Volume 59(2):127-137, 2015
Acta Biologica Szegediensis
http://www2.sci.u-szeged.hu/ABS

ARTICLE

Genetic and morphological diversity in *Chara vulgaris* L. (Characeae)

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ABSTRACT *Chara vulgaris* L. (Characeae) is a highly polymorphic species that plays an important ecological role in aquatic ecosystems. It grows in different regions of Iran and forms several geographical populations. Genetic diversity studies are very limited in algal taxa of the country and there is no detailed information about the genetic diversity present in *C. vulgaris*. Therefore, the present investigation was performed to study the population structure of 89 plant specimen collected from 11 geographical populations of *C. vulgaris* in Iran. Genetic diversity parameters were determined in each population based on ISSR molecular markers. AMOVA test revealed significant genetic difference among the studied populations. Mantel test revealed significant correlation between genetic distance and geographical distance of the studied populations. However, STRUCTURE analysis revealed that some common ancestral alleles exist among these populations. ANOVA test revealed significant differences in quantitative morphological characters among the studied populations. UPGMA tree and PCoA plot revealed morphological variability of these populations as the members of each population were scattered in different groups. Therefore, in spite of genetic differences of the studied populations, they are not morphologically differentiated.
Acta Biol Szeged 59(2):127-137 (2015)

KEY WORDS
gene flow
ISSR molecular markers
population structure

Introduction

The Charales (Characeae), commonly called stoneworts is a group of highly complex green algae that comprises six genera (Wood 1965). They have a close evolutionary history to land plants (Karol et al. 2001; McCourt et al. 2004) and play an important ecological role in aquatic ecosystems throughout the world except Antarctica (Wood 1965). The presence of Characeae indicates a pristine aquatic ecosystem. They support the other biological components of the water ecosystems (Carpenter and Lodge 1986; Noordhuis et al. 2002) and make water clean with filtering mud particles between the whorls of their branchlets. Charophytes have been used for fish-culture, polishing-paste, mud-bathing, therapy, clarification of sugar and luring noxious insects (Scheffer 1998). Charophytes are sensitive to environmental changes such as eutrophication (Blindow 1992), therefore, many Charophytes become rare or endangered in recent decades (Baastrup-Spohr et al. 2013; Auderset and Rey-Boissezon 2015).

High morphological variability has been reported in *Chara* species (Wood and Imahori 1965; Corillion 1972) due to variation in their habitats (Blindow and Schutte 2006; Schneider et al. 2006) and the genetic alterations inside species (Mannschreck et al. 2002).

Chara vulgaris L. has worldwide distribution from South America, Africa, Asia to Europe (Caisova and Gabka 2009). It is also a highly polymorphic species with many forms and varieties (Wood and Imahory 1965; Caisova and Gabka 2009). This species grows under different environmental conditions in many geographical areas of Iran. *C. vulgaris* is the most commonly found taxon and probably the most abundant Charophytes in Iran and can be collected in a wide variety of habitats from most of the provinces of the country. Specimens show variability in morphological characters. They typically grow on sandy or sandy-mud substrates with relatively low organic content. This species occurs at nearly every altitude and latitude and can be found in streams, river channels, in artificial, natural, permanent and temporal small water bodies between 0.1-2 m depth. It also roots at the bottom of artificial basins covered with a thin film of silt, nonetheless, it has a higher abundance in running water.

Previous genetic diversity investigations have been performed in different *Chara* species (*e.g.*, Mannschreck et al. 2002; Schaible et al. 2009). However, there is no detailed study about the degree of genetic variability within and among geographical populations of *C. vulgaris* in Iran. Therefore, in the present study we investigated the genetic diversity and the population structure of *C. vulgaris* in 11

Submitted May 19, 2015; Accepted November 11, 2015
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Table 1. *C. vulgaris* populations studied, their localities and voucher numbers.

Populations	Number of samples	Altitude (m)	Longitude	Latitude	Voucher No.
Razavi Khorasan	43	1201	36°48′33″	58°50′47″	2011405
South Khorasan	2	777	33°47′57″	56°49′21″	2011413
Yazd	7	1645	31°43′36″	54°09′25″	2011514
Kerman	5	2024	29°56′54″	56°33′43″	2011482
Isfahan	4	1606	33°36′51″	51°43′32″	2011402
Mazandaran	9	1294	36°11′44″	52°10′17″	2011510
Gilan	4	625	36°40′24″	49°31′31″	2011493
Tehran	3	1881	35°44′34″	52°40′29″	2011409
Golestan	2	1306	37°25′46″	56°34′41″	2011490
Khuzestan	9	353	30°25′53″	50°19′37″	2011464
Semnan	1	1157	35°34′18″	53°22′27″	2011476

geographical populations by using morphological and inter-simple sequence repeat molecular markers (ISSRs). These molecular markers are easy to use, simple and cost effective along with high degree of reproducibility (Sheidai et al. 2012, 2013, 2014; Azizi et al. 2014).

