# CHARACTERIZATION OF GENETIC DIVERSITY IN THREE CHARA L. SPECIES (CHARACEAE) IN IRAN USING RAPD-PCR TECHNIQUE

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Received 14.04.2012. Accepted for publication 07.11.2012.

Ghaemmaghami, S. S., Afsharzadeh, S. & Balali, G. R. 2012 12 31: Characterization of genetic diversity in three *Chara* L. species (*Characeae*) in Iran using RAPD-PCR technique. -*Iran. J. Bot. 18 (2): 38- 326.* Tehran.

Species separation in the family *Characeae* is generally based on morphological characters, but these traits used to identify species are influenced by habitat characteristics. The green algal genus *Chara* Linnaeus comprises of several species, which are morphologically variable. RAPD-PCR technique was used to characterize genetic diversity in three *Chara* L. species collected from Iran. Five primers were used in this study. Different loci were detected using a total of 17 samples of *Chara vulgaris* L., *C. gymnophylla* A. Braun and *C. zeylanica* C. L. Willdenow. The present study is the first on the molecular characterization of these species using RAPD-PCR marker in Iran. The method seems to be suitable for characterization of the genus at species level.

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*Key words. Genetic diversity, Chara, Iran, Characeae, RAPD-PCR.*

**تعیین تنوع ژنتیکی سه گونه از جنس کارا در ایران با استفاده از تکنیک رپید**

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تمایز گونه‌ها در خانواده کاراسه عموما بر پایه صفات ریخت‌شناسی است، ولی این صفات تحت تاثیر خصوصیات محیطی قرار می‌گیرند. جنس کارا شامل تعداد زیادی گونه می‌باشد که تغییرپذیری مورفولوژیکی زیادی را از خود به نمایش می‌گذارند. تکنیک مولکولی رپید برای تعیین تنوع ژنتیکی در سه گونه از جنس کارا در ایران مورد استفاده قرار گرفت. در این مطالعه پنج پرایمر استفاده شدند. لوکوس‌های مختلفی برای ۱۷ نمونه از گونه‌های *Chara vulgaris*، *C. gymnophylla* و *C. zeylanica* شناسایی شد. مطالعه حاضر برای اولین بار استفاده از تکنیک مولکولی رپید را در تمایز این گونه‌ها در ایران شرح می‌دهد. به نظر می‌رسد این تکنیک برای تمایز گونه‌های این جنس مفید و موثر باشد.

## INTRODUCTION

*Characeae* (stoneworts) are macrophytic green algae with whorls of branches at nodes. They form a significant part of the submerged vegetation of lakes and rivers (Mannschreck *et al.,* 2002; Urbaniak, 2010). Investigations show that this group of organisms are most closely related to land plants and from this point of view they hold a unique phylogenetic position (Bhattacharya and Medlin 1998; Karol *et al.,* 2001). The members of the genus *Chara* have unforked whorled branchlets in which the female reproductive organs (oogonia) positioned above male ones (antheridia), and this character distinguishes them from other genera in the family *Characeae* (Wood and Imahori, 1965; Sakayama *et al.,* 2009; Kato *et al.,* 2010). The genus *Chara* like other genera in *Characeae* in its taxonomic history has followed a line of development.

Many *Chara* species have strong similarities in their morphological characteristics (Wood and Imahori, 1965; Urbaniak, 2010). The real causes of morphological variability are difficult to explain (because of the function of several factors such as environmental adaptations and genetically defined characters), and for these reasons the genus *Chara* has been defined differently (Wood and Imahori, 1965; Mannschreck *et al.,* 2002; Boegle *et al.,* 2010). Different authors have treated the genus in different

manners. According to Wood and Imahori (1965), the genus comprises only few species, which have a wide morphological spectrum, so the species are very polymorph (Wood and Imahori, 1965; Mannschreck *et al.,* 2002). In contrast, some authors such as Corillion (1957) and Krause (1997), believe that the genus comprises many species that are not morphologically variable (Meiers *et al.,* 1999; Mannschreck *et al.,* 2002; Boegle *et al.,* 2007). These different interpretations are mainly due to a lack of objective methods to distinguish between environmental modifications and genetically anchored features. The objectivity of molecular techniques offers new possibilities for the study of taxonomy and genetic diversity in the genus *Chara*. On the other hand, some excellent literature are available on the SEM study of oospore wall ornamentation in *Characeae* taxonomy (e.g. Casanova, 1997, 2005; Urbaniak, 2010, 2011). Sundaralingam (2002) who carried a cytological, cytogenetical and biochemical research on *Charophyta* stated that only molecular marker systems could show the biodiversity of these green algae (Sundaralingam, 2002).

