Separation and the study of genetic diversity of a number of strains of *Dunaliella* microalgae using PCR-RFLP molecular marker Cleaved Amplified Polymorphic Sequence (CAPS).

Azam Panahpour*, Reza Mohammadi, Nader Chaparzadeh

Department of Biology, Azarbayjan University of Shahid Madani, Tabriz, Iran

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Abstract

Today, *Dunaliella* algae has received a lot of attention due to its high ability to produce beta-carotene, its applications in the food, pharmaceutical, cosmetic and research industries such as genetic engineering. Therefore, further identification of this genus and its productive species will be very useful. Common taxonomy of the *Donalilla* genus is usually based on its morphological and physiological traits, since these traits can change under different growth conditions due to the lack of a hard cell wall. For this reason, physiological and morphological studies have not been sufficient and have caused confusion in the systematic nature of this genus. Therefore, in this study, molecular markers were used to identify this species better and more accurately.

The present study conducted at the northwestern and western agricultural biotechnology research institute in Tabriz which contains a good collection of *Dunaliella* microalgae. In this experiment, the genetic diversity of a number of Iranian *Dunaliella* microalgae strains has been investigated using the PCR-RFLP (CAPS) molecular marker. After DNA extraction, the polymerase chain reaction was performed using a pair of protected primers in the ITS fragment, then enzymatic cutting was performed on PCR products of different strains using MseI, Bsp143I and NcoI restricted enzymes. By further examining the pattern of banding of different strains and the value of bands, the diversity of the ITS area of these strains was investigated. The results of the CAPS markers showed that the G45, Q1, Q41 strains and the standard *D. salina* sample (3.19) had 100% similarity. Given the role of ITS sequence in species identification, this can be said to be categorized in *D. salina* group.

Keywords: Dunaliella, Beta-carotene, Taxonomy, ITS fragment.

Introduction

The use of microalgae is increasing in the production of human food, animal feed, food additives, pharmaceuticals and chemicals. Algae breeding as a research tool is also expanding rapidly as modern research in recent years has shifted to various branches of science, including physiology, molecular biology and medicine. While chemicals isolates from microalgae have reached the level of economic production, there are a large number of microalgae that have not yet been studied. Among these algae, *Donalilla*, which has been known for about a hundred years, has been the subject of numerous studies in physiology, biochemistry, ecology and commercial applications. For more than a century since its official description, *Donalilla* has become an ideal model for studying the adaptability to salinity in algae. Adequate knowledge of the organic matter of the compatible solution to maintain osmotic balance is mainly based on the study of *Donalilla* species. In addition, the accumulation of beta-carotene masses under certain conditions has led to interesting applications in biotechnology by some breeds [1].

Due to the high production of beta-carotene and glycerol, *Donalila* algae has a high degree of adaptability to environmental osmotic changes. By cultivating the algae on a large scale, the high production capacity of beta-carotene in it has been well used. In case of nutrient deficiencies, high salinity and severe light, *Donalilla's* single-celled algae can produce about 14% of its beta-carotene dry weight.

Many microalgae have a high nutritional value due to their protein, vitamins, minerals and unsaturated fats. However, commercial use of this food source is limited for human and animal nutrition and the two microalgae, Spirulina and *Donalilla salina*, are grown as single-celled isolates and in large quantities. Natural Beta-carotene is a fat-soluble that makes it an effective anti-cancer agent, heart disease and a cholesterol-lowering agent [2].

Donalilla algae, due to its monocellular and haploid nature, lack of cell wall and phagocytic properties, has many advantages in the field of genetic engineering. There are many studies around the world about genetic engineering on the genus *Donalilla* and especially the Salina species. These include the transfer of some mutated genes for beta-carotene production and some genes involved in glycerol production and photosynthetic pathways [3].

The common taxonomy of the *Donalilla* genus is based on its morphological and physiological traits, since these traits can change under different growth conditions due to the lack of a

hard cell wall. For this reason, physiological and morphological studies have not been sufficient and have caused confusion in the systematic nature of this genus.

Donalilla species are among the most famous green algae that have undergone physiological and biochemical studies. Launched in 1966 as an industrial producer of beta-carotene, *Donalilla* algae have been the subject of much research to identify higher-yielding species and better production mechanisms. However, the taxonomy of this genus still needs extensive study and review. This is because classification based on physiological and morphological traits has led to errors of naming in the genus *Donalilla* and different naming of species of the same species in different collections, so that sometimes these strains do not differ in molecular identification and different strains are introduced by different researchers as a result of uncertainty in today's *Donatilla* system [4].

Using the pair of MA1-MA2 and MA1-MA3 initiators on *Donalilla parva* (LB1983), *Donalilla bardaville* (LB 2538), *Donalilla Tritiolecta* (LB 999) and *Donalilla salina* (LB 1644). It was well preserved among the *donald* species. He also concluded that the difference in the size of these bands was due to differences in the number of introns in the studied species. On the other hand, the products obtained with the help of the MA1-MA3 starting pair showed that the MA3 sequence is also well protected in this genus. The team went on to design and use proprietary initiators DSs to identify the species of *Donald salina*, DPs for *Donalia parva* and DBs for *Donalilla bardaville* [5].

In recent years, the use of available molecular markers has made it possible to identify some species. The use of molecular methods as well as classification based on common ancestors will be important for a better understanding of the classification, systematics and phylogeny of this genus. Molecular studies need to be conducted simultaneously with taxonomic studies based on morphology and physiology, not only because of the obvious link between past letters and species concepts, but because of the possibility of better and improved classification [6].

Types of species, according to the Linnean binary naming system, provide powerful tools for better communication and understanding of information when they are based on accurate taxonomic studies. DNA sequences, along with phylogenetic analyzes, allow classifications to better reflect evolutionary relationships. Specific DNA sequences (markers) can also be used to identify cultured species and area specimens. However, before a DNA sequence can be linked to a species, the species must be properly identified and described. Description and naming, in order to avoid confusion, should be done according to the accepted code of the list of words and specific scientific terms [7].

Molecular studies have given new impetus to the discussion of what constitutes a species. The ability and some of the challenges of morphological and molecular composition studies for microalgae classification have been well elucidated by several recent studies, such as the study of *Selenastraceae* and the term *Volvocalean chlorogonium*. Many authors have commented on the irregularity of names in literatures. However, this confusion cannot be resolved unless there is a systematic study of many species and strains using morphological and physiological characteristics in conjunction with more information provided by molecular biology. Also, giving the number to the studied items will help to transfer the information. The need for accurate recording of the strains used in each study because this genetic variation may be due to differences in physiological and biochemical characteristics [8].

In 2002, the simultaneous production and extraction of beta-carotene from living cells of *Donald salina* in a two-phase system, including a water phase and an organic phase. In general, a comparison of final beta-carotene production in the presence and absence of solvent showed that the presence of a second phase of soluble biological solvent in the culture medium could increase beta-carotene production [9].

Hejazi and his colleagues continued their study in 2004 with a new method for producing beta-carotene from *Donald salina*, in which cells were first grown at low light intensity and then transferred to high-intensity bioreactor. The results showed that a direct contact between cellular and organic solvent is an accelerator for faster production and extraction although it is not a requirement for beta-carotene extraction. In order to investigate the sustainability and therefore utility of the morphological and physiological features used in the diagnosis of the *Donalilla* species, cultured all their available isolates in a range of ecological councils and recognized changes in their pigment composition. They used this information to identify or re-identify strains [10].

Due to the uncertainty in the current systematic nature of the *Donalilla* genus and the apparent diversity of strains that have been proven by various researchers, it was decided to use genotypic traits, instead of phenotypic traits, in this study to solve this problem. In fact, molecular identification is used as a powerful tool for the detection of inter-species and intra-species microorganisms similar in appearance and in mixed populations. In addition, among the positions in the genome that are commonly used in taxonomic and systematic molecular studies of algae, we can point to the distances between nuclear ribosomal genes which are less protected during evolution. The genes encoding ribosomal RNA and ITS (Internal Transcriptional Spacer) are duplicated and consecutive throughout the genome and there are thousands of copies. Due to easy replication of small amounts of DNA (due to the large number of replicas of rRNA genes) and also due to the diversity even in closely related species, comparison of ITS sequence is greatly used in taxonomic and molecular phylogeny studies and in determining the diversity within and between species [11].

There are many molecular techniques that can be used to study genetic diversity. A simple and inexpensive way to detect ITS polymorphism is to use PCR-RFLP (CAPS) analysis. This method is widely used in the study of taxonomy, genetics and population biology. The PCR-RFLP method, also known as

CAPS (Cleaved Amplified Polymorphic Sequence), is a very common technique for genetic analysis. This technique has been used many times to investigate genetic variation within species as well as between species. It is also a suitable and common technique for genotypic studies. This technique detects SNPs, MNPs and microindel (adding or losing 50 nucleotides) through changes that create or eliminate the position of identifying restricted enzymes. The first step in this method of propagation is to have a variety of parts, after which the propagated parts are treated by shear enzymes. Since the presence or absence of the detection site of restricted enzymes leads to the formation of different cut-off parts, it is possible to isolate the electrophoresis of the cut-off parts with different sizes of allele identification [12].

In this study, PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) marker was used to evaluate the genetic diversity and better classification of Iranian *Donalila* genus in the algae collection of agricultural biotechnology research institute in the northwest and west of the country.

Materials and Methods

The study was conducted using the *Donalilla* algae collection at the northwestern and west agricultural biotechnology research institute. This unique collection includes samples of algae isolated from Iran's aquatic lakes, including Urmia, Maharloo, Qom and Gavkhoni swamps. The algae collection in this research institute includes isolates of new strains from Algae called *Donalilla*.

Johnson's modified culture medium was used for culture in the 1.5 M NaCl medium (once every 14 days). Due to the lack of organic matter in it, the Donalila culture medium usually becomes clear to secondary contamination after several reactions. After repeated reactions, DNA was extracted from the samples. At this stage, the modified CTAB extraction method was used.

Steps to perform PCR-RFLP reaction

In this method, ITSF and ITSR primers were used to propagate the ITS gene. The primers, which were frozen dry, were double-sterilized to 100 µmol with distilled water and then diluted in another microtube to a concentration of 1 µmol. These microtubules were stored at -20°C and melted only during use and quickly transferred back to the freezer (Table 1) [13].

Initiator	Nucleotide sequence	The length of the multiplied piece
ITS F	AATCTATCAATAACCAC ACCG	Bp 710
ITS R	TTTCATTCGCCATTACT AAGG	

Table 1. *Name and sequence of ITS initiators.*

Optimizing PCR conditions

Signage PCR kit was used for polymerase chain reaction. The base solution of this kit contained all the materials needed for the reaction except the initiators and the sample DNA. These substances contained in the nucleotide deoxyphosphate (dATP, dCTP, dGTP and dTTP), magnesium chloride, PCR buffer and DNA polymerase tag enzyme. Each reaction was performed at a volume of 25 µl [14].

Initially, 1 microliters of DNA from each sample were poured into PCR-specific microtubules and transferred to the refrigerator. Immediately, 24 microliters of the base solution mixture and the primer pair were added to each microbe containing DNA. The thermocoupler was used for propagation. According to the program given to the device, the polymerase chain reaction was performed using the desired pair of primers [15].

In the last heat cycle and after the final expansion stage, the thermocycler temperature was set at 40°C for 24 h, which maintained the PCR product. With the completion of the final phase, expansion of electrophoresis of PCR products were performed. If necessary, the PCR product was stored at -20°C for several days to be electrophoresed at the appