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Using ISSR Genomic Fingerprinting to Study the Genetic Differentiation of *Artemia* Leach, 1819 (Crustacea: Anostraca) from Iran and Neighbor Regions with the Focus on the Invasive American *Artemia franciscana*

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Abstract: Due to the rapid developments in the aquaculture industry, *Artemia franciscana*, originally an American species, has been introduced to Eurasia, Africa and Australia. In the present study, we used a partial sequence of the mitochondrial DNA *Cytochrome Oxidase subunit I* (*mt*-DNA *COI*) gene and genomic fingerprinting by Inter-Simple Sequence Repeats (ISSRs) to determine the genetic variability and population structure of *Artemia* populations (indigenous and introduced) from 14 different geographical locations in Western Asia. Based on the haplotype spanning network, *Artemia urmiana* has exhibited higher genetic variation than native parthenogenetic populations. Although *A. urmiana* represented a completely private haplotype distribution, no apparent genetic structure was recognized among the native parthenogenetic and invasive *A. franciscana* populations. Our ISSR findings have documented that despite that invasive populations have lower variation than the source population in Great Salt Lake (Utah, USA), they have significantly revealed higher genetic variability compared to the native populations in Western Asia. According to the ISSR results, the native populations were not fully differentiated by the PCoA analysis, but the exotic *A. franciscana* populations were geographically divided into four genetic groups. We believe that during the colonization, invasive populations have experienced substantial genetic divergences, under new ecological conditions in the non-indigenous regions.

Keywords: genetic variation; brine shrimp *Artemia*; invasive species; *mt*-DNA COI; Inter-Simple Sequence Repeats (ISSRs) genomic fingerprinting; Western Asia

1. Introduction

The brine shrimp *Artemia* is a unique zooplankton that has a limited number of species, distributed globally, except in Antarctica [1]. This tiny crustacean has potentially adapted to live in extreme environmental conditions, such as hypersaline environments [2,3].

Artemia has been mainly used as live food in larviculture and fishery industries, especially in Asia [4]. *Artemia* has been used to improve the quality of sodium chloride production in solar salt-fields [5,6]. It was also introduced as a model organism in many bioscience studies, including

cellular and molecular biology [7], phylogeography and asexual evolution [8], bioencapsulation [9] and toxicity assessment [10].

The genus *Artemia* consists of seven bisexual species and a large number of parthenogenetic populations with different ploidy levels [3,11]. It has been assumed that Asia is the origin of *Artemia urmiana* Günther, 1899 (Lake Urmia, Iran), *Artemia sinica* Cai, 1989 (China), *Artemia tibetiana* Abatzopoulos, Zhang and Sorgeloos, 1998 (Qinghai-Tibetan Plateau, China), and corresponding parthenogenetic populations [3,12]. Recently, two new species, *Artemia frameshifta* and *Artemia murae* have been described from Mongolia [13]. These species have been described using a single mitochondrial DNA protein-coding *Cytochrome Oxidase subunit I* (*COI*) gene sequence without confirmation by any morphometric and population genetic analyses [11]. A main problem in the submitted *COI* sequence of *A. frameshifta* (LC195588) was detected in the protein sequence with several stop codon(s). In a protein coding gene, this is an indication for a nuclear copy of this mtDNA gene. Sometimes, the *COI* marker provides a sharp PCR amplified band on the agarose gel, but the sequence information presents heterogeneities [14]. These kinds of results could mislead the interpretation of downstream phylogenetic analyses. Therefore, the population would need more biosystematic evidences to determine its taxonomical status. In *A. murae*, neither the existence of males nor the reproductive mode has been revealed [13]. Based on the current information, we assume that *A. murae* is a parthenogenetic population, which needs more investigations to confirm the biological status of the species.

Generally, the long-distance translocations of the American species *Artemia franciscana* to other non-indigenous regions have occurred as a result of commercial activities, which have been fully documented previously [2,15–18]. *Artemia franciscana* is a successful invader in saltwater ecosystems due to its faster filter-feeding rate, a high potential of reproduction [15,19], and a better physiological immune system, which is associated with nutritional behavior against cestode parasites [15] than the native species. Asem et al. [17] have suggested that these biological characteristics could afford a high level of adaptive potential of *A. franciscana* in the new non-indigenous habitats, which would eventually result in the replacement with native species.

Lee [20] has documented that the genetic structure of introduced populations to the non-indigenous habitats is one of the most determinative parameters in their successful establishment. Generally, genetic diversity of the species could determine the potential of an exotic species to acclimatize in the new environmental conditions [21].

Previous studies on *A. franciscana* have documented that invasive populations demonstrated genetic variations relative to the native American source populations [2,17,18,22–24]. The low genetic diversity in the non-indigenous populations has been attributed to the founder effect [22] or population bottleneck due to the decreasing of population size in introduced populations during the process of establishment [17]. Moreover, high genetic variation could be a result of adaptive capacity and physiological flexibility as a special biological trait observed in invasive populations [2,6,24–26].

Eimanifar et al. [2] have reported the existence of invasive *A. franciscana* in four sites from Iran (three sites) and Iraq (one site) using the mitochondrial *COI* sequence marker. The aim of the present study was to further perform an analysis based on population genetic approaches to determine the intra- and inter-specific genetic variations of native and invasive *Artemia* populations from Iran and neighboring regions (14 sites) using Inter-Simple Sequence Repeats (ISSRs) genomic fingerprinting. Genomic fingerprinting by ISSR has been demonstrated to be a useful molecular tool to recognize DNA polymorphisms among *Artemia* taxa [27–30]. We hypothesize that the establishment of an exotic species in the new geographical habitats should be accompanied by intra-species genetic divergence to better adapt to the new environmental conditions. Here, we utilized high-resolution ISSR genomic fingerprinting to compare the genetic differentiation in native and colonizing populations of American *A. franciscana* in the indigenous environments.

2. Material and Methods

2.1. Sample Collection and DNA Extraction

Artemia cyst specimens were collected from 14 geographical localities across Iran and neighbor countries (Figure 1). All studied populations had been confirmed to be bisexual or parthenogenetic according to Asem et al. [3]. The sample localities with their geographical coordinates, abbreviations and IPMB voucher are summarized in Table 1. Total genomic DNA was extracted from part of the antenna of adult males and females using the Chelex® 100 Resin method (6%, Bio-Rad Laboratories, Hercules, CA, USA) [16]. All extracted DNA was stored at −80 °C for subsequent genetic characterization.