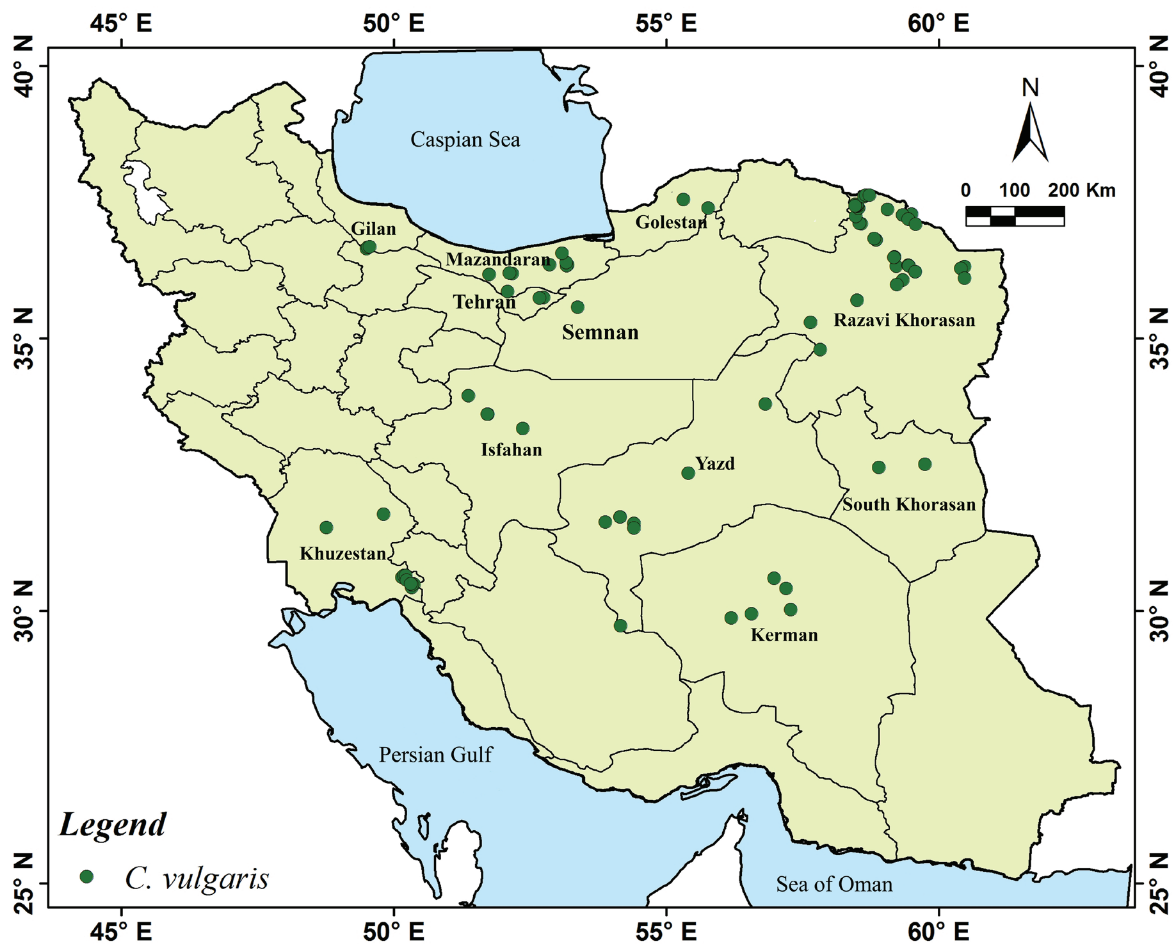

Figure 1. Distribution map of *C. vulgaris* populations.

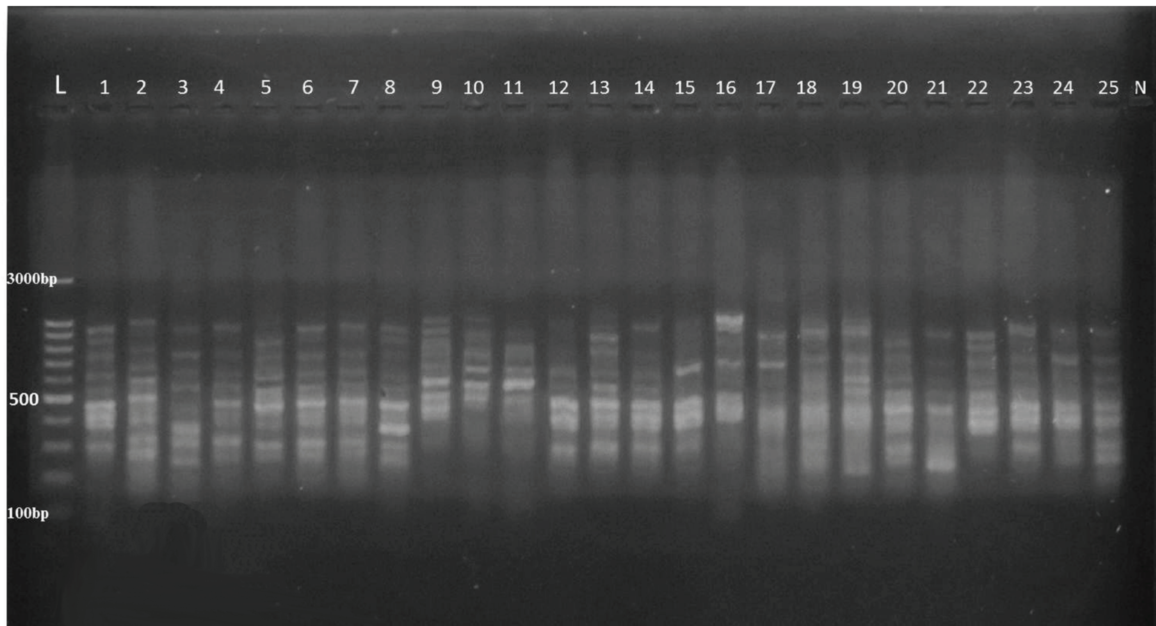

Figure 2. ISSR marker profiles of 15 individuals of *C. vulgaris* population generated by primer $(AGC)_5GT$ in 2% agarose gel. N: negative control; 1-15: individuals; L: 100 kb molecular-weight size marker (Fermentas, Germany).

