling of more regions in different part of the Caspian Sea basin (particularly in Anzali watershed and Aras) are helpful for describing sections for conservation and management

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