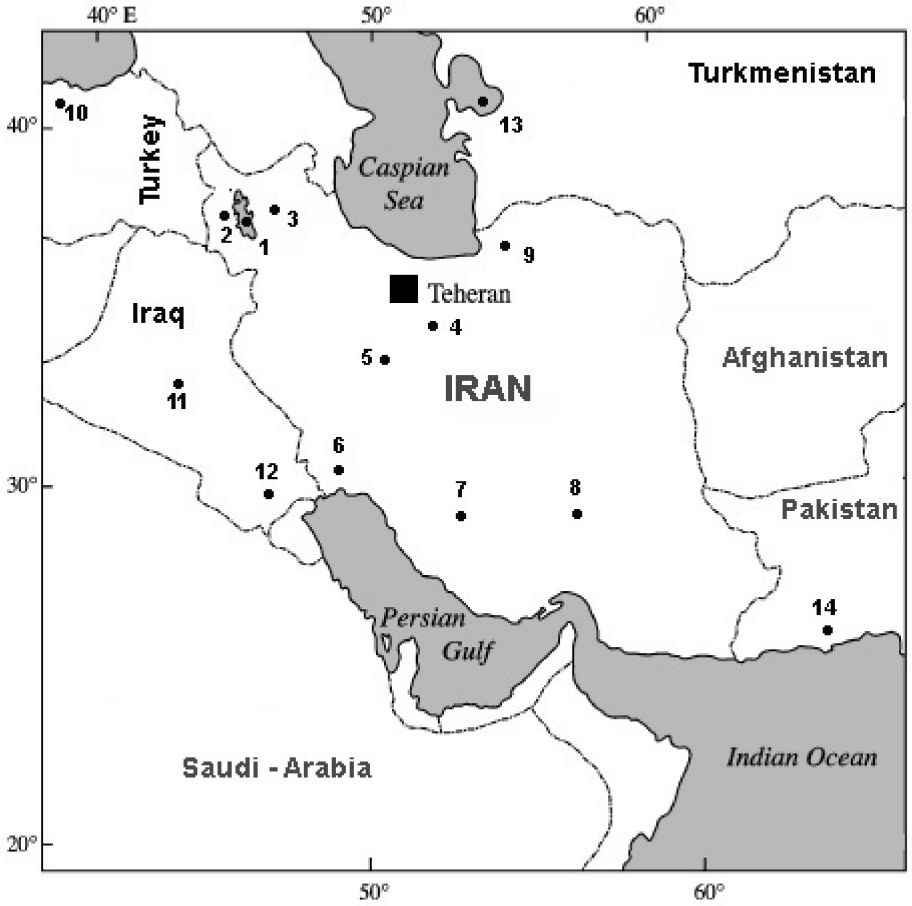

Figure 1. Map of *Artemia* sampling sites (1 = URM, 2 = LAGW, 3 = LAGE, 4 = QOM, 5 = MIG, 6 = MSH, 7 = MAH, 8 = NOG, 9 = INC, 10 = CAM, 11 = ABG, 12 = GAA, 13 = KBG, 14 = KOC; Abbreviations are listed in Table 1).

2.2. Population Identification and Phylogenetic Analyses

A partial sequence of the mitochondrial marker *cytochrome c oxidase subunit I* (*COI*) was utilized to identify the taxonomical status of the studied populations using phylogenetic analyses as implemented in the MEGA X program (Temple University, Philadelphia, USA) [2,17,18]. To identify the taxonomical status of the studied populations, the *COI* reference sequences from the recognized bisexual species and parthenogenetic populations were downloaded from GenBank (Table 2). Sequences were aligned using MEGA X with default settings [31]. Phylogenetic trees were reconstructed based on the Maximum Likelihood approach included in the MEGA X program. To reveal the genealogical and geographical relationships, a median haplotype network was established, following the median-joining algorithm in the Network program ver. 5.0.1.1 (Universität Hamburg, Hamburg, Germany) [32].

Table 1. Origin of *Artemia* samples from Iran and neighbor regions. (IPMB = Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Abb. = Abbreviation).

No.	Voucher Number (IPMB)	Abb.	Species/Population	Locality	Country	Geographic Coordinates	*COI* Accession Numbers
1	57211	URM	*A. urmiana*	Urmia Lake	Iran	37°20′ E–45°40′ N	JX512748-808 [28]
2	57223	LAGW	Parthenogenetic	Western Lagoon around Urmia Lake	Iran	37°15′ E–45°85′ N	KF691338-342 [2]
3	57224	LAGE	Parthenogenetic	Eastern Lagoon around Urmia Lake	Iran	37°50′ E–46°40′ N	KF691343-345 [2]
4	57225	QOM	Parthenogenetic	Qom Salt Lake	Iran	34°40′ E–51°80′ N	KF691367-372 [2]
5	57226	MIG	Parthenogenetic	Mighan Salt Lake	Iran	34°20′ E–49°80′ N	KF691357-361 [2]
6	57230	MSH	*A. franciscana*	Mahshar port	Iran	49°11′ E–30°33′ N	KF691351-356 [2]
7	57228	MAHR	*A. franciscana*	Maharlu Lake	Iran	29°57′ E–52°14′ N	KF691347, 349-350 [2]
8	57229	NOG	*A. franciscana*	Nough Catchment	Iran	30°60′ E–56°50′ N	KF691362-366 [2]
9	57227	INC	Parthenogenetic	Incheh Lake	Iran	37°24′ E–54°36′ N	KF691333-337 [2]
10	57292	CAM	Parthenogenetic	Camalti Lake	Turkey	27°08′ E–38°25′ N	KF691520-525; 527-529 [2]
11	57255	ABG	Parthenogenetic	Abu-Ghraib	Iraq	44°30′ E–33°20′ N	KF691373-375 [2]
12	57256	GAA	*A. franciscana*	Garmat Ali	Iraq	47°49′ E–30°30′ N	KF691376-383 [2]
13	57258	KBG	Parthenogenetic	Kara Bogaz Gol	Turkmenistan	53°33′ E–41°17′ N	KF691530-532,534 [2]
14	57257	KOC	Parthenogenetic	Korangi Creek (Karachi coast)	Pakistan	67°10′ E–24°48′ N	KF691442-445; 447-448 JX512748 [2]

[28] Eimanifar and Wink (2013); [2] Eimanifar et al. (2014).

Table 2. Species information and GenBank accession numbers for *COI* reference sequences.

Species/Population	Abbreviation	Individual	Accession Numbers	Ref.
A. urmiana	URM	4	JX512748-751	[28]
A. sinica	SIN	4	KF691298-301	[2]
A. tibetiana	TIB	4	KF691215-218	[2]
A. salina	SAL	4	KF691512-515	[2]
A. persimilis	PER	4	DQ119647 HM998992 EF615594 EF615593	[27] [33] [14] [14]
A. franciscana	FRA	4	KJ863440-443	[2]
Diploid Pop.	DI	4	KU183949-952	[3]
Triploid Pop.	TRE	3	HM998997-999	[33]
Tetraploid Pop.	TETR	4	KU183954-957	[3]
Pentaploid Pop.	PEN	4	KU183968-971	[3]

Ref: [28] Eimanifar and Wink 2013; [2] Eimanifar et al. 2014; [27] Hou et al. 2006; [33] Maniatsi et al. 2011; [14] Wang et al. 2008; [3] Asem et al. 2016.

2.3. Genomic Fingerprinting by ISSR-PCR

Genomic variability was examined by inter simple sequence repeat ISSR-PCR using the same DNA template used for phylogenetic analyses. Initially, 15 ISSR primers were analyzed to distinguish the intra- and inter-specific genetic variability within and among 83 randomly selected individuals, belonging to 14 geographically different localities of *Artemia*. Out of 15 tested ISSR primers, five were selected because of unambiguous banding patterns of the PCR products (Table 3).

Table 3. List of primers screened for Inter-Simple Sequence Repeats (ISSR) analysis.