Materials and Methods

Plant materials

Eighty-nine samples were collected from 11 different geographical populations (Razavi Khorasan, South Khorasan, Yazd, Kerman, Isfahan, Mazandaran, Gilan, Tehran, Golestan, Khuzestan, and Semnan). Details of localities are provided in Table 1 and Figure 1. Specimens are deposited in Herbarium of Shahid Beheshti University (HSBU).

DNA extraction and ISSR assay

Fresh thalli were collected randomly from 10 plants derived from each of the studied populations and mixed, then dried in silica gel powder. These thalli were used for DNA extraction. Genomic DNA was extracted using CTAB activated charcoal protocol (Sheidai et al. 2013). The quality of extracted DNA was examined electrophoretically by running on a 0.8% agarose gel.

Ten ISSR primers were used: (AGC)5GT, (GA)9C, UBC807, UBC811, (CA)7GT, (GA)9A, (GA)9T, UBC834, UBC810, and UBC823. They were commercialized by UBC (the University of British Columbia). Polymerase chain reactions (PCR) were performed in a volume of 25 µl containing: 10 mM Tris-HCl buffer (pH 8); 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng of genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The reactions were performed in Techne thermocycler (Germany) using the following cycling conditions: 5 min initial denaturation step at 94 °C, 45 cycles of 30 s at 94 °C; 30 s at 50 °C/52.6 °C/53.3 °C/55.3 °C/58.2 °C/ and 1min at 72 °C. The reaction was completed by final extension step of 10 min at 72 °C. Five different annealing temperatures were used as follows: 58.2 °C for the primer ((AGC)5GT); 55.3 °C for ((GA)9C and (GA)9T); 53.3 °C for (UBC807), 52.6°C for (UBC811) and 50 °C for the other primers.

The amplicons were visualized electrophoretically by running on a 2% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular-weight size marker (Fermentas, Germany).

Morphological study

Specimens (5-10) were collected randomly in each location for morphological studies. In total, 24 characters (quantitative and qualitative) were studied and coded accordingly for multivariate statistical analyses (Table 4).

Data analyses

ISSR bands obtained (Fig. 2) were coded as binary characters (presence = 1, absence = 0). Genetic diversity parameters were determined for dominant molecular markers in each

Table 2. Genetic diversity parameters in the studied populations.

Population	Number of samples	Ne	I	He	UHe	%P
Razavi Khorasan	43	1.324	0.333	0.209	0.211	85.51%
South Khorasan	2	1.164	0.140	0.096	0.128	23.19%
Yazd	7	1.334	0.314	0.203	0.219	68.12%
Kerman	5	1.187	0.166	0.110	0.123	31.88%
Isfahan	4	1.308	0.266	0.179	0.205	47.83%
Mazandaran	9	1.318	0.287	0.190	0.201	57.97%
Gilan	4	1.333	0.278	0.189	0.216	49.28%
Tehran	3	1.244	0.216	0.145	0.173	39.13%
Golestan	2	1.061	0.053	0.036	0.048	8.70%
Khuzestan	9	1.316	0.312	0.200	0.212	66.67%
Semnan	1	1.000	0.000	0.000	0.000	0.00%

Ne: number of effective alleles; I: Shannon's Information Index; He: gene diversity; UHe: unbiased gene diversity; %P: percentage of polymorphic loci.

population. These parameters were Nei's gene diversity (H), Shannon information index (I), number of effective alleles and percentage of polymorphism (Weising et al. 2005; Freeland et al. 2011).

Nei's genetic distance was determined among the studied populations and used for clustering. For grouping specimens, Neighbor Joining (NJ) clustering methods as well as Neighbor Net method of networking were performed after 100 times of bootstrapping (Huson and Bryant 2006; Freeland et al. 2011). DARwin (ver. 5; 2012) was used for clustering, while SplitsTree4 (V4.6; 2006) was used for network analysis.

Mantel test was performed to check correlation between geographical distance and genetic distance of the studied populations (Podani 2000). PAST (ver. 2.17; Hamer et al. 2012) program was used for Mantel test.

Significant genetic difference among the studied populations and provinces were determined by AMOVA (Analysis of molecular variance) test (with 1000 permutations) for dominant molecular markers as implemented in GenAlex 6.4 (Peakall and Smouse 2006). Furthermore, Nei's Gst analysis of dominant markers as implemented in GenoDive (ver.2) (Meirmans and Van Tienderen 2004) was also carried out. Finally, genetic differentiation of the populations was also studied by G'st-est (standardized measure of genetic differentiation, Hedrick 2005), and D-st (Jost measure of differentiation, Jost 2008). These parameters were determined in case if the studied populations do not follow normal distribution.

In order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program, Hickory (ver. 1.0; Holsinger and Lewis 2003), was used to estimate parameters related to genetic structure (Theta B value).

The genetic structure of geographical populations and provinces were studied by structure analysis (Pritchard et al. 2000) for dominant markers (Falush et al. 2007).

Model-based clustering was carried out to group the studied populations based on genetic affinity using STRUCTURE software (ver. 2.3; Pritchard et al. 2000). This program was also used to reveal the genetic admixture of the studied populations. For this analysis, the admixture ancestry model under the correlated allele frequency model was used. The Markov chain Monte Carlo simulation was run 20 times for each value of K (2-11) for 20 iterations after a burn-in period of 10^5. All other parameters were set at their default values. Data were scored as dominant markers and analyzed according to the method suggested by Falush et al. (2007). STRUCTURE Harvester web site (Earl and von Holdt 2012) was used to visualize the STRUCTURE results and also to perform Evanno method to identify the proper number of K (Evanno et al. 2005).

