

Genetic variation in the narrow-clawed crayfish (*Astacus leptodactylus*) populations as assessed by PCR-RFLP of mitochondrial *COI* gene

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ABSTRACT

The genetic variation and population structure of narrow-clawed crayfish (*Astacus leptodactylus*) was examined by means of polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis of the cytochrome oxidase subunit I (*COI*) of mitochondrial DNA. A total of 194 adult specimens were collected from seven sample sites including, two in the south Caspian Sea and one each in Anzali wetland and Aras reservoir and three rivers Chafrood, Masule Rudkhan and Siah Darvishan. The PCR products were digested with 19 restriction enzymes and five enzymes revealed polymorphism patterns (*DdeI*, *MboI*, *TaqI*, *RsaI* and *HinfI*). Twenty eight composite haplotypes were showed with the number of haplotypes in each population sample ranging from 8 to 13. Private haplotypes were found at very low frequencies. Two regional (Siah Darvishan River and Astara) groups were clearly recognized by cluster and molecular variance model (AMOVA) analyses ($P < 0.0001$). Each of these groups revealed dominant haplotypes while these haplotypes play less important rule in population structures of the other geographic areas. Intrapopulation haplotype (h) and nucleotide (π) diversities were high for each locality, ranging $h = 0.7560 \pm 0.030$ and $\pi = 0.00334 \pm 0.00301$, respectively. Results of this study discerned two genetically divergent populations of narrow-clawed crayfish including Siah Darvishan River and Astara. Thus, the population structure of the narrow-clawed crayfish, as inferred from mtDNA analysis, is constituted by genetically separate groups that nearly reflect their geographic distribution.

Key words: *Astacus leptodactylus*; Mitochondrial DNA; PCR-RFLP; Genetic diversity

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INTRODUCTION

The narrow-clawed crayfish *Astacus leptodactylus* is naturally and widely distributed in lakes, ponds and rivers throughout of Iran. *A. leptodactylus* is a widespread species and can be found throughout Europe, Eastern Russia, and the Middle East. However it is absent from some of the Northern European countries such as Norway and Sweden, and the Southern European countries such as Spain and Portugal [1, 2]. The narrow-clawed crayfish is considered indigenous in the Eastern part of its range, but has been introduced into many of the Western European countries [2, 3]. This species is found in both fresh and brackish waters, e.g. lagoons, estuaries, as well as running freshwater rivers in the Ponto-Caspian Basin [4, 5]. Iranian native crayfish includes one crayfish species, *Astacus leptodactylus*, with two subspecies, *A. l. leptodactylus* and *A. l. eichwaldi*. Only *A. leptodactylus* is commonly distributed in Iranian water resources. *A. leptodactylus* was found not only in the Caspian Sea up to 5–10 km offshore [6, 7]. The narrow-clawed crayfish is tolerant to changes in temperature, low oxygen content, and low water transparency, and is known to occur in saline conditions such as estuaries. Tolerance experiments indicated that juveniles and adults are well adapted for surviving salinities of at least 21ppt in the long term, and will tolerate being transferred directly back into freshwater. However, their ability to colonize the estuarine environment may be restricted to areas of low salinity (i.e. 7ppt) due to the adverse effects of seawater on egg development and hatching [4].