Primer	Sequence	GC (%)	Annealing Temperature (°C)	Amplification Pattern
ISSR1	(AC)8T	47.1	48–54	Smear
ISSR2	(CAC)5	66.7	48–54	Smear
ISSR3	(GACA)4	50	48–54	Smear
ISSR4	(AG)12	50	48–54	Poor
ISSR5	(TC)9	50	48–54	Poor
ISSR6	(GT)10	50	48–54	Smear
ISSR7	(CA)10A	47.6	48–54	Poor
ISSR8	(GAA)5	33.3	48–54	No amplification
ISSR9	(CAG)6	66.7	48–54	No amplification
ISSR10	(GCCG)4	100	48–54	No amplification
ISSR11	(AG)8C	52.9	48	Good and sharp
ISSR12	(AG)8YTa	50	48	Good and sharp
ISSR13	(GA)9T	47.4	50	Good and sharp
ISSR14	(TG)8G	52.9	50	Good and sharp
ISSR15	(AC)8C	52.9	49	Good and sharp

PCR was carried out in a 25 µl volume consisting of 40 ng template DNA, 2.5 µl 10× PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween-20, 25 mM $MgCl_2$), 10 pmol primer, 2 µg/µl bovine serum albumin (BSA), 0.5 units *Taq* DNA polymerase (Bioron), 0.1 mM dGTP, dCTP, and dTTP, 0.045 mM dATP, 1 µCi [α-33P]-dATP (Perkin Elmer, LAS, Rodgau, Germany). PCR amplifications were executed in a thermal cycler based on the following conditions: 94 °C denaturation for 1 min, 35 cycles of 46–54 °C annealing for 50 s and 72 °C extension for 2 min. The final cycle was continued for 7-min at 72 °C. Final PCR products were mixed with 8 µl bromophenol blue and run on high-resolution denaturing polyacrylamide gels 6% (0.2 mm) for 3 h at 65 W (size 45 × 30 cm) including 1× TBE buffer. The gels were dried and exposed for 2 days to X-ray hyperfilm (Kodak, Taufkirchen, Germany) and subsequently developed. Finally, the autoradiograms were scanned to identify the polymorphic bands [25].

2.4. ISSR Statistics

The quality and quantity of ISSR bands were inspected visually. Ambiguous and smeared bands were excluded from the analysis, and only unequivocally reproducible bands were scored for each individual as present (1) or absent (0). The binary data matrix (presence = 1; absence = 0) was formulated in MS Excel v.2016 and used for subsequent genetic analyses.

ISSR data were analyzed via the Bayesian model-based clustering algorithm as implemented in the STRUCTURE v. 2.3 program (University of Oxford, Oxford, United Kingdom) [34,35]. We analyzed the genetic structure among populations by assigning individuals into potential numbers of clusters ($K = 1 - 10$). ISSR genotypes were processed with a period of burn-in 50,000 and 100,000 MCMC repetitions [34]. The CLUMPAK online program was employed to identify the pattern of clustering modes and packaging population structure [35]. The online programs, CLUMPAK [36] and STRUCTURE HARVESTER [37] were implemented to assess and visualize the most appropriate number of *K* by calculating the likelihood of the posterior probability [38].

The binary data matrix was employed to calculate the genetic diversity parameters of each population using GenAlex ver. 6.5 (Australian National University, Acton, Australia) [39]. The population genetic parameters were as follows: *Na* (number of different alleles), *Ne* (number of effective alleles), *I* (Shannon's information index), *He* (expected heterozygosity), *uHe* (unbiased expected heterozygosity), PPL (percentage of polymorphic loci), NB (number of bands) and NPB (number of private bands) and pairwise population matrix of *Nei* genetic distance [28,29].

Intra- and inter-specific molecular variations and genetic relationships among populations were implemented by Principal Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA) as utilized by GenAlex ver. 6.5, respectively [39].

To better understand the population genetic variations, ISSR analyses were performed on three platforms separately, as follows: whole populations, native populations and invasive American *A. franciscana*.

3. Results

3.1. Phylogenetic Analyses and Haplotype Distribution

Our phylogenetic analyses provide evidence that the studied bisexual specimens from three localities of Iran, Nough Catchment (NOG), Mahshar port (MSH) and Maharlu Lake (MAHR), and a locality from Iraq, Garmat Ali (GAA), clustered in the clade of *A. franciscana* (Figure 2). In addition, all parthenogenetic populations clustered in two separated clades that shared a common ancestor with *urmiana*. Although most of the parthenogenetic populations were located in clade P1, the majority of CAM specimens (eight out of nine). The clade P1 contained two sub-clades, consisting of diploids and triploid populations.

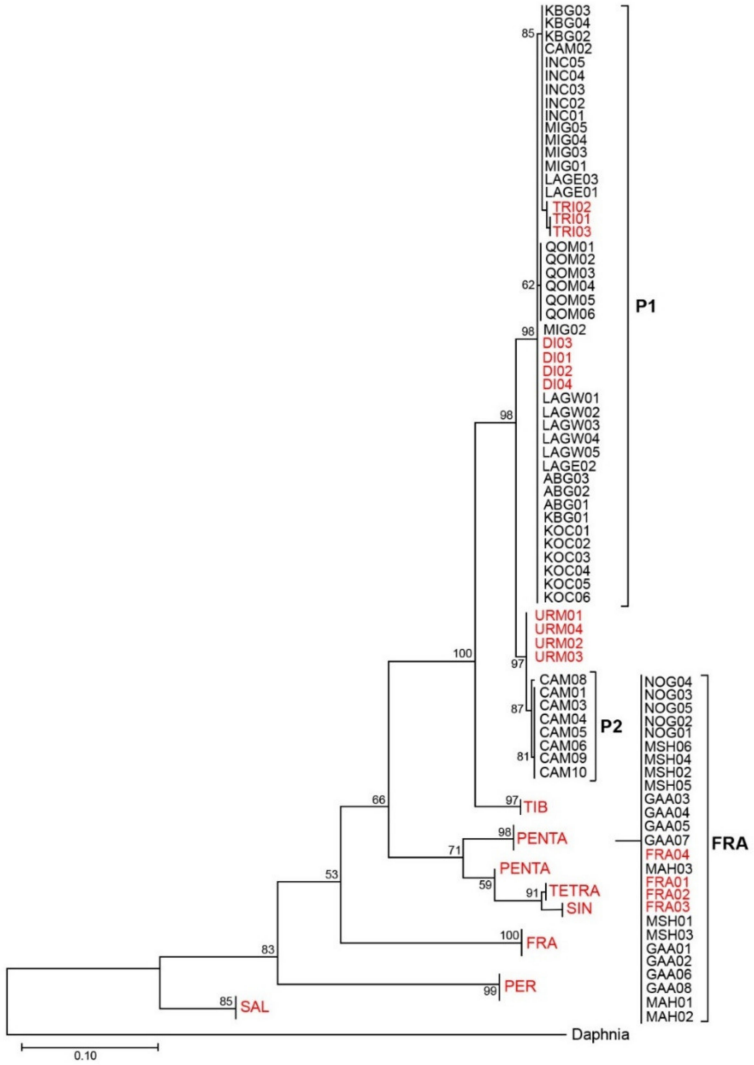

Figure 2. Phylogenetic tree of *Artemia* using *COI* sequences based on the ML approach. The number behind major nodes denotes bootstrap confidential values. *Daphnia tenebrosa* (HQ972028) was used as an outgroup. (URM: *Artemia urmiana*, TIB: *Artemia tibetiana*, SIN: *Artemia sinica*, FRA: *Artemia franciscana*, PER: *Artemia prersimilis*, SAL: *Artemia salina*, DI: Diploid parthenogenetic population, TRI: Triploid parthenogenetic population, TETRA: Tetraploid parthenogenetic population, PENTA: Pentaploid parthenogenetic population; abbreviations listed in Table 1).

Figure 3 represents the haplotype spanning network of *COI* among native *A. urmiana* and parthenogenetic populations. Results demonstrated that *A. urmiana* has wider genetic variation compared to parthenogenetic ones. Genetic differentiation and a close relationship of Camalti Lake (CAM) population (Turkey) with *A. urmiana* was clearly revealed in the tree. While *COI* sequences of nine parthenogenetic populations were distributed in five Haplotypes (H2–6), *A. urmiana* showed private haplotypes without a shared haplotype in other populations. The *COI* sequences of invasive *A. franciscana* were grouped into six distinct haplotypes. No population with private haplotypes was observed (Figure 4). The numbers of individuals and population composition were calculated for each haplotype (Appendix A, Tables A1 and A2).

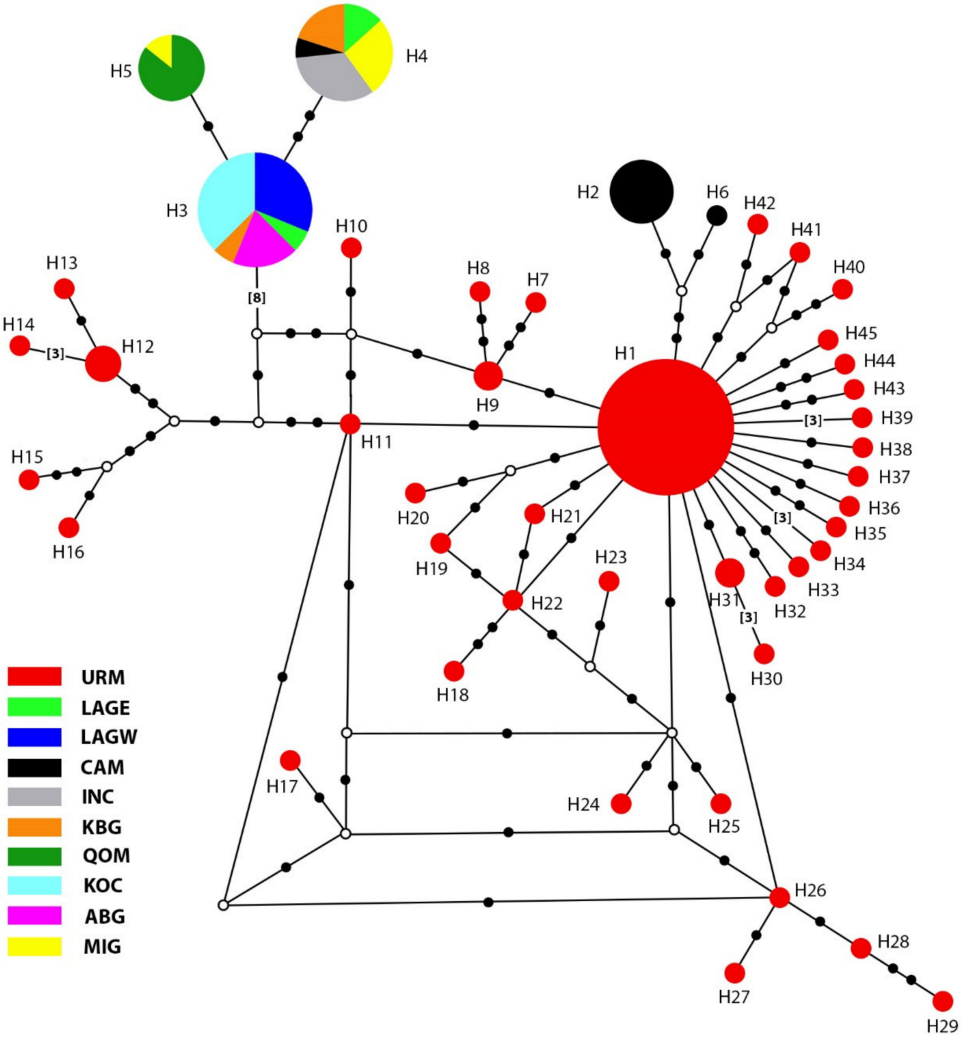

Figure 3. The relationship of *COI* haplotypes distribution among native populations (abbreviations listed in Table 1).

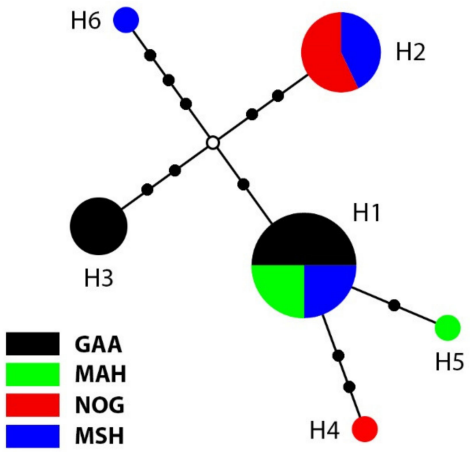

Figure 4. The relationship of *COI* haplotypes distribution among invasive *A. franciscana* populations (Abbreviations listed in Table 1).

3.2. ISSR Profiling

We have shown previously that ISSR can detect substantial genetic variation in the genus *Artemia* [28,29]. ISSR fingerprints can differ within and among populations. Altogether, 152 observed and unambiguously identified ISSR bands were analyzed. The total number of polymorphic loci showed a mean value of 25.42 ± 2.28%; the lowest number was seen in Camalti Lake (CAM) (7.24%) and the highest in Nough Catchment (NOG) (38.82%). Generally, the lowest values of genetic indices belonged to the parthenogenetic CAM, while the highest values were shared between the native parthenogenetic population from Eastern lagoon around Urmia Lake (LAGE) (*Ne* = 1.256 ± 0.031, *I* = 0.208 ± 0.024, *He* = 0.143 ± 0.017, *uHe* = 0.171 ± 0.020) and invasive *A. franciscana* from NOG (*Na* = 1.013 ± 0.071, *I* = 0.208 ± 0.023) (Table 4). In summary, 134 and 126 distinguished ISSR bands were examined for ten native and four American *A. franciscana* populations, respectively (Tables 5 and 6). According to the results of the *Nei* genetic matrix, parthenogenetic CAM from Turkey and invasive GAA from Iraq showed the high genetic distance with parthenogenetic and invasive populations, respectively (Tables 7 and 8).

Table 4. Genetic indices among examined populations according to ISSR markers.

Pop.	N	*Na*	*Ne*	*I*	*He*	*uHe*	*PPL*	*NB*	*NPB*
KOC	7	0.645 (0.062)	1.102 (0.021)	0.090 (0.017)	0.059 (0.012)	0.064 (0.012)	17.76	71	0
ABG	7	0.743 (0.066)	1.149 (0.024)	0.131 (0.020)	0.088 (0.013)	0.095 (0.014)	23.68	77	0
LAGE	3	0.914 (0.072)	1.256 (0.031)	0.208 (0.024)	0.143 (0.017)	0.171 (0.020)	34.87	86	0
LAGW	7	0.829 (0.066)	1.157 (0.024)	0.140 (0.020)	0.093 (0.014)	0.100 (0.015)	25.66	87	0
KBG	7	0.743 (0.068)	1.161 (0.025)	0.139 (0.020)	0.094 (0.014)	0.101 (0.015)	25.00	75	0
QOM	7	0.638 (0.062)	1.097 (0.020)	0.087 (0.016)	0.058 (0.011)	0.062 (0.012)	17.11	71	0
MIG	5	0.678 (0.067)	1.150 (0.025)	0.128 (0.020)	0.087 (0.014)	0.096 (0.015)	23.03	68	0
CAM	7	0.559 (0.051)	1.041 (0.014)	0.036 (0.011)	0.024 (0.008)	0.026 (0.008)	7.24	74	0
INC	7	0.658 (0.064)	1.125 (0.022)	0.110 (0.018)	0.074 (0.013)	0.080 (0.014)	19.74	70	0
URM	5	0.783 (0.072)	1.200 (0.027)	0.173 (0.022)	0.117 (0.015)	0.130 (0.017)	30.26	73	0
MAH	2	0.822 (0.066)	1.181 (0.025)	0.155 (0.021)	0.106 (0.015)	0.142 (0.020)	25.66	86	1
NOG	7	1.013 (0.071)	1.244 (0.030)	0.208 (0.023)	0.140 (0.016)	0.151 (0.017)	38.82	95	0
MSH	7	1.066 (0.066)	1.223 (0.028)	0.193 (0.022)	0.129 (0.015)	0.139 (0.016)	36.84	106	0
GAA	5	0.921 (0.067)	1.206 (0.028)	0.172 (0.022)	0.117 (0.015)	0.130 (0.017)	30.26	94	0
Mean	5.9 (0.035)	0.787 (0.018)	1.164 (0.007)	0.141 (0.005)	0.095 (0.004)	0.106 (0.004)	25.42 (2.28%)	-	-

Pop. = Population, N = number of individuals, *Na* = number of Different Alleles, *Ne* = number of Effective Alleles, *I* = Shannon's Information Index, *He* = Expected Heterozygosity, *uHe* = Unbiased Expected Heterozygosity, *PPL* = Percentage of Polymorphic Loci, *NB* = number of bands, *NPB* = number of private bands.

Table 5. Genetic indices among native populations according to ISSR markers.

Population	N	*Na*	*Ne*	*I*	*He*	*uHe*	*PPL*	*NB*	*NPB*
KOC	7.000	0.731 (0.067)	1.115 (0.023)	0.102 (0.019)	0.067 (0.013)	0.073 (0.014)	20.15	71	2
ABG	7.000	0.843 (0.071)	1.169 (0.027)	0.148 (0.022)	0.100 (0.015)	0.107 (0.016)	26.87	77	4
LAGE	3.000	1.037 (0.075)	1.291 (0.034)	0.236 (0.026)	0.162 (0.018)	0.195 (0.022)	39.55	86	1
LAGW	7.000	0.940 (0.069)	1.178 (0.027)	0.158 (0.022)	0.106 (0.015)	0.114 (0.016)	29.10	87	4
KBG	7.000	0.843 (0.073)	1.183 (0.028)	0.158 (0.023)	0.107 (0.015)	0.115 (0.017)	28.36	75	0
QOM	7.000	0.724 (0.066)	1.110 (0.023)	0.099 (0.018)	0.065 (0.013)	0.070 (0.014)	19.40	71	0
MIG	5.000	0.769 (0.073)	1.170 (0.027)	0.145 (0.022)	0.098 (0.015)	0.109 (0.017)	26.12	68	3
CAM	7.000	0.634 (0.055)	1.046 (0.016)	0.041 (0.013)	0.027 (0.009)	0.029 (0.009)	8.21	74	0
INC	7.000	0.746 (0.069)	1.142 (0.025)	0.125 (0.021)	0.084 (0.014)	0.091 (0.015)	22.39	70	1
URM	5.000	0.888 (0.077)	1.227 (0.029)	0.196 (0.024)	0.133 (0.017)	0.148 (0.018)	34.33	73	3
Mean	6.200 (0.036)	0.816 (0.022)	1.163 (0.008)	0.141 (0.007)	0.095 (0.005)	0.105 (0.005)	25.45 (2.74)	-	-

Pop. = Population, N = number of individuals, *Na* = number of Different Alleles, *Ne* = number of Effective Alleles, *I* = Shannon's Information Index, *He* = Expected Heterozygosity, *uHe* = Unbiased Expected Heterozygosity, *PPL* = Percentage of Polymorphic Loci, *NB* = number of bands, *NPB* = number of private bands.

Table 6. Genetic indices among invasive *A. franciscana* populations according to ISSR markers.

Population	N	*Na*	*Ne*	*I*	*He*	*uHe*	*PPL*	*NB*	*NPB*
MAH	2.000	0.992 (0.071)	1.219 (0.029)	0.187 (0.025)	0.128 (0.017)	0.171 (0.023)	30.95	86	3
NOG	7.000	1.222 (0.073)	1.295 (0.034)	0.251 (0.026)	0.169 (0.018)	0.182 (0.019)	46.83	95	6
MSH	7.000	1.286 (0.065)	1.269 (0.033)	0.233 (0.025)	0.156 (0.018)	0.168 (0.019)	44.44	106	8
GAA	5.000	1.111 (0.070)	1.249 (0.033)	0.207 (0.025)	0.141 (0.018)	0.157 (0.020)	36.51	94	5
Mean	5.250 (0.091)	1.153 (0.035)	1.258 (0.016)	0.220 (0.013)	0.149 (0.009)	0.169 (0.010)	39.68 (3.65)	-	-

Pop. = Population, N = number of individuals, *Na* = number of Different Alleles, *Ne* = number of Effective Alleles, *I* = Shannon's Information Index, *He* = Expected Heterozygosity, *uHe* = Unbiased Expected Heterozygosity, *PPL* = Percentage of Polymorphic Loci, *NB* = number of bands, *NPB* = number of private bands.

Table 7. Pairwise Population Matrix of *Nei* Genetic Distance among invasive *A. franciscana* populations.

Population	KOC	ABG	LAGE	LAGW	KBG	QOM	MIG	CAM	INC
ABG	0.162	-	-	-	-	-	-	-	-
LAGE	0.167	0.175	-	-	-	-	-	-	-
LAGW	0.193	0.127	0.190	-	-	-	-	-	-
KBG1	0.156	0.117	0.142	0.111	-	-	-	-	-
QOM	0.182	0.131	0.171	0.135	0.099	-	-	-	-
MIG	0.217	0.185	0.171	0.212	0.124	0.148	-	-	-
CAM	0.362	0.346	0.249	0.358	0.279	0.354	0.331	-	-
INC	0.233	0.165	0.184	0.223	0.149	0.176	0.091	0.351	-
URM	0.219	0.194	0.165	0.190	0.141	0.174	0.194	0.351	0.211

Table 8. Pairwise Population Matrix of *Nei* Genetic Distance among native populations.

Population	MAH	NOG	MAH
NOG	0.143	-	-
MSH	0.145	0.144	-
GAA	0.198	0.170	0.152

Based on the total results of ISSR, the AMOVA analysis documented that most of the genetic variations were attributed among native and invasive populations more than within populations (69% vs. 31%). There was no indicative genetic variability observed in the midst of among- and within variation (55% vs. 45%) in native populations. In contrast, the high differentiation was represented within populations (71% vs. 29%) of non-indigenous *A. franciscana* in Asia (Table 9, Figure 5A–C).

Table 9. Molecular variation (within and among populations) for examined populations by AMOVA based on ISSR markers.

Whole Populations					
Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	13	1673.318	128.717	20.265	69%
Within Pops	69	639.405	9.267	9.267	31%
Total	82	2312.723	-	29.532	100%
Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Native Populations					
Among Pops	9	627.912	69.768	10.006	55%
Within Pops	52	418.362	8.045	8.045	45%
Total	61	1046.274	-	18.052	100%
A. franciscana					
Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	3	117.338	39.113	5.239	29%
Within Pops	17	221.043	13.003	13.003	71%
Total	20	338.381	-	18.241	100%

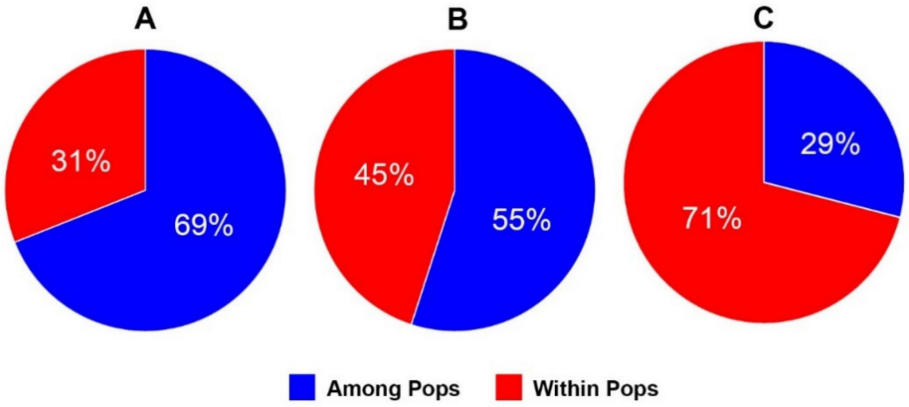

Figure 5. Contribution of genetic variation within and among populations for the examined populations by AMOVA, based on ISSR markers (**A** = whole examined populations, **B** = native populations, **C** = invasive *A. franciscana*).

Bayesian clustering analysis using STRUCTURE was performed to investigate the genetic patterns of the studied populations. The optimum *K* was obtained at *K* = 2 for the whole 14 populations and ten native populations, and *K* = 9 for four invasive *A. franciscana*, respectively. Figure 6 showed the clustering of genetic structures, where the first highest posterior probability (*K*) was represented by different colors for each population. With regard to the genetic patterns of ISSR, native and exotic populations could be completely divided into two groups (Figure 6A). The results of the analysis for native populations documented that parthenogenetic CAM is a distinct population with a differing clustering pattern. The STRUCTURE analysis could not fully distinguish *A. urmiana* and parthenogenetic populations (Figure 6B). The high value of optimum *K* (*K* = 9) was prominent in the clustering analysis for *A. franciscana* populations, which generally revealed a complex pattern (Figure 6C). Proportions of genetic clusters (percentage) for each locality were summarized in Figure 7 (see also Tables A3–A5).

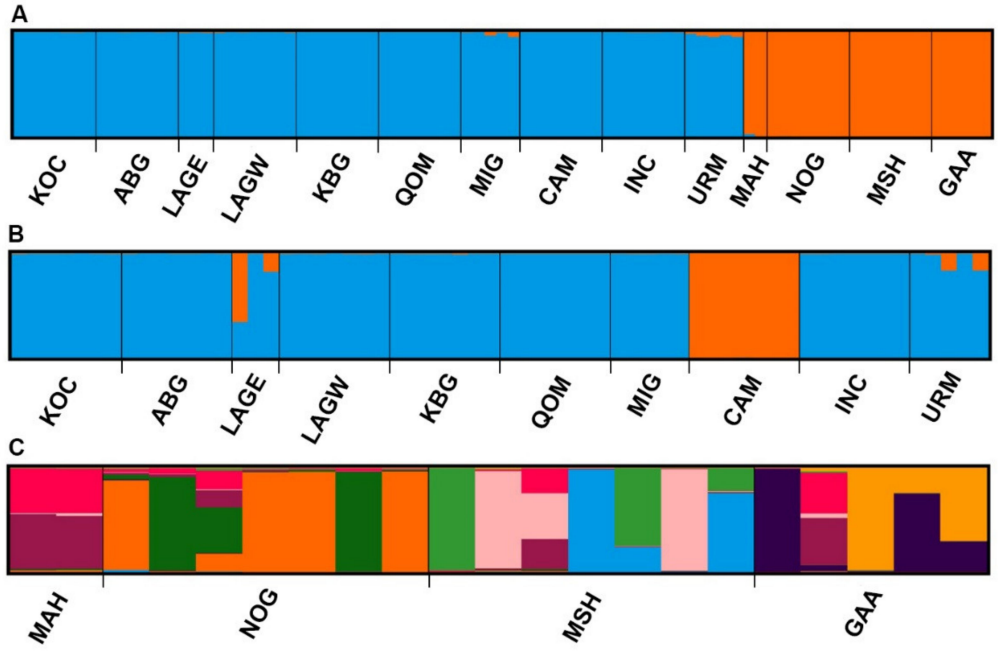

Figure 6. Clustering of genetic structures based on ISSR markers (**A** = whole examined populations, **B** = native populations, **C** = invasive *A. franciscana*; abbreviations listed in Table 1).

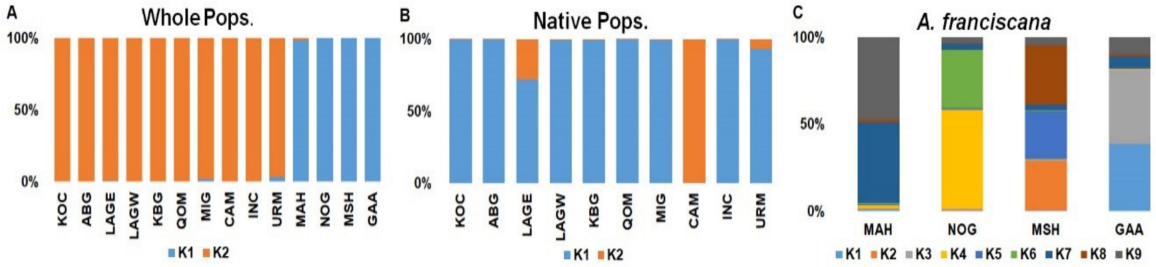

Figure 7. Proportion of genetic clusters for each locality in the STRUCTURE analysis (**A** = whole examined populations, **B** = native populations, **C** = invasive *A. franciscana;* abbreviations listed in Table 1).

The first and second PCoA coordinates explained 64.66% and 7.00% of the variance, respectively (overall, 71.66% of total variation). The results showed all populations clustered into three groups, where invasive *A. franciscana* has been significantly separated from the native populations based on the first coordinate. Ten native populations (including *A. urmiana* and parthenogenetics) were divided into two distinct groups, G2 and G3. G2 included all of the CAM population and a single individual of Eastern lagoon around Urmia Lake (LAGE). Bisexual *A. urmiana* and other parthenogenetics were placed in G3 (Figure 8). The results of the separate analyses of PCoA for native and invasive populations are shown in Figures 9 and 10, which include overall 42.55% and 41.55% of the total variation, respectively. Although native populations produced almost the same result with the "whole populations" analysis (Figure 9), the separated analyses of PCoA for invasive *A. franciscana* could separate all four populations in isolated groups (Figure 10).

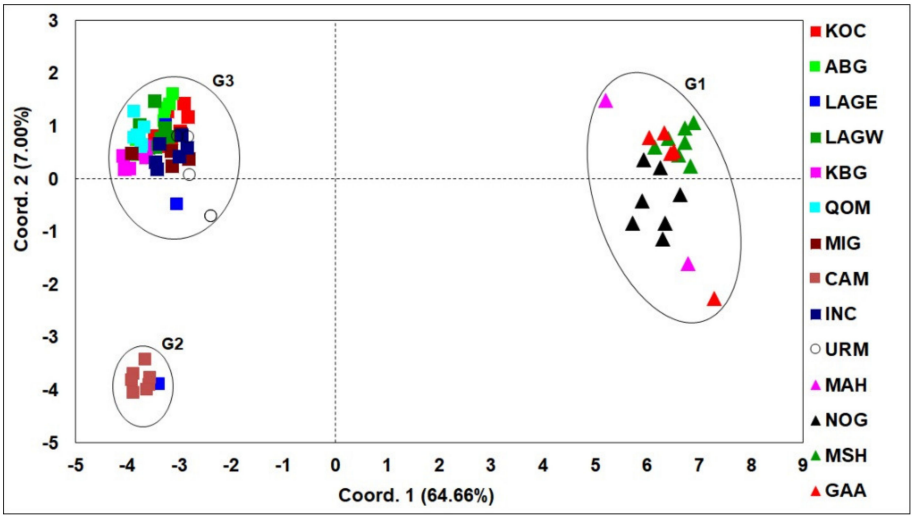

Figure 8. Principal Coordinates Analysis (PCoA) showing differentiation patterns among whole examined populations based on ISSR markers (abbreviations listed in Table 1).

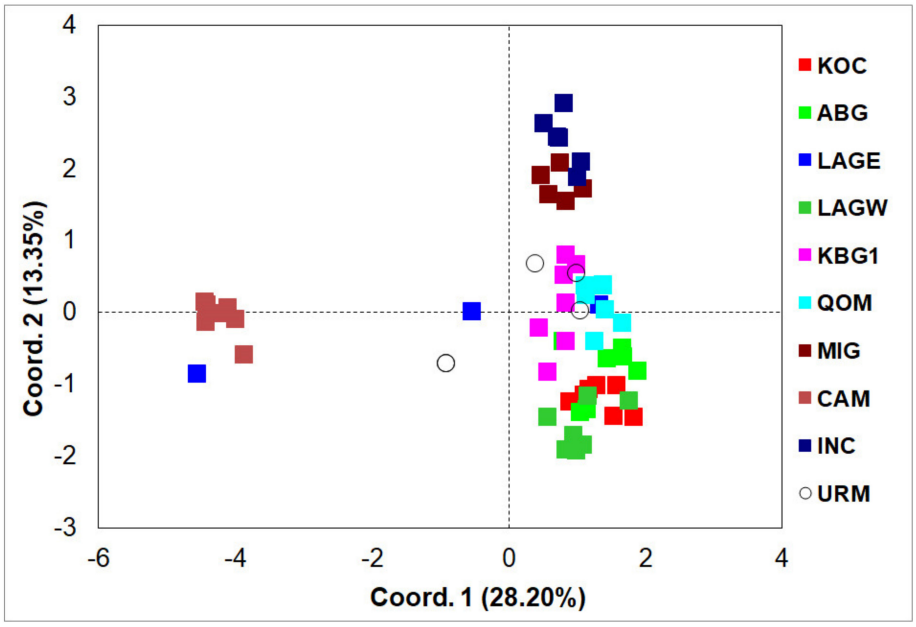

Figure 9. Principal Coordinates Analysis (PCoA) showing differentiation patterns among native populations based on ISSR markers (abbreviations listed in Table 1).

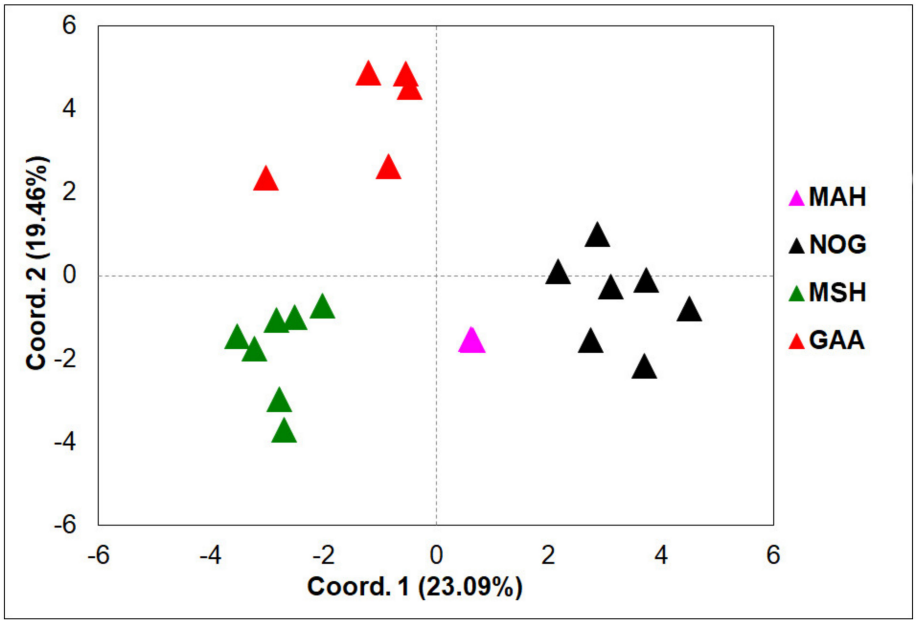

Figure 10. Principal Coordinates Analysis (PCoA) showing differentiation patterns among invasive *A. franciscana* populations based on ISSR markers (abbreviations listed in Table 1).

Similar to the phylogeny based on *COI* sequences, clustering analysis of ISSR sequences by STRUCTURE has revealed a distinguished structure for non-indigenous *A. franciscana* populations. In this analysis, all native populations (bisexual *A. urmiana* and parthenogenetic ones) have displayed almost similar patterns, but a separated analysis for native populations has revealed a non-identical structure for the CAM population. Additionally, an inconsistent pattern of the Eastern lagoon around Urmia Lake (LAGE) was also observed. A separated analysis for invasive populations could not reveal different patterns among examined populations by STRUCTURE (Figure 6A–C). On the other hand, PCoA has divided *A. franciscana* from native populations. Although PCoAs could not branch off natives by localities, the separated PCoA has divided invasive populations based on localities in the

four groups. Contrary to *COI* haplotype distribution, the ISSR marker was unable to reveal a private pattern for bisexual *A. urmiana* in comparison to native parthenogenetic populations.

4. Discussion

The present study was performed to compare the population structure and genetic differentiation of native and invasive *Artemia* populations. The mitochondrial *COI* gene has been established as a useful molecular marker to determine the intra- and inter-specific evolutionary associations [2,3,17,18]. Asem et al. [2] have documented that di- and triploid parthenogenetic brine shrimps are maternally related to *A. urmiana*, while tetra- and pentaploid lineages shared a common maternal ancestor with *A. sinica*. Based on the mitochondrial *COI* dataset, all examined parthenogenetic individuals have grouped in a close evolutionary relationship with *A. urmiana* (Figure 2). Our results have demonstrated that they should include di- and/or triploids. This observation has also been confirmed by the phylogenetic tree. Although individuals of eight *Artemia* populations have been exactly located in sub-clades of di- and/or triploid, eight out of nine specimens of CAM from Turkey have been placed in a particular clade (P2), in a close connection with *A. urmiana* (Figure 2). This finding has also been confirmed by haplotype distribution (Figure 3). Previously, Sayg [40] has confirmed that triploid and pentaploid parthenogenetic populations coexisted in Camalti Lake (CAM). Our observation has confirmed that Camalti Lake (CAM) populations had a parthenogenetic reproductive mode and those examined specimens should be considered as a diploid and/or triploid population (see [3]). Despite the fact that *A. urmiana* shared a common ancestor with di- and triploids, it has presented an unexpectedly high level of haplotype diversity of the *COI* marker. These results have also been documented by Eimanifar and Wink [28]. The high level of haplotype variation might be attributed to the evolutionary life history of *A. urmiana* [30] and/or its large population size [28]. Urmia Lake has undergone considerable changes in environmental conditions, such as salinity and temperature [41,42], which could have influenced genetic variation and population size during evolution.

Although *COI* sequences should reveal a phylogeographic structure in the closely related species [43], our results could not determine a level of geographical differentiation among native as well as among invasive populations. Contrary to the results of the mitochondrial marker, genomic fingerprinting ISSR could not reveal a significant high level of genetic variation in *A. urmiana*. This result might be due to differences in the rate of variation of mitochondrial and nuclear genes and potential hybridization events in the past.

The ISSR method had been utilized to study ten diploid parthenogenetic *Artemia* populations from China by Hou et al. [27]. Their genetic variation was significantly higher than our examined parthenogenetic populations. For example, the percentage of polymorphic loci ranged from 54.12–87.06% vs. 8.21–39.55%. Western Asia is the origin of di- and triploid parthenogenetic populations [3], so high genetic diversity of Chinese populations could be the result of their adaptation during colonization through biological dispersal to the new environments in East Asia under historical evolutionary progress.

In general, it was reported that the invasive populations have lower genetic variation in the new environments as compared with their origin populations [44]. The colonized population of *A. franciscana* in Vietnam has a lower genetic diversity than its native source population from San Francisco Bay (SFB) [22]. In contrast, Eimanifar et al. [2] have documented that Asian invasive *A. franciscana* populations had higher genetic variation than the American Great Salt Lake (GSL) population and native Asian species. Similar results have been reported for some invasive populations from Mediterranean regions [23,24]. A recent study assumed that invasive populations of *A. franciscana* show a wide degree of genetic differentiation in Australia [17].

Overall, our ISSR results have documented that invasive *A. franciscana* populations had distinctly higher genetic variation than Western Asian native parthenogenetic populations. On the other hand, native *A. franciscana* from Great Salt Lake (GSL) have represented higher variation than examined invasive populations in this study, as the percentage of polymorphic loci differed from 67–81% vs.

30.95–46.83% (see [29]). Additionally, all four invasive *A. franciscana* populations clearly revealed a different genetic structure. Observation of low genetic diversity in native populations might be attributed to the effect of asexual reproduction in parthenogenetic populations and/or critical climatic conditions in West Asia, especially Urmia Lake in the last two decades (see [3,30]). We believe that interactions between different ecological conditions in the new environments and the high potential of physiological plasticity and genetic adaptation of *A. franciscana* could exert different evolutionary pathways during the introduction of exotic populations, which would have ultimately caused intra-specific variations and genetic divergence in the examined invasive populations.

In conclusion, it is expected that the non-indigenous species should have a lower genetic variation than their source populations [25,44,45]. However, non-indigenous *A. franciscana* populations gave opposite results in comparison with native populations from GSL and SFB. Since there is neither a taxonomical identification key nor morphological identifications to distinguish bisexual species and parthenogenetic populations [11,46], it would not be possible to identify the exotic population at the earliest time of invasion. Therefore, there is a lack of information to regularly determine the colonization progress and evolutionary development of *A. franciscana* in the new habitats. We assume that differences in the genetic variation of non-indigenous populations could be due to the study on different invasion periods consisting of i) introduced, ii) establishing/colonizing, iii) established/colonized populations.

Author Contributions: Research design, material preparation, and data collection were performed by A.E. Data analysis was carried out by A.A. The first draft of the manuscript was written by A.A. and A.E. P.-Z.W., W.L. and M.W. reviewed the draft. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. M.W. is Editor-in-Chief of Diversity.

Appendix A

Table A1. Information of network haplotype composition of native populations.

Haplotype	Ind.	Pop. (Ind.)	Haplotype	Ind.	Pop. (Ind.)
H1	18	URM (18)	**H24**	1	URM (1)
H2	7	CAM (7)	**H25**	1	URM (1)
H3	16	KOC (6), LAGW (5)ABG (3), LAGE (1), KBG (1)	**H26**	1	URM (1)
H4	15	ING (5), MIG (4), KBG (3), LAGE (2)	**H27**	1	URM (1)
H5	7	QOM (6), MIG (1)	**H28**	1	URM (1)
H6	1	CAM (1)	**H29**	1	URM (1)
H7	1	URM (1)	**H30**	1	URM (1)
H8	1	URM (1)	**H31**	2	URM (2)
H9	2	URM (2)	**H32**	1	URM (1)
H10	1	URM (1)	**H33**	1	URM (1)
H11	1	URM (1)	**H34**	1	URM (1)
H12	3	URM (3)	**H35**	1	URM (1)

Table A1. *Cont.*

Haplotype	Ind.	Pop. (Ind.)	Haplotype	Ind.	Pop. (Ind.)
H13	1	URM (1)	**H36**	1	URM (1)
H14	1	URM (1)	**H37**	1	URM (1)
H15	1	URM (1)	**H38**	1	URM (1)
H16	1	URM (1)	**H39**	1	URM (1)
H17	1	URM (1)	**H40**	1	URM (1)
H18	1	URM (1)	**H41**	1	URM (1)
H19	1	URM (1)	**H42**	1	URM (1)
H20	1	URM (1)	**H43**	1	URM (1)
H21	1	URM (1)	**H44**	1	URM (1)
H22	1	URM (1)	**H45**	1	URM (1)
H23	1	URM (1)	-	-	-

Ind. = Individual, Pop. = Population.

Table A2. Information of network haplotype composition of invasive *A. franciscana* populations.

Haplotype	Ind.	Pop. (Ind.)
H1	8	GAA (4), MAH (2), MSH (2)
H2	7	NOG (4), MSH (3)
H3	4	GAA (4)
H4	1	NOG (1)
H5	1	MAH (1)
H6	1	MSH (1)

Ind. = Individual, Pop. = Population.

Table A3. Proportion of genetic clusters for each locality in STRUCTURE analysis among whole examined populations.

Population	K1 (%)	K2 (%)
KOC	0.1	99.9
ABG	0.2	99.8
LAGE	0.7	99.3
LAGW	0.3	99.7
KBG	0.1	99.9
QOM	0.1	99.9
MIG	1.9	98.1
CAM	0.1	99.9
INC	0.2	99.8
URM	3.6	96.4
MAH	98.7	1.3

Table A3. *Cont.*

Population	K1 (%)	K2 (%)
NOG	99.9	0.1
MSH	99.9	0.1
GAA	99.9	0.1

Table A4. Proportion of genetic clusters for each locality in STRUCTURE analysis among native populations.

Population	K1 (%)	K2 (%)
KOC	000099.7	0.3
ABG	99.8	0.2
LAGE	72.1	27.9
LAGW	99.6	0.4
KBG	99.5	0.5
QOM	99.8	0.2
MIG	99.6	0.4
CAM	0.2	99.8
INC	99.7	0.3
URM	93.1	6.9

Table A5. Proportion of genetic clusters for each locality in STRUCTURE analysis among invasive *A. franciscana* populations.

Population	K1 (%)	K2 (%)	K3 (%)	K4 (%)	K5 (%)	K6 (%)	K7 (%)	K8 (%)	K9 (%)
MAH	1.1	0.4	0.3	1.7	0.4	1.1	46	1.8	47.1
NOG	0.6	0.5	0.4	57	0.6	33.6	3.6	0.3	3.4
MSH	0.5	28.6	0.3	0.4	27.9	0.4	3.4	33.9	4.6
GAA	38.8	0.2	42.3	0.3	0.4	0.2	7	1.1	9.7

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