The occurrence of gene flow among populations was checked by different methods. First, we performed indirect Nm analysis using POPGENE (ver. 2) for ISSR loci studied according to the following formulae:

Nm = estimate of gene flow from Gst, $Nm = 0.5(1 - Gst)/Gst$.

Then we used reticulation (Legendre and Makarenko 2002) and NeighborNet analyses (Huson and Bryant 2006). Finally, the population assignment test was performed by using maximum likelihood method as implemented in GenoDive (ver.2; 2013) (Meirmans and Van Tienderen 2004).

Morphological data were standardized (mean = 0, variance = 1) and used to estimate Euclidean distance among the studied populations. UPGMA (unweighted group mean using average) and PCoA (principal coordinate analysis) as well as PCA (principal components analysis) were used for grouping the populations and for the identification of the most variable morphological characters among the studied populations (Podani 2000). Mantel test was used to determine the correlation between genetic distance and morphological distance.

Table 3. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the studied populations.

Population	RK	SK	Yz	Kr	Is	Mz	Gi	Th	Gl	Kh	Sm
RK	-	0.8642	0.9704	0.9588	**0.9805***	0.9658	0.9648	0.9054	0.9436	0.9732	0.8632
SK	0.1460	-	0.8781	0.7953	0.8561	0.8569	0.8519	0.7981	0.8395	0.8386	**0.7543**
Yz	0.0301	0.1300	-	0.9340	0.9683	0.9417	0.9425	0.8935	0.9338	0.9360	0.8211
Kr	0.0420	0.2290	0.0683	-	0.9557	0.9447	0.9284	0.8577	0.9541	0.9292	0.8115
Is	0.0197	0.1554	0.0323	0.0453	-	0.9581	0.9587	0.9119	0.9372	0.9551	0.8389
Mz	0.0347	0.1545	0.0600	0.0569	0.0428	-	0.9518	0.9059	0.9386	0.9406	0.8270
Gi	0.0358	0.1603	0.0592	0.0743	0.0422	0.0494	-	0.9078	0.9235	0.9324	0.8419
Th	0.0994	0.2255	0.1126	0.1534	0.0922	0.0988	0.0968	-	0.8464	0.8603	**0.7673**
Gl	0.0580	0.1750	0.0685	0.0470	0.0648	0.0634	0.0796	0.1668	-	0.9041	0.7884
Kh	0.0271	0.1761	0.0661	0.0734	0.0459	0.0612	0.0700	0.1505	0.1008	-	0.8550
Sm	0.1471	0.2819	0.1971	0.2088	0.1756	0.1899	0.1721	0.2649	0.2377	0.1567	-

*bold numbers indicate significant values RK: Razavi Khorasan; SK: South Khorasan; Yz: Yazd; Kr: Kerman; Is: Isfahan; Mz: Mazandaran; Gi: Gilan; Th: Tehran; Gl: Golestan; Kh: Khuzesta; Sm: Semnan.

Results

Populations' genetic diversity and structuring

In total, 69 ISSR bands were obtained, from which all were polymorphic (Fig. 2). Genetic diversity parameters determined in 11 geographical populations of *C. vulgaris* are presented in Table 2. The highest value for polymorphism percentage (85.51%), gene diversity (0.209) and Shanon' information index (0.333) occurred in Razavi Khorasan population. The highest value for polymorphism percentage (85.51%), gene diversity (0.209) and Shanon' information index (0.333) occurred in Razavi Khorasan population. Golestan and Semnan populations had the lowest value for the same parameters: 8.700, 0.053, 0.036, and 0.00, 0.00, 0.00, respectively.

AMOVA test revealed the presence of significant molecular difference among the studied populations (P = 0.01). It also revealed that 11% of total genetic variability occurred among the studied populations, while 89% occurred within these populations. These results indicate the presence of high level of genetic variability within *C. vulgaris* populations. Gst analysis (0.148, P = 0.001) and Hickory test (Theta B = 0.40) also supported the AMOVA test results and revealed signifi-

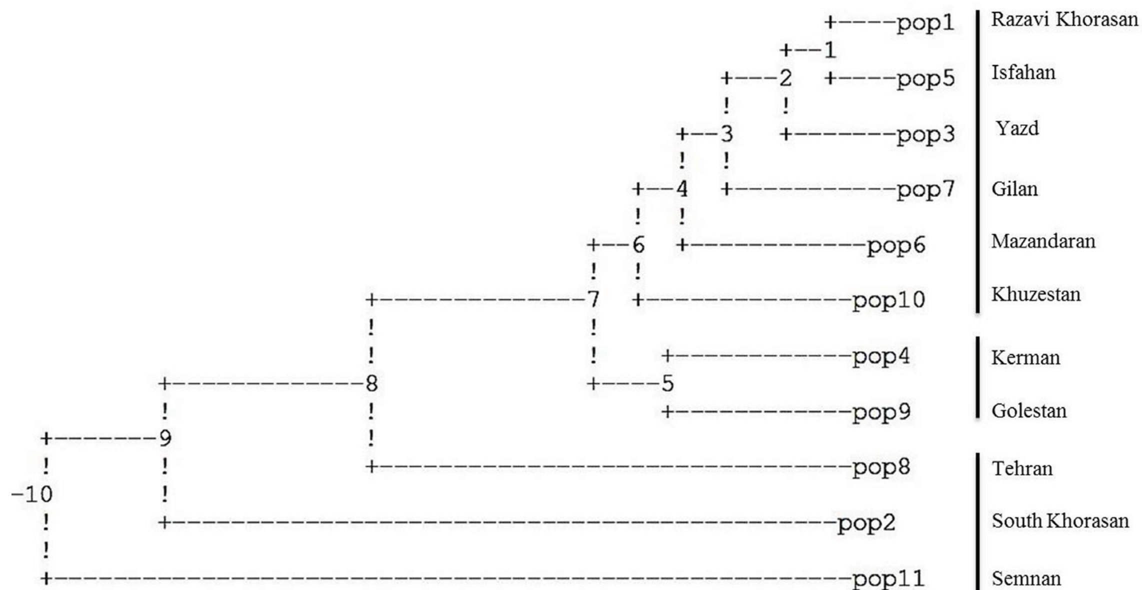

Figure 3. NJ tree of populations based on genetic data.