Amplified fragment length polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD) and $SSR_S$ are the most common genetic marker systems that have been used in biosystematics. These markers are useful for detection of genetic diversity at lower taxonomic levels. Ramos an co-workers (2008), indicated that molecular markers in recent years have faced good advances, but molecular biologist can retain RAPD as a useful marker yet (Ramos *et al.,* 2008). RAPD marker was the method of our selection; there are many organisms, which no specific marker has been developed for them, but RAPD method could be used for screening of genetic diversity; RAPD could be done in a simple laboratory with minimums in performing PCR. This method is useful for detection of genetic diversity between morphologically similar species and it has been used in some algal genera, such as *Alexandrium*, *Dunaliella* and some seaweed genera (Abrol *et al.,* 2006; Zhao *et al.,* 2008).

There is no report on DNA isolation and the use of RAPD markers for molecular identification and genetic diversity characterization of *Chara* taxa in Iran.

In this study the isolation, amplification, and characterization of genomic DNA of three *Chara* taxa in Iran using five oligonucleotide primers was carried out.

## MATERIALS AND METHODS

Collection and preparation of algal samples for DNA extraction

Sampling of *Chara* specimens was carried out in the years 2009 and 2010 from different freshwater regions (such as ponds, pools and rivers) of Iran. Collection site of algal samples are listed in table 1. In this study, 17 populations have been collected and investigated. The collected samples were studied using morphological means, and three species *Chara vulgaris* Linnaeus, *C. gymnophylla* A. Braun and *C. zeylanica* C. L. Willdenow were identified using valid identification keys (Wood and Imahori, 1965; Moore, 1986).

DNA extraction and determination of DNA quality and quantity

After sample collection, the algae were thoroughly washed, microscopic examinations revealed that there were no epiphytes attached to the samples. After that the samples decolorized in dark.

DNA extraction was carried out using a modified CTAB method (Murray and Thompson, 1980).

Spectrophotometry and agarose gels were used for determination of quality and purity of DNA before amplification.

The suitability of the isolated DNA for PCR procedures was tested on 1% agarose gels. Figure 1 shows a gel that isolated DNA from our samples has been runned on it for qualitative test. Only those samples, which showed an intact band and no shearing/ degradation of DNA were used in subsequent analysis and the rest were rejected.

For determination of DNA concentration, we used biophotometer system. Absorbance of DNA and protein were measured in OD 260 nm and 280 nm wavelengths respectively.

If the calculates OD ratio 260/280 were between 1.7-2, so DNA has a good quality and concentration and is suitable for PCR procedures.

RAPD-PCR procedures

The random primers selected from an OPERON series based on GC content (Abrol *et al.,* 2006) and synthesized by Farayand Danesh Co. The primers used in this research are listed in table 2.

PCR reactions were carried out in a total volume of 20 µl, containing 2 µl of template DNA solution (100-200 ng), 1 µl random oligonucleotide primers (0.4 µM), 1 µl $MgCl_2$ (2 mM), 1 µl dNTP mix (0.4 µM), 2.5 µl 10X PCR buffer, Taq DNA polymerase 0.35 µl (1 unit). The final volume must be reach to 20 µl using D.D $H_2O$.

Amplification reactions were conducted in a master cycler (Eppendorf, Germany). The thermal cycler was programmed 5 min at 940 C for an initial denaturation followed by 40 cycles of 1 min at 940C to denaturate, 1 min at 370C for annealing, 2 min at 720C for extension, followed by a termination step of 10 min at 720C, and cooling to 40C. Bands were resolved by electrophoresis in a 2 % agarose gels in 1x TAE (Tris-EDTA) buffer and stained with ethidium bromide.

Table 1: The collection sites for 17 populations mentioned in dendrograms.

| Population | Location and height | Longitude and latitude | Date (y/mm) |
|---|---|---|---|
| *Chara vulgaris* population 1 | Lorestan, Mahuk River, 1469m | N 33º 41′ 26′′<br>E 49 º 05′ 16′′ | 2009/6 |
| *C. vulgaris* population 2 | Kermanshah road to Bisotoon, 1304m | N34º 28′ 26′′<br>E 47º 37′ 14′′ | 2009/6 |
| *C. vulgaris* population 3 | Hamedan, Malayer 1887m | N 34º 30′ 02′′<br>E 47º 36′ 12′′ | 2009/6 |
| *C. vulgaris* population 4 | Chahar Mahal and Bakhtiari, Boroujen road to Lordegan, Bijgard village, 2229m | N 32º 00′ 55′′<br>E 51º 16′ 41′′ | 2010/7 |
| *C. vulgaris* population 5 | Semnan, Shahrood, artifitial waterfall, 1407m | N 36º 39′ 33′′<br>E 55º 05′ 06′′ | 2009/8 |
| *C. vulgaris* population 6 | Isfahan, Naeen, Mollaahmad spring, 2358m | N 33º 49′ 33′′<br>E 50º 00′ 14′′ | 2009/8 |
| *C. vulgaris* population 7 | Chahar Mahal and Bakhtiari, Lordegan, 2135m | N 32º 06′ 32′′<br>E 51º 12′ 54′′ | 2010/9 |
| *C. vulgaris* population 8 | Chahar Mahal and Bakhtiari, Lordegan, Sini Village, 1852m | N 31º 48′ 24′′<br>E 51º 03′16′′ | 2009/9 |
| *C. vulgaris* population 9 | Isfahan, Noghan, 2400m | N 33º23′ 55′′<br>E 50º 08′ 23′′ | 2009/10 |
| *C. vulgaris* population 10 | Isfahan, Moorchehkhort, Miran Garden, 1590m | N 31º 01′ 12′′<br>E 51º 32′ 33′′ | 2010/4 |
| *C. gymnophylla* population 1 | Isfahan, Semirom, Rood-Abad river, 1712m | N 31º 09′ 15′′<br>E 51º 25′ 36′′ | 2010/3 |
| *C. gymnophylla* population 2 | Isfahan, Noghan, 2400m | N 33º23′ 55′′<br>E 50º 08′ 23′′ | 2009/10 |
| *C. gymnophylla* population 3 | Hamedan, Malayer, 1887m | N 34º 30′ 02′′<br>E 47º 36′ 12′′ | 2009/5 |
| *C. gymnophylla* population 4 | Hamedan, Malayer, 1864 m | N 34º 13′ 42′′<br>E 49º 24′ 53′′ | 2009/5 |
| *C. gymnophylla* population 5 | Chahar Mahal and Bakhtiari, Boldaji, 2280m | N 31º 58′ 22′′<br>E 51º 12′ 49′′ | 2009/7 |
| *C. zeylanica* popualation 1 | Isfahan, Natanz, Targhrood | N 33º 21′ 01′′<br>E 51º 47′ 53′′ | 2009/8 |
| *C. zeylanica* popualation 2 | Isfahan, Natanz, Targhrood | N 33º 21′ 32′′<br>E 51º 45′ 50′′ | 2011/11 |

RAPD-PCR optimization for investigated *Chara* species

Yu and Paula (1992), stated that optimization of conditions used in PCR procedures (for example by changing the amount of Taq DNA polymerase and $MgCl_2$) could increase the efficiency of the RAPD-PCR technique. In present study, we found that the amount of 0.35 µl and 1 µl of Taq DNA polymerase and $MgCl_2$ respectively, is suitable and optimal for PCR.

The PC-ORD software was used for construction of dendrograms.

## RESULTS AND DISCUSSION

In this study morphological characters of the collected samples were worked out. Three species were identified using valid identification keys: *Chara vulgaris*, *C. gymnophylla* and *C. zeylanica*.

For the first time isolation, amplification, and characterization of genomic DNA of three *Chara* species in Iran using five oligonucleotide primers was carried out.

## RAPD-PCR analysis

RAPD-PCR amplification of DNA from *Chara* samples was carried out with five oligonucleotide random primers. After this, 2% agarose gel was used for PCR products run on it. 100 bp gene ruler was used for estimation of molecular size. Tables 3-6 shows various amplified products using random

Table 2. Oligonucleotide random primers with their GC content used in this study.

| Primer | Nucleotide sequence | GC content (%) |
|---|---|---|
| OPF-07 | 5′ CCGATATCCC 3′ | 60 |
| OPG-02 | 5′GGCACTGAGG 3′ | 70 |
| OPG-03 | 5′GAGCCCTCCA 3′ | 70′ |
| OPB-04 | 5′GGACTGGAGT 3′ | 60 |
| 0PA-09 | 5′GGGTAACGCC 3′ | 70 |

Table 3: Loci produced for *Chara vulgaris*, *C. gymnophylla* and *C. zeylanica* using primer OPA-09.

| | Molecular size of the band (bp) | 140 | 160 | 240 | 250 | 290 | 300 | 320 | 380 | 400 | 420 | 580 | 700 | 900 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| species | *Chara vulgaris* | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 |
| | *C. gymnophylla* | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 |
| | *C. zeylanica* | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 4: Loci produced for *Chara vulgaris*, *C. gymnophylla* and *C. zeylanica* using primer OPB-04.

| Molecular size of the band (bp) | 150 | 270 | 300 | 350 | 370 | 380 | 410 | 450 | 500 | 550 | 600 | 850 | 1000 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. vulgaris* | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *C. gymnophylla* | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| *C. zeylanica* | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Table 5: Loci produced for *Chara vulgaris*, *C. gymnophylla* and *C. zeylanica* using primer OPG-02.

| Molecular size of the band (bp) | 200 | 220 | 270 | 280 | 400 | 600 | 880 | 900 | 1500 |
|---|---|---|---|---|---|---|---|---|---|
| *C. vulgaris* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 |
| *C. gymnophylla* | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| *C. zeylanica* | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |

Table 6: Loci produced for *Chara vulgaris*, *C. gymnophylla* and *C. zeylanica* using primer OPG-03.

| Molecular size of the band (bp) | 120 | 150 | 190 | 200 | 270 | 300 | 500 | 560 | 600 | 620 | 1100 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. vulgaris* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| *C. gymnophylla* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| *C. zeylanica* | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |

oligonucleotide primers in three *Chara* species. The RAPD-PCR technique successfully differentiated three species treated in this research with high efficiency.

Primers showed high polymorphism in *Chara* species in this research. The most polymorphism was created by primer OPF-07 (Fig. 2). The size of the loci that has been amplified using these five primers is between 100-1800 bp. Primer OPG-02 amplified a 200 bp locus in all populations of these three species in the present study and this locus is conserved within 3 species here. Figure 3 shows RAPD-PCR products for primer OPG-02.

Primer OPA-09 produced a very sharp and distinctive band for *Chara vulgaris* at 380 bp. It shows that the number of copies for this band is much higher than other bands. Other primers produced such strong bands for this species or other two remaining species. For example OPB-04 produced a sharp band at 300 bp region for *Chara vulgaris* and *C. gymnophylla* and at 270 bp region for *C. gymnophylla* and *C. zeylanica*. Based on the information of 5 primers used in this study a dendrogram was created using PC-ORD. Four dendrograms of the five created, showed that *C. vulgaris* and *C. gymnophylla* are more related together than each of them to *C. zeylanica* (Figs. 5-8). In each dendrogram we have two main clades, and each of them consists of subclades. For example, in OPB-04 dendrogram, one main clade at 60% similarity level divides into two other subclades, which one comprises populations 1-10 of *Chara vulgaris*. The first subclade

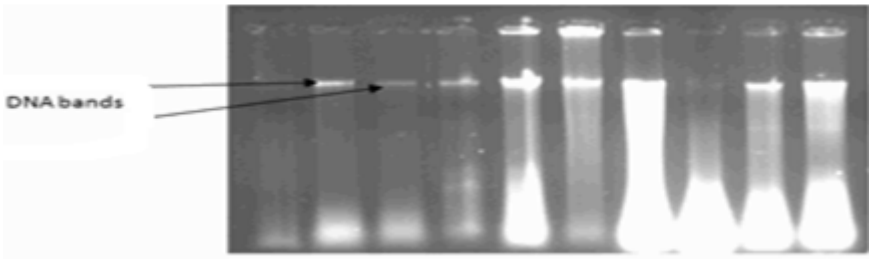


Fig. 1: Qualitative test of isolated DNA from various *Chara* taxa on 1% agarose gel.

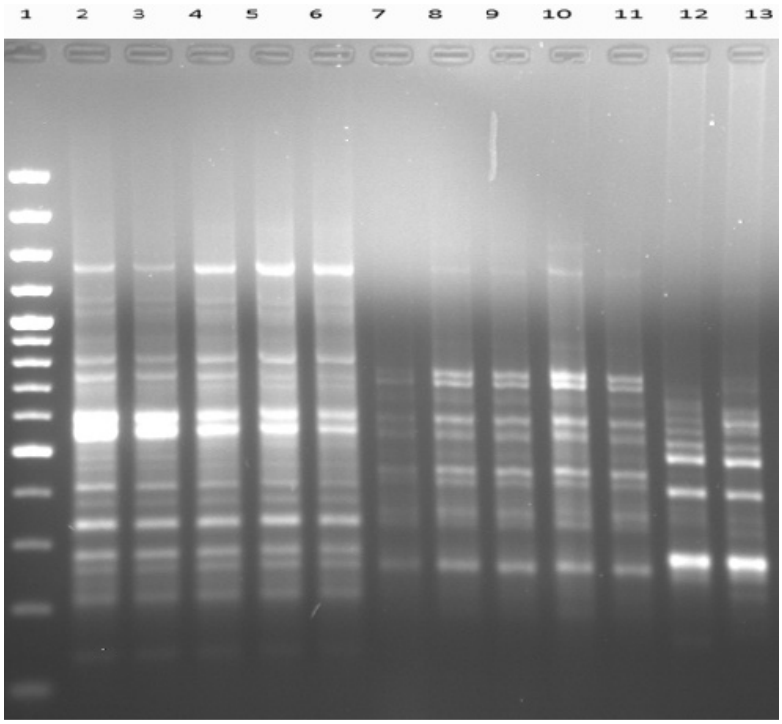


Fig. 2: RAPD-PCR products for primer OPF-07: lane 1: 100 bp ladder (Molecular weight marker), Lanes 2-6: *Chara vulgaris* (5 populations), Lane 7-11: *C. gymnophylla* (5 populations) and lanes 12-13: 2 populations of *C. zeylanica.*