The advantages of mtDNA as a tool for population genetics have been extensively reviewed [8], as well as the ability of mtDNA to retain a history of past isolation, even in the event of contemporary admixture of groups that evolved in allopatry [8-10]. Geographical investigations of mtDNA within several freshwater species have also demonstrated the significance of both historical biogeography and contemporary gene flow in shaping intraspecific population genetic structure [11, 12]. Further, mtDNA can aid in determining the taxonomic distinctiveness of individual populations and therefore assist in setting precedence for conservation programs [13]. Mitochondrial DNA has proved to be an superior tool for examining population genetics, above or below the species level [8, 14]. It has appeared as a genetic marker able to distinguish stocks [15-20]. Because of the fast evolution and maternal mode of inheritance, mitochondrial DNA (mtDNA) has been widely used to survey genetic differences and evolutionary history between species and within species [8, 13, 21]. Variations of the complete mtDNA genome, individual genes, or restriction fragment length polymorphisms (RFLPs) have been applicable in characterizing taxa, establishing phylogenetic relationships, clarifying conspecific hybridizations, identification of hatchery and wild stocks and estimating stocks in many aquatic species [11, 13, 14, 19, 22, 23]. Many scientists have attempted to depict the genetic structure of freshwater crayfish species. A variety of methods have been applied. Early studies based on protein electrophoresis have shown only low levels of variation between populations of European freshwater crayfish [24, 25]. During the last decade molecular techniques with a higher degree of variability have been developed and applied successfully in population studies of freshwater crayfish. Genetic differentiation of populations had been assessed using

mitochondrial DNA [17, 26-28], RAPD-PCR [29-31], AFLP [32] and microsatellites [31, 33-36]. Phylogenetic studies [37, 38], and reconstruction of the phylogeography [39] were possible using mitochondrial DNA. Regarding crayfish species, most of these studies focused on the *Austropotamobius pallipes* and information on genetic structure of *A. leptodactylus* is still rare. Assessing the relative levels of genetic diversity within and divergence among narrow-clawed populations in Iran can provide the first step towards conserving narrow-clawed genetic resources here and optimizing them for future breed improvement programs. This study is the first molecular-based study of *A. leptodactylus* covering a large part of its distribution range, including river catchments of the south part of the Caspian Sea, Anzali lagoon, Southwest Caspian Sea and Aras reservoir in Northwest of Iran. The purpose of this study is to appraise the genetic status of the narrow-clawed crayfish breeds, by estimating both their relative levels of genetic diversity and the scope of genetic divergence among them using clustering procedure.

MATERIALS AND METHODS

Adults of *A. leptodactylus* were collected from seven localities (Table 1, Fig. 1). From each locality between 14 and 37 individuals were collected and analyzed (Table 1). Tissues samples from walking legs (pereiopods) or abdomen were dissected on site, immediately fixed in 95% ethanol, and transferred to the laboratory.

The genomic DNA was isolated from each sample using standard phenol-chloroform extractions and ethanol precipitations following the method described previously [14, 40]. Extracted DNA was verified for concentration using spectrophotometer (Nanodrop ND1000) and the DNA was following standardized to a specific concentration (i.e. 50 ng/ μ l for PCR reactions and 100 ng/ μ l for permanent storage in a DNA archive). The quality of each DNA specimen was approved visually on a 0.8% 0.5 \times TBE agarose gel containing ethidium bromide against a known standard.

The mtDNA cytochrome oxidase subunit I was amplified using the polymerase chain reaction (PCR). Primers were designed manually specifically for *A. leptodactylus* (alignment of primer sequences against the noble crayfish *A. astacus* accession number: I0DEU4). Primers 5'-GGT CAA CAA ATC ATA AAG ATA TTG-3' (forward) and 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (reverse) were designed to amplify an approximate 1535 bp of the mtDNA *COI* gene. For amplification, the following reagents were added to each microtube: 2 μ l of template DNA; 5 μ l of 10 \times buffer (100 mM Tris-HCl, pH 8.3, 15 mM MgCl₂, 500 mM KCl); 1 μ l of each primer (10 pmol); 5 μ l of a 5 mM solution of each deoxyribonucleoside triphosphate (dNTP); 2.5 units of Taq DNA polymerase. Enough ultrapure water was added to each sample to make a solution of 50 μ l. Polymerase chain reaction conditions consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min (5 min for the last extension only). The amplicons were exposed to endonuclease digestion using the four-base recognition enzymes *Rsa* I, *Hinf* I and *Mbo*I and the five-base *Dde*I and *Taq*I. All restriction digestions followed the standard procedure provided by the manufacturer. Restriction digestion was carried out in a 10 μ l volume containing

2 μ l of PCR product, 2 units of restriction enzyme, 1 μ l of the appropriate buffer and 7 μ l of ultrapure water. RFLP digestion was performed in an incubator at 37°C for at least 16 h. Restriction fragments were segregated on a 6% acryl amid gel, stained with silver staining technique and photographed.