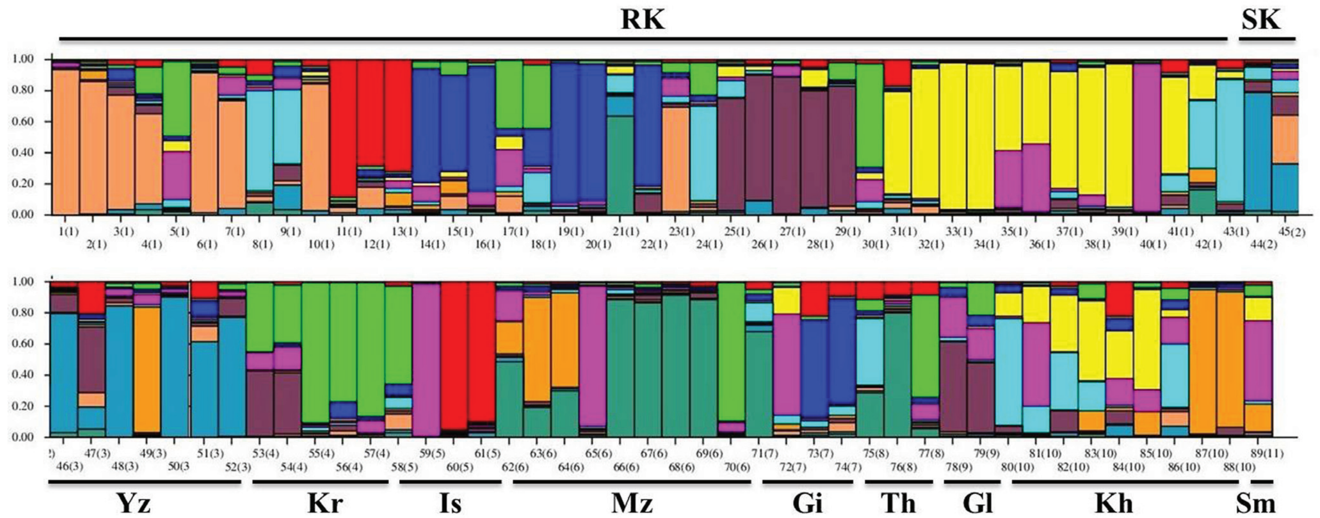

Figure 4. STRUCTURE plot of *C. vulgaris* populations studied. RK: Razavi Khorasan; SK: South Khorasan; Yz: Yazd; Kr: Kerman; Is: Isfahan; Mz: Mazandaran; Gi: Gilan; Th: Tehran; Gl: Golestan; Kh: Khuzestan; Sm: Semnan.

cant genetic differences among the studied populations.

Hedrick's standardized fixation index (G'st = 0.161, P = 0.001) and Jost's differentiation index (D-est = 0.062, P = 0.001) revealed that the studied geographical populations of *C. vulgaris* are genetically differentiated.

Nei's genetic identity and genetic distance of the studied populations are presented in Table 3. The highest value for genetic identity (0.9805) occurred between Razavi Khorasan and Isfahan populations, while the lowest value of the same (0.7543) occurred between South Khorasan and Semnan populations.

The NJ tree of ISSR data is presented in Fig. 3. It produced 3 major clusters. Population numbers 1, 3, 5, 6, 7 and 10 (Razavi Khorasan, Yazd, Isfahan, Mazandaran, Gilan and Khuzestan Province, respectively) comprised the first major cluster. In this cluster, Razavi Khorasan and Isfahan populations (1 and 5) showed higher genetic similarity. Yazd, Mazandaran, Gilan and Khuzestan Province populations (3, 6, 7 and 10, respectively) joined them with some distance. Kerman and Golestan populations (4 and 9) formed the second major cluster. Populations Tehran, South Khorasan, and Semnan (8, 2 and 11, respectively) formed the third major cluster.

Pair-wise AMOVA revealed that all paired populations differed significantly from each other.

Mantel test performed between populations' genetic distance and their geographical distance produced significant positive correlation (r=0.20, P=0.05). Therefore, *C. vulgaris* populations showed isolation by distance (IBD) phenomenon, and with increase in geographical distance, a lower degree of gene flow occurred between them.

The STRUCTURE plot (Fig. 4) revealed some degree of genetic admixture in the studied populations. This is due to shared ancestral alleles, or ongoing gene flow. These results showed high degree of genetic variability both within and among the studied populations supporting our results obtained from AMOVA.

The Neighbor Net diagram (Fig. 5) produced similar grouping to NJ tree and The STRUCTURE plot. It also revealed some degree of gene flow between populations, and also showed intra-population genetic diversity of populations. Members of many populations were placed intermixed with other populations due to genetic variability possibly caused by inter-population gene flow. This is supported by the mean Nm = 0.85 value obtained.