at 90% similarity level gives rise to two subclades, one is consisted of populations 1-8 and the other comprises populations 9 and 10. The second subclade comprises populations 1-5 of *C. gymnophylla*. The other main clade consists of 2 populations of *C. zeylanica* (Fig. 5). The situation of other dendrograms (except for dendrogram of primer OPA-09) is more or less similar to this dendrogram (Figs. 5-8) . In dendrogram of OPA-09 data (Fig. 4) one main clade comprises *C. vulgaris* and *C. zeylanica* populations and the other comprises *C. gymnophylla* populations which is in conflict with the results of morphological Chararacters.

Abrol and co-workers (2006) used the same technique for molecular characterization of five Indian *Chara* taxa and have supposed some changes in the status of *C. wallichii* A. Braun and *C. erythrogyna* W. Griffith (Abrol *et al.,* 2006). There is no previous work using RAPD method for phylogenetic approachs in the genus *Chara*.

The application of molecular markers in differentiation of *Chara* (and other genera in family *Characeae*) species has a fast-growing interest. Boegle et al., (2007), used AFLP for molecular differentiation of *C. intermedia* A. Braun, *C. baltica* A. Bruzelius and *C. hispida* L.. Kato et al. (2008), have used sequence data from the gene encoding the large subunit of Rubisco (rbcL) and intergenic spacer regions between the beta subunit of the ATP synthase for construction of the intraspecific phylogeny of *C. braunii*. Boegle et al., (2010), based on physiological, morphological and AFLP analysis concluded that *C. intermedia* and *C. baltica* are only partially differentiable and the two

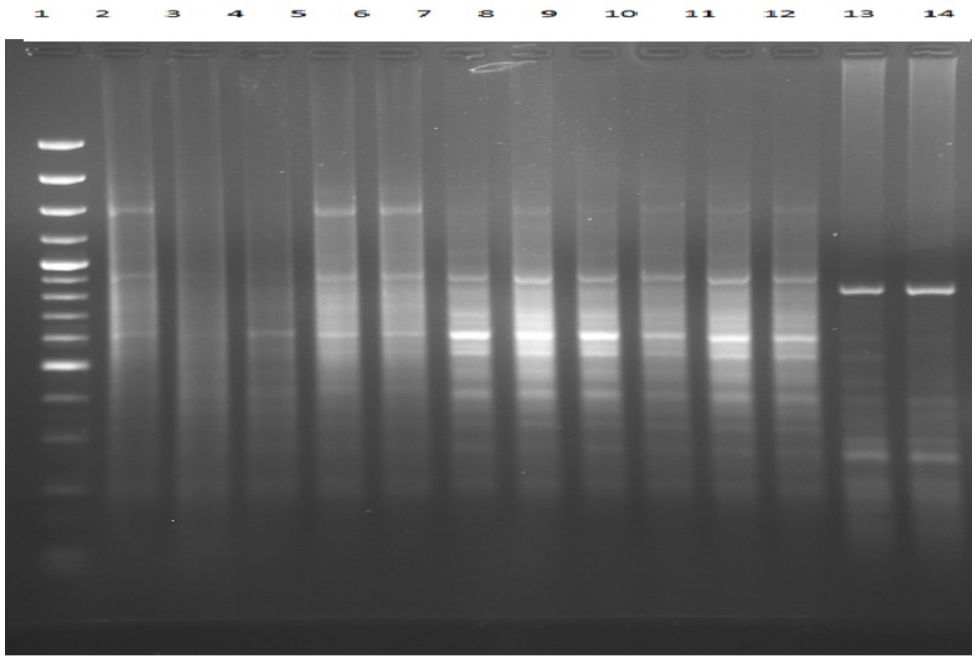


Fig. 3: RAPD-PCR products for primer OPG-02: lane 1: 100 bp ladder (Molecular weight marker), Lanes 2-6: *Chara vulgaris* (5 populations), Lane 7-12: *C. gymnophylla* (6 populations) and lanes 12-13: 2 populations of *C. zeylanica.*

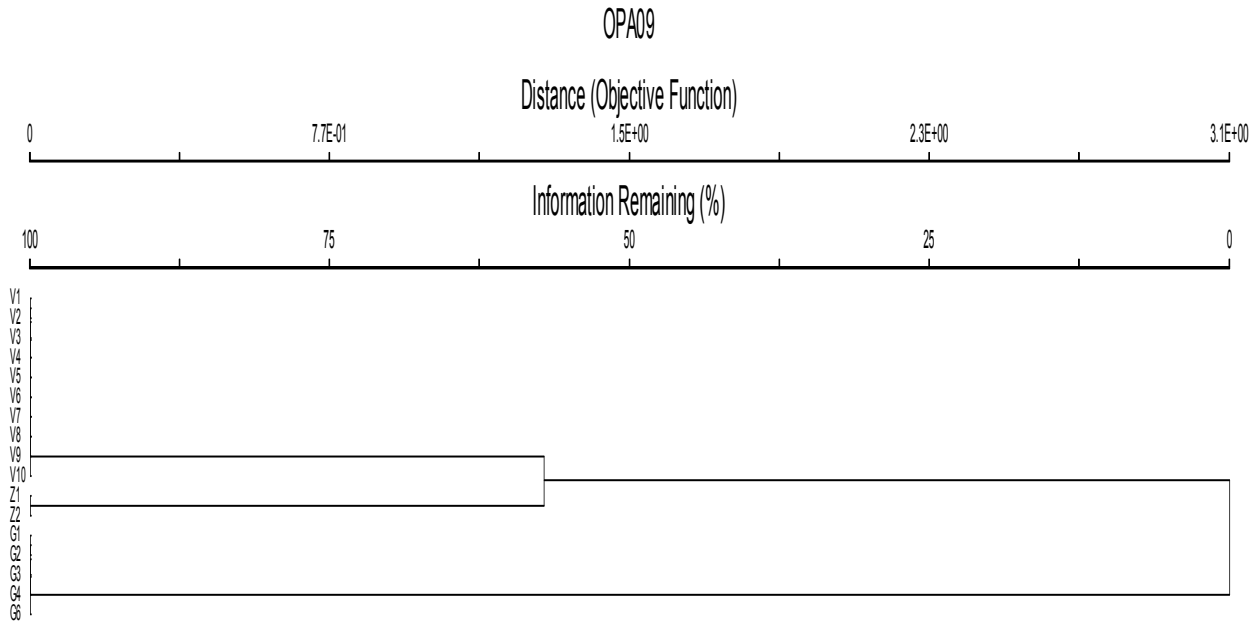


Fig. 4: Dendrogram created by UPGMA algorithm using Jaccard similarity coefficient on data from primer OPA-09 for 17 populations of *Chara* (V1-V10: 10 populations of *Chara vulgaris*, G1-G5: five populations of *C. gymnophylla* and Z1-Z2: two populations of *C. zeylanica*).

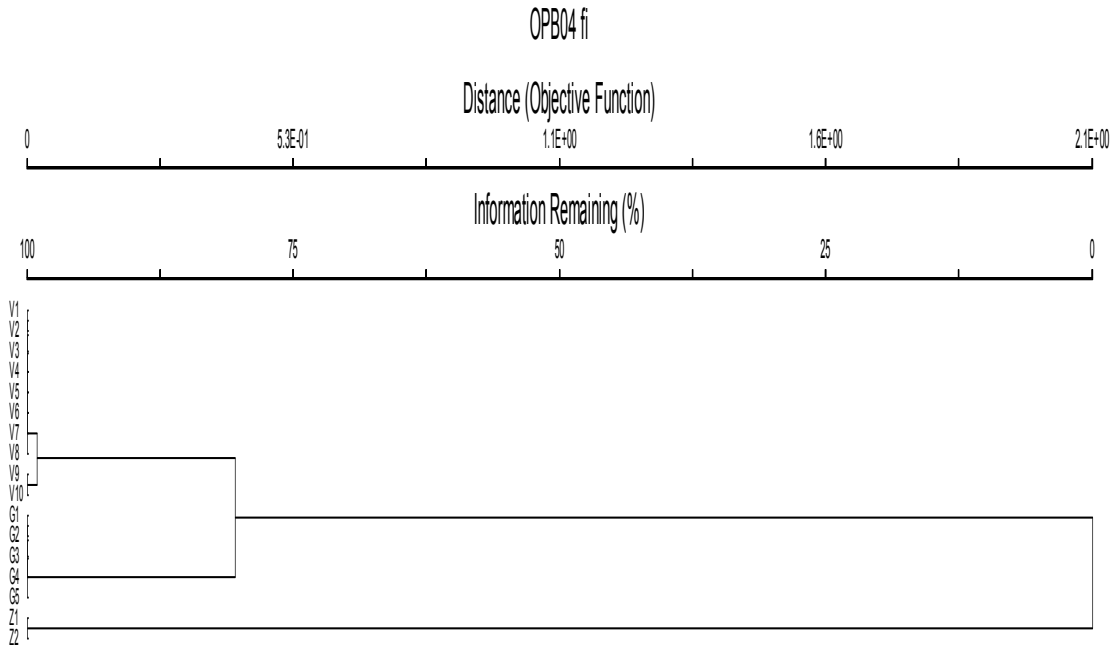


Fig. 5: Dendrogram created by UPGMA algorithm using Jaccard similarity coefficient on data from primer OPB-04 for 17 populations of *Chara* (V1-V10: 10 populations of *Chara vulgaris*, G1-G5: five populations of *C. gymnophylla* and Z1-Z2: two populations of *C. zeylanica*).

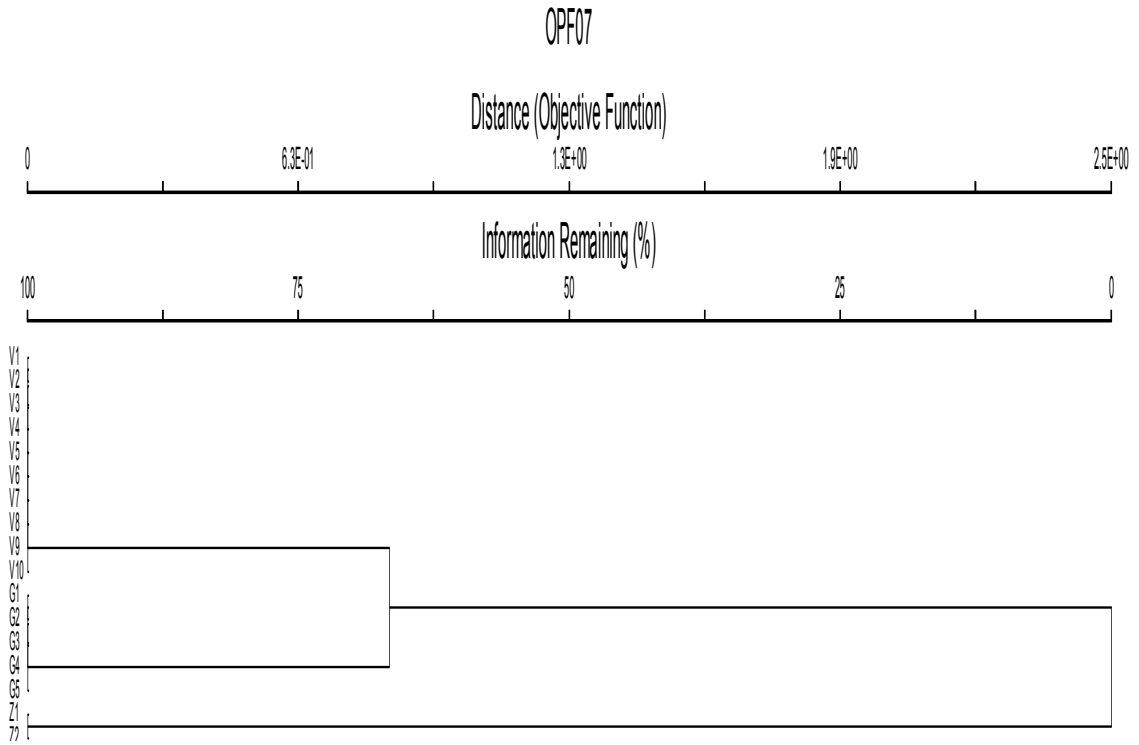


Fig. 6: Dendrogram created by UPGMA algorithm using Jaccard similarity coefficient on data from primer OPF-07 for 17 populations of *Chara* (V1-V10: 10 populations of *C. vulgaris*, G1-G5: five populations of *C. gymnophylla* and Z1-Z2: two populations of *C. zeylanica*).

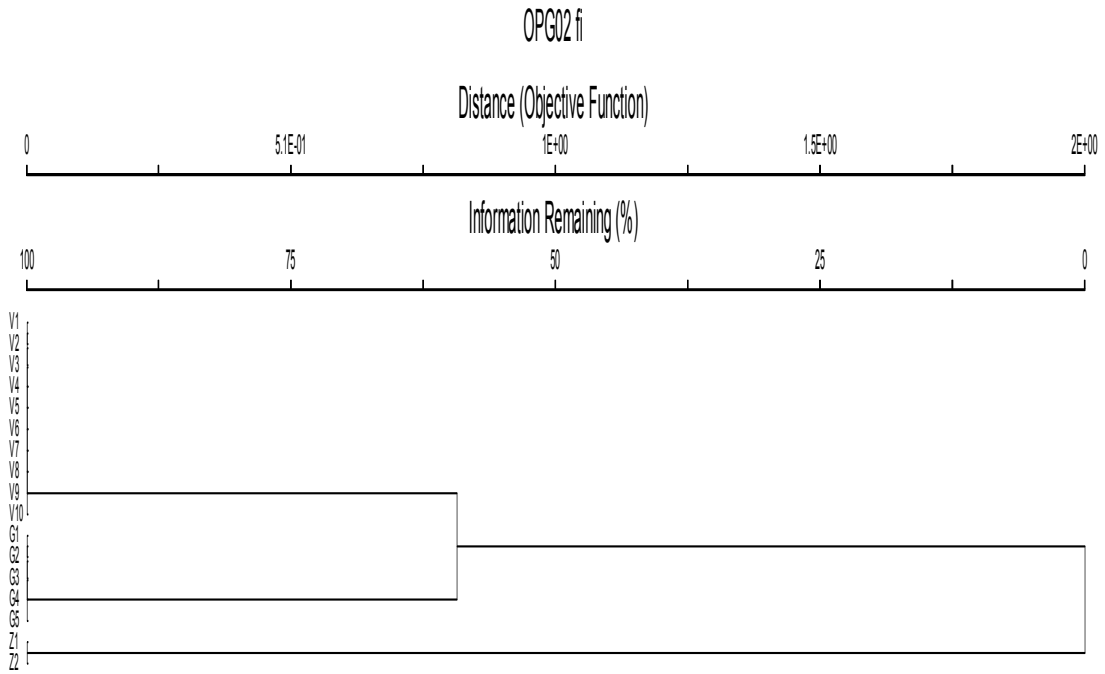


Fig. 7: Dendrogram created by UPGMA algorithm using Jaccard similarity coefficient on data from primer OPG-02 for 17 populations of *Chara* (V1-V10: 10 populations of *Chara vulgaris*, G1-G5: five populations of *C. gymnophylla* and Z1-Z2: two populations of *C. zeylanica*).

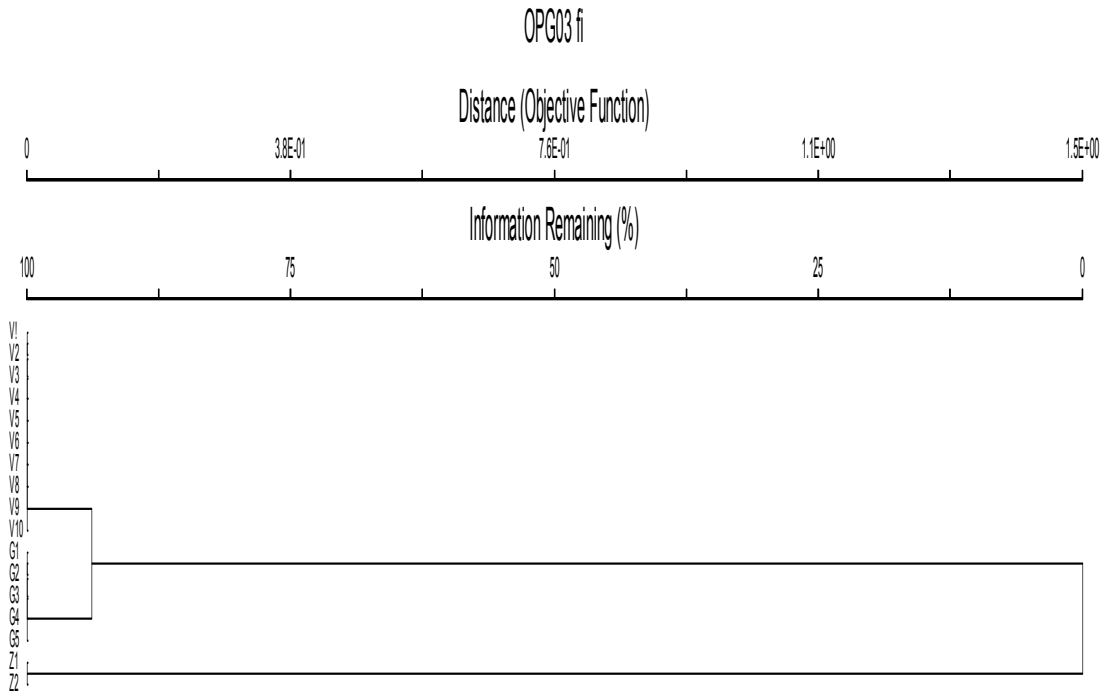


Fig. 8: Dendrogram created by UPGMA algorithm using Jaccard similarity coefficient on data from primer OPG-03 for 17 populations of *Chara* (10 populations of *Chara vulgaris*, 5 of *C. gymnophylla* and 2 populations of *C. zeylanica*).

species form a continuum. Kato *et al.,* (2010), carried out molecular phylogenetic analysis on *C. altaica* using sequence data from large subunit of Rubisco (*rbc*L).

A more comprehensive and fundamental systematic assay is needed to answer the questions about the diversity and evolution of this exceptional green alga and other charophytes in Iran. These assays could be based on a comprehensive collection of *Chara* species from Iran and testing other molecular marker systems such as AFLP, ISSR, or even sequencing.

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