Table 1: Sampling localities, numbers, and number of individuals sampled (*n*)

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

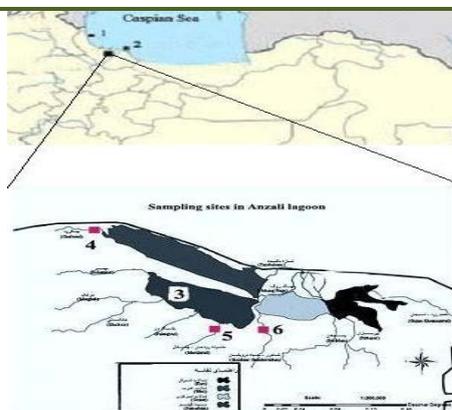


Figure1: Sampling sites of the narrow-clawed crayfish, *A. leptodactylus*. The localities are labeled with numbers. Detailed information about the sites can be found in Table 1.

A composite mtDNA haplotype, consisting of five letters that represent the fragment pattern generated by each of the restriction endonucleases, was compiled for each individual. The nucleotide diversity (π) and haplotype diversity (h) in a population were computed according to known equation [41, 42]. The estimates of nucleotide divergence [42] between the mtDNA haplotypes and the samples examined were taken as standard genetic distances. The estimates were used for phylogenetic analysis performed with an unweighted pair group method with arithmetic mean (UPGMA) algorithm. Clustering robustness was calculated using bootstrap (100 iterations) [43, 44]. Estimates of nucleotide divergence and dendrogram topology were made using the PAUP version 4.0b10 software package [45]. The main genetic variation indices were estimated using the REAP [46] and ARLEQUIN version 2.0 [28] software packages. Heterogeneity of haplotype frequencies between each sample pair was evaluated using the Monte Carlo method (1000 pseudorandom replicates [47]). Quantitative estimates of the geographic subdivision of mtDNA variation were performed using the AMOVA method, where molecular variance was partitioned into three hierarchical levels, including the between-groups, between-population within groups, and among haplotype

within population components [48, 49]. F-statistics criteria were calculated to test statistical significance of the hierarchic components of variance [50].

RESULTS

Variation within samples in all 194 individuals amplified successfully in the mtDNA *COI* gene. Twenty eight different composite haplotypes were revealed with the number of haplotypes in each population sample ranging from 8 to 13 and 13 of them were private, that is, present in only one population sample (Table 2). Private haplotypes were found at very low frequencies, which were almost completely absent in other geographic areas ($P < 0.005$).

Table 2: Nucleotide diversity (π) and haplotype diversity (h) values and geographic distribution of *A. leptodactylus* mtDNA RFLP composite haplotypes derived in this study

	Astara (34)	Kiashahr (31)	Anzali (27)	Chafrood (28)	Masuleh (14)	Siah darvishan (23)	Aras (37)
π		0.0035	0.0012	0.0017	0.0046	0.0032	0.0054
h		0.762	0.687	0.718	0.816	0.782	0.702
No.	Haplotypes						
1	AAAAA	5	11	8	7	3	4
2	AABAA	3	3	6	2	2	1
3	ABAAB	2	4	2	-	1	-
4	ABCAB	-	1	2	2	-	9
5	AABBC	13	3	1	-	3	-
6	ACBAB	-	-	-	-	-	-
7	BBBAC	1	-	-	-	-	-
8	BABCA	-	1	2	2	-	-
9	ACABD	-	-	-	-	-	-
10	ACCAA	3	4	-	1	-	-
11	BACBC	1	-	-	-	-	-
12	BACAC	-	-	-	2	-	1
13	ABB CD	-	-	-	-	-	1
14	ABAAD	-	-	1	1	-	-
15	ACACC	-	-	-	-	1	-
16	ABCAB	-	-	-	-	1	-
17	AACBD	-	-	-	-	-	1
18	ABABB	-	3	3	1	-	-
19	ABCAA	1	-	-	-	-	1
20	BAABA	3	-	1	2	-	-
21	BBACD	-	-	-	1	-	-
22	BBBAC	-	-	-	1	1	-
23	BAABD	-	-	-	-	-	-
24	BAABC	2	1	1	5	-	-
25	BACBC	-	-	-	-	-	1
26	BACCB	-	-	-	1	-	-
27	CCAAB	-	-	-	-	-	2
28	ABBCC	-	-	-	-	-	1

Note: Haplotypes are composite scores for fragment patterns produced by digestion with *RsaI*, *HinfI*, *DdeI*, *MboI* and *TaqI*, respectively. Numbers in parentheses indicate numbers of individuals.

The geographic distributions of all haplotypes in throughout of sampling areas are given in Table 2. The most prevalent composite haplotype was haplotype 1 (AAAAA) with a frequency of 0.2577 in the all the samples. Frequencies of the non-private composite haplotypes varied among geographic areas. For example, haplotype 1 was found in all samples (Table 2). In the remaining geographic areas, it varied from 0.07 in Masuleh River to 0.32 in the Aras River. Haplotype 2 (AABAA) showed a frequency distribution with 0.11 in all geographic areas.

In the present study the mean haplotype diversity (h) and nucleotide diversity (π) were 0.7560 ± 0.030 and 0.00334 ± 0.00301 , respectively. The samples with the highest haplotype diversity were from Siah Darvishan River (0.829 ± 0.046) and Chafrood River (0.816 ± 0.032). On the other hand, the population samples with the lowest variability were from the southern part of the Caspian Sea in Kiashahr with haplotype diversity 0.687 ± 0.025 as well as the Aras River (0.702 ± 0.038) (Table 2). Table 3 demonstrates the results of nucleotide divergence methods to estimate the genetic differentiation of the populations examined. The estimation was based on the number of nucleotide substitutions. Nucleotide divergence ranged between 0.00149 and 0.01853 (Table 3).

Table 3: Pairwise estimates of mtDNA divergence (% of nucleotide substitutions) in *A. leptodactylus*

Locations	Astara	Kiashahr	Anzali	Chafrood	Masuleh	Siah darvishan
Kiashahr	0.00614	-				
Anzali	0.00851	0.00149	-			
Chafrood	0.00615	0.00317	0.00268	-		
Masuleh	0.00178	0.00933	0.00832	0.00384	-	
Siah	0.01853	0.01624	0.00856	0.00981	0.01608	-
Aras	0.00727	0.00295	0.00382	0.00498	0.00694	0.01473

The lowest Nucleotide divergence was seen between Kiashahr and Anzali samples. The results of the chi square test of *A. leptodactylus* populations test showed significant genetic structures in 8 of the 21 pairwise comparisons, most involving the Siah Darvishan River and Astara samples (Table 4). The χ^2 test of haplotype frequencies also revealed that the population of Siah Darvishan River and the population of Astara are significantly different from one another ($P < 0.0001$). It was shown that the main contribution to the heterogeneity of the population set was made by the *A. leptodactylus* samples from the Siah Darvishan River and Astara populations. Thus, three distinct genetic groups were identified (Siah Darvishan River Astara and all others). The cluster analysis also supported the existence of these three groups.

Table 4: Pairwise comparison of Monte Carlo based chi-square values for *A. leptodactylus* populations from 7 locations (P -values in parentheses).

Locations	Astara	Kiashahr	Anzali	Chafrood	Masuleh	Siah darvishan
Kiashahr	58.32	-				
Anzali	89.66	22.12	-			
Chafrood	74.62	28.65	19.12	-		
Masuleh	98.54	16.03	22.09	11.78	-	
Siah darvishan	116.85	96.8	83.75	69.26	52.43	-
Aras	49.17	31.14	11.8	36.9	18.4	87.00

There were no clear subclustering and no correlation between geographic distribution and pairwise χ^2 values between the two southern populations of Caspian Sea and other locations (Fig. 2). The most differentiated cluster included populations of the Astara and Siah Darvishan River populations. Aras and Anzali lagoon populations formed one population. Furthermore, the population positions on the dendrogram inferred from the mtDNA variation did not show clear differentiation among the *A. leptodactylus* populations from Kiashahr and Anzali lagoon. Thus, the UPGMA tree suggested three

distinct groups; Siah darvishan River, Astara, Aras - Anzali lagoon- Chafroud River - Masuleh River (Fig. 2).

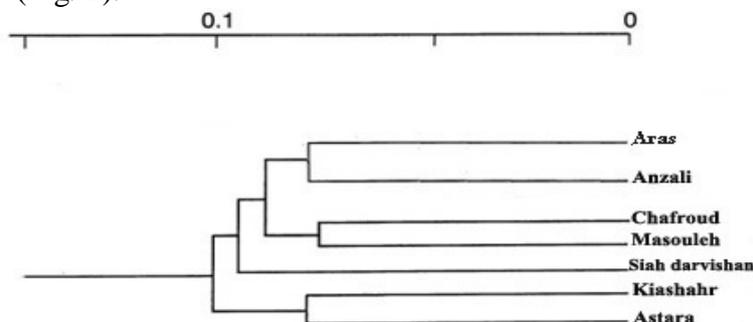


Figure 2: An UPGMA tree of the population genetic distances for the mtDNA *COI* data from *A. leptodactylus*. Bootstrap values are given at each node.

The degree of genetic differentiation among all sample pairs was tested with analysis of molecular variance (AMOVA). The results of AMOVA demonstrated that collections between Astara in the southwest Caspian Sea and also Siah Darvishan River are significant ($P < 0.0001$). Therefore heterogeneity test of the narrow-clawed crayfish populations for haplotype frequencies and Monte-Carlo with 1000 replicates in RFLP analysis of *COI* gene showed significant differences ($P < 0.0001$) and these results showed that haplotype distribution in different location were significant and populations of Siah darvishan River and Astara statistically were significant ($P < 0.0001$).

To make quantitative estimates of the values of genetic differences, total molecular variance of haplotype frequencies was subdivided into three hierarchical levels (Table 5). An analysis showed that molecular divergence of *A. leptodactylus* was mostly distributed among haplotypes within populations (56.85%, Table 5). The AMOVA also segmented of total 38.79% genetic variation among the groups and had the low level of variance among populations within region (4.36%, $P = 0.143$). However, according to the data acquired, the hierarchical levels of geographically isolated subdivisions were statistically significant ($P < 0.05$). Thus concluded that the most part of the mtDNA intraspecific variation is determined by the differences among the haplotypes within a single population.

Table 5: Hierarchic search for haplotype differences in *A. leptodactylus*

Source of variation	df	Percentage of variation	Fixation indices	<i>P</i>
Among groups	2	38.79	$F_{SC} = 0.0228$	< 0.001
Among populations within groups	4	4.36	$F_{CT} = 0.0017$	0.143
Among haplotype within population	188	56.85	$F_{ST} = 0.0332$	< 0.001
Total	194			

DISCUSSION

Results obtained from the analysis of mtDNA variation proved that most narrow-clawed crayfish samples display a wealth of diversity, presenting values of haplotype

diversity. We found that the large mtDNA gene region (*COI* gene) showed the high haplotype diversity. Detecting genetic variation in mtDNA between organisms depends chiefly on the evolutionary rate of genes and the number of nucleotide bases surveyed [11, 51]. As molecular variance of Astara and Siah Darvishan River are significant, two distinct populations were identified. Our result is contrary to allozyme data which showed a very low level of genetic variability in several populations of *A. leptodactylus* [24] white-clawed crayfish, *Austropotamobius pallipes pallipes* [52], noble crayfish, *Astacus astacus* [25]. These studies mentioned that severe population size reduction due to several factors (such as overfishing, pollution, habitat destruction, etc.) tended to increase stochastic genetic effects leading to a loss of genetic variability within populations. UPGMA analysis built from haplotype frequencies clearly revealed a geographical structuring of populations, with two main clusters in *A. leptodactylus* corresponding to the southern part of Caspian Sea and Siah Darvishan River. This result supports the hypothesis that each of these clusters include genetically differentiated populations. Most of the variation is distributed within populations, which may be explained by the existence of many shared haplotypes between populations. Analyses of the genetic structure of crustacean species with Caspian Sea tributaries distribution are relatively few and have mostly dealt with fish species [e.g. 15, 20, 23, 53]. In majority of the studies so far, a moderate to strong genetic cline between sampling sites was observed, for example, Persian sturgeon [12, 14]. The geographical pattern of the genetic distribution, supported by the present data, is similar to those obtained in other freshwater organisms such as Persian sturgeon, *Acipenser persicus* [12, 19], ship sturgeon, *A. nudiventris*, [50], and Russian sturgeon *A. gueldenstaedtii* [54, 55].

This study, based on the *COI* mtDNA marker, revealed a high number of coexisting haplotypes (28) in a restricted geographic range, indicating a strong genetic structure, with most localities containing just one or few 'private' haplotypes. Private haplotypes were, however, detected in low frequencies and therefore could not be used as population markers [14, 16, 26]. To discover the origin of the private haplotypes found within river catchments, additional populations nearby need to be examined.

The differences between stocks located close together were smaller than those between more distant stocks. However, it was not possible to determine whether low genetic variation found among individuals within a given stock was due to environmental constraints or genetic drift as result of a small population size. Nevertheless, the artificial translocation of individuals can be observed in the disjunct distribution of some shared haplotypes between different drainage areas (Fig. 1) Five of 7 populations showed no genetic diversity, while the most sample sites contained moderate level of genetic variability, especially because of the presence of private haplotypes. These levels of genetic variability are in accordance with the data of Grandjean and Souty-Grosset (2000) [26]. According to Avise et al., (1994) [2], contemporary intraspecific mtDNA variability is controlled by stochastic lineage extinction, which in turn is a function of long-term effective population size. Thus, the high genetic variability recorded within southern populations of Caspian Sea are not in accordance with ancient bottleneck events but suggested that these populations have had large effective populations sizes in the past [57, 58]. In the same manner, the genetic

homogeneity revealed among Aras and Anzali lagoon populations suggests that these populations could be designated as one unique unit for conservation [11, 14, 33, 36, 39]. Several factors may be responsible for the observed pattern. For example, Overfishing, which increases the risks of effective population size reduction, and extinction of intermediate populations, may be one possibility. In addition, anthropogenic degradation of habitat could be an impediment to dispersal and final settling of larvae [1, 31, 33]. Data from the present study would be useful in assisting management and conservation, as well as notifying a recovery plan for wild populations. This is important specifically for a unique population such as Astar population, since the majority of the *A. leptodactylus* broodstocks for the hatcheries in Iran depend on wild populations [2, 59, 60]. Additional techniques such as microsatellite analysis and mtDNA sequences should be conducted to evaluate genetic variation within populations of narrow-clawed crayfish to give particular attention to diverse populations and to more identify artificially stocked and naturally scattered populations.

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Conflict of Interest: The authors declare that they have no competing interest.

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