Evanno method produced K=8 genetic groups. Eight out of 11 studied populations revealed almost complete lack of genetic fragmentation and the occurrence of genetic continuity among the studied populations. This is well supported by the STRUCTURE plot based on K=8. High degree of intra-population genetic variability and inter-population genetic admixture was observed in this plot too. For example, members of Khorasan Razavi population varied in their genetic structure (differently colored segments). This also held true for Khorasan and Mazandaran populations.

Some members of these populations contained alleles from the other populations (similarly colored segments). For example, members of Khorasan Razavi population contained similar alleles (colored segments) from both Khorasan and Mazandaran populations.

Morphometry

The mean of morphological characters of the studied populations is provided in Table 4. The studied populations varied

Table 4. Mean values of morphological characters studied in C. *vulgaris* populations.

Morphological characters	Population											P value
	RK	RS	Yz	Kr	Is	Mz	Gi	Th	Gl	Kh	Sm	
Length of bract cell inside	3.02	1.54	2.79	3.68	2.64	2.43	2.89	3.67	3.75	1.87	1.97	0.01
Length of bract cell outside	4.47	2.38	5.06	6.18	5.00	4.65	4.33	5.59	6.13	3.11	4.20	0.01
Number of cells in end segment	3.20	3.00	3.57	3.20	3.00	3.00	2.75	3.00	3.00	3.00	3.00	0.01
Length of end segment of branchlet (mm)	13.89	15.75	15.34	12.50	11.64	11.80	10.43	10.51	10.71	9.93	8.50	0.01
Length of first segment of branchlet (mm)	1.78	1.75	2.21	2.49	1.91	1.98	1.49	3.00	3.00	2.18	2.25	0.01
Length of end cell in end segment (mm)	2.33	3.40	2.02	1.84	1.53	1.94	2.26	1.83	1.78	1.86	**2.66**	0.01
Length of branchlet (mm)	20.33	20.75	21.69	20.01	16.63	18.53	15.42	20.45	22.11	17.41	**14**	0.01
Length of tips of the axis (mm)	14.88	25	16.19	15.43	16.83	18.17	14.99	18.16	22.42	17.74	**18.2**	0.01
Internode length (μm)	15.05	35	17.83	20.05	13.79	19.36	14.57	26.86	29.52	16.87	**24.2**	0.01
Diameter of antheridium (μm)	392	370	393.1	437	446.7	430.6	424	471	440	444.8	**391**	0.01
Oogonium wide (μm)	440.2	450	422.3	409	383.7	380.8	457	421.3	399.7	423.8	**425**	0.01
Oogonium length (μm)	670.2	712	691.4	663.2	598.5	635.2	715.7	712	718.7	694.8	**762**	0.01
Corona wide (μm)	190.6	200	177.3	193.2	197.5	186.7	216.5	236.6	199.5	194.9	200	0.01
Corona length (μm)	128.4	150	107.2	107.5	105	110	135.2	128.6	133.5	153.9	125	0.01
Number of corticate segment	2.40	3.00	2.72	2.20	2.50	3.60	3.00	3.00	3.50	3.40	3.00	0.01
Number of ecorticate segment	3.20	4.00	3.57	3.20	3.00	2.60	2.50	3.00	3.00	3.00	3.00	0.01
Internode diameter (mm)	0.63	0.65	0.72	0.82	0.87	0.75	0.72	1.06	0.81	0.71	0.75	0.01
Number of branchlets in each node	10.00	9.00	10.14	10.00	10.25	10.20	10.00	10.00	10.00	10.20	10.0	0.01
Oospore length (μm)	518.6	738.2	493.1	514.8	543.2	471.8	557.9	547.4	440.3	509.1	596.3	0.01
Oospore wide (μm)	330.6	370.1	328.5	344.3	329.9	301.9	383.9	346.9	278.1	329.3	324.2	0.01
Oospore length/wide ratio (μm)	1.56	1.99	1.51	1.49	1.65	1.56	1.45	1.57	1.58	1.54	1.84	0.01
Internode diameter (mm)	0.63	0.65	0.72	0.82	0.86	0.75	0.71	1.06	0.81	0.70	0.75	0.01
Number of branchlets in node	10.00	9.00	10.14	10.00	10.25	10.20	10.00	10.00	10.00	10.20	10.00	0.01
Oospore length (μm)	518.6	738.1	493.1	514.8	543.2	471.8	557.9	547.3	440.3	509.1	596.3	0.01
Oospore wide (μm)	330.6	370.0	328.5	344.3	329.9	301.9	383.9	346.9	278.1	329.3	324.1	0.01
Oospore length/wide ratio (μm)	1.56	1.99	1.51	1.49	1.65	1.56	1.45	1.57	1.58	1.54	1.83	0.01
Fossa breath (μm)	51.29	67.24	51.12	57.66	49.03	45.30	56.84	50.94	42.14	54.33	54.19	0.01
Number of striae	11.20	11.00	11.57	10.40	11.75	11.00	11.50	12.00	10.00	10.60	11.00	0.01
Length of plant (cm)	18.80	60.00	35.28	35.00	17.25	36.00	23.75	66.66	40.00	22.00	25.00	0.01

RK: Razavi Khorasan; SK: South Khorasan; Yz: Yazd; Kr: Kerman; Is: Isfahan; Mz: Mazandaran; Gi: Gilan; Th: Tehran; Gl: Golestan; Kh: Khuzestan; Sm: Semnan

in the studied quantitative morphological characters. For example, Golestan and Tehran populations had the highest value for length of bract cell inside (3.75 and 3.65, respectively). Similarly, Kerman and Golestan populations had the highest value for length of bract cell outside (6.16 and 6.13, respectively). South Khorasan populations and Yazd population had the highest value for Length of end segment of branchlet (15.8 and 15.3, respectively). ANOVA (analysis of variance) test revealed significant difference for quantitative morphological characters among the studied populations ($P<0.01$).

UPGMA dendrogram of morphological characters and PCoA plot (Fig. 6) produced similar results. Therefore, only PCoA plot is given and discussed here. PCoA plot revealed morphological variability within the studied populations. The members of each population were scattered in the plot and did not form a separate group. These populations differed in degree of morphological variability; a higher degree of variability was observed among plant specimens of populations Razavi Khorasan and Yazd (1 and 3).

In general, some agreement occurred between genetic similarity and morphological similarity among the studied populations. Plant specimens of populations Razavi Khorasan, Yazd, Kerman and Khuzestan (1, 3, 4 and 10, respectively) are in many places close to each other in both analyses. However, we did not get a complete agreement between the two types of data. In fact, Mantel test did not show significant correlation between morphological distance and genetic distance in these populations ($r = 0.04$, $P = 0.3$).

PCA analysis of morphological data revealed that the first 3 PCA components comprised about 70% of total variation among the studied populations. It showed that three morphological characters (length of the bract cell from inside and

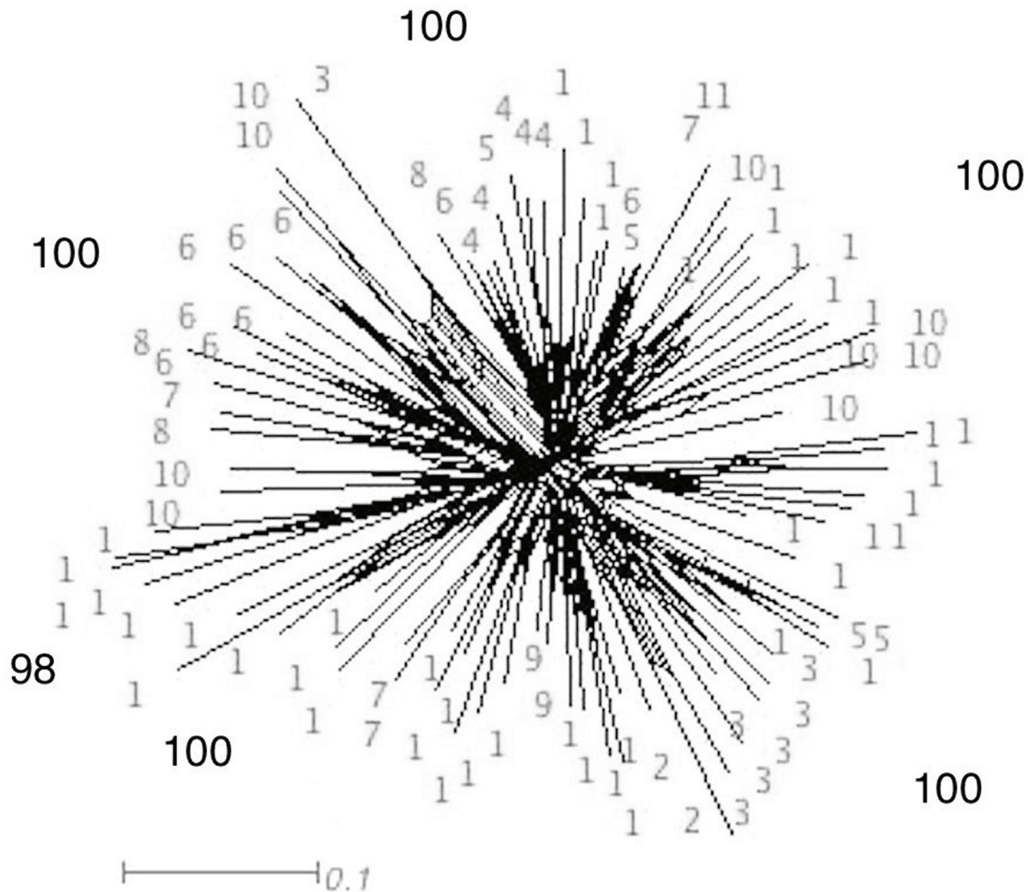

Figure 5. Neighbor Net diagram of ISSR data. Populations Razavi Khorasan, South Khorasan, Yazd, Kerman, Isfahan, Mazandaran, Gilan, Tehran, Golestan, Khuzestan, and Semnan, are marked with numbers 1-11, respectively.

from outside as well as the length of branchlet) possessed r= >0.80 with the first axis and are the most variable characters among the studied populations.

Discussion

Plant species that grow in different environmental conditions diversify in their genetic and morphological features due to local adaptations, genetic drift and species expansion (Sheidai et al. 2012, 2013). According to Knaus (2008), if we take the species to be the unit of distinction, the infra-taxa (the subspecies, the variety and the ecotype) are consequently non-distinct. The process by which a group of organisms diverge from being one cohesive group to becoming two or more distinct groups is the process of speciation. Stebbins (1993) also included the idea that species are systems of populations, which resemble each other, yet contain genetically different ecotypes that could be arranged in a continuous series. These allopatric infra-specific categories are usually recognized as infra-taxa.

The extent of polymorphism detected in the populations investigated in this study (up to 85.51%) suggests high intraspecific genetic diversity within *C. vulgaris* populations, which is also reflected in high morphological variation. This study is in agreement with previous reports finding very high levels of genetic diversity between *Chara* populations of a single taxon. Allozyme studies by Grant and Proctor (1980) and molecular marker studies by Mannschreck et al. (2002) and O'Reilly et al. (2007) found both high inter- and intraspecific genetic diversity in *Chara*.

Mannschreck et al. (2002) reported 99% AFLP band polymorphism among *Chara* species, and 91% variation between populations of a single taxon. Genetic variation in *Chara* populations may result from gene duplication via polyploidy, as presumed in Grant & Proctor (1980). Polyploidy is only widespread amongst monoecious species of *Chara* (Proctor 1976), such as *C. vulgaris*. Reported chromosome counts for *C. vulgaris* are n = 14, 18, 28, 42 (Sato 1959; Guerlesquin

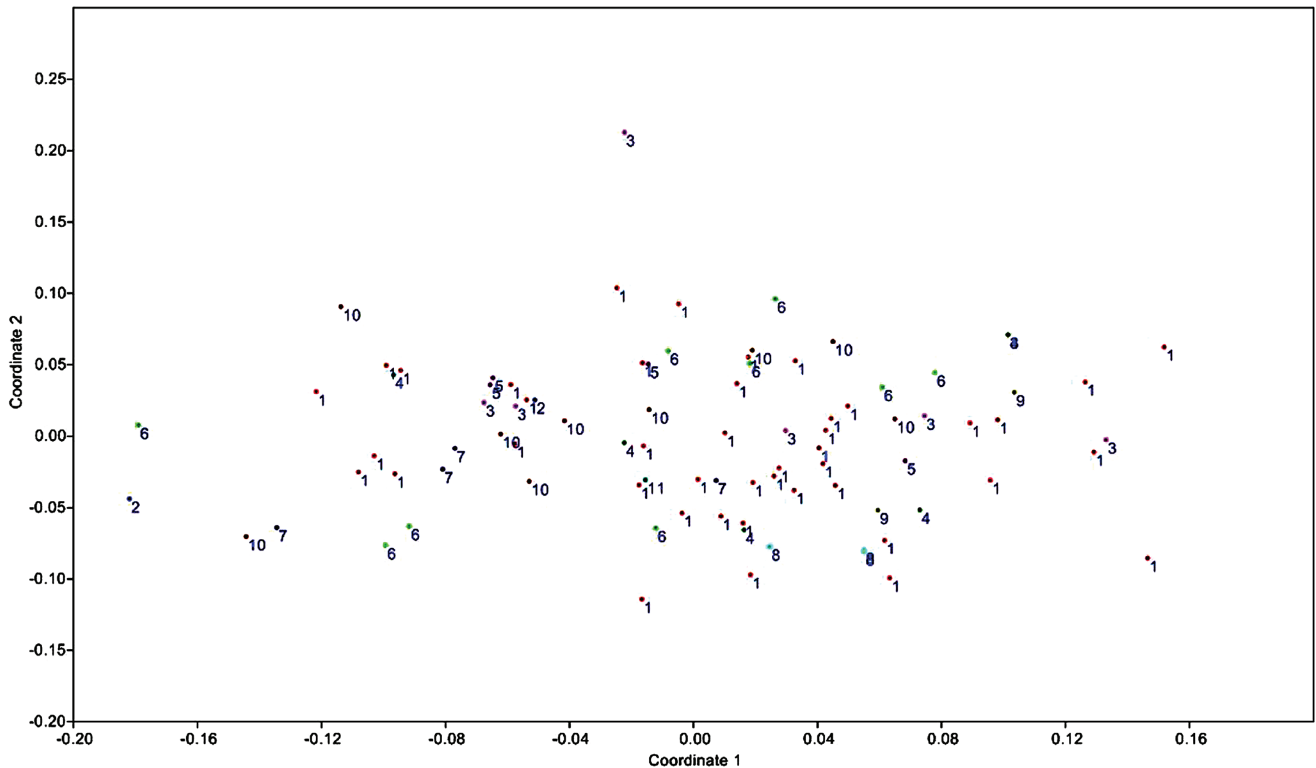

Figure 6. PCoA plot of morphological characters in *C. vulgaris* populations. Populations Razavi Khorasan, South Khorasan, Yazd, Kerman, Isfahan, Mazandaran, Gilan, Tehran, Golestan, Khuzestan, and Semnan, are marked with numbers 1-11, respectively.

1966, 1967; Mirasidov 1971; Grant and Proctor 1972; Khatun et al. 2009).

The present study showed genetic divergence of the studied *C. vulgaris* populations, but did not show their morphological divergence. A Mantel test showed no significant correlation between the genetic data and the morphological data, supporting the hypothesis that phenotypic variability in *Chara* L. is either some extent environmentally induced or represents developmental stages. Absence of association between the genetic data and the morphological data within and between the populations of *C. curta* and *C. aspera* was also observed by O'Reilly et al. (2007). They suggest that genetic variation in *Chara* populations may result from polyploidy. Variation in ISSR bands results in sequence changes due to either insertion/deletion or sequence rearrangements (Sheidai et al. 2012, 2013; Noormohammadi et al. 2012). It seems that gene flow/presence of ancestral alleles in the studied *C. vulgaris* populations resulted in both genetic and morphological overlap/similarities among them and we cannot completely differentiate these populations from each other. Studies of putative phenotypic plasticity in other algae have shown that morphological variation may be at least partly genetically and partly environmentally controlled (Guiry 1992). Very few experimental investigations of phenotypic plasticity or developmental differentiation in the Charales have been published, despite plasticity having long been hypothesized for this group (Willdenow 1805; Wood and Imahori 1965; Proctor 1975). Therefore, in spite of significant genetic difference among the studied populations we do not attempt to consider them as separate ecotypes or varieties that are known to exist in *C. vulgaris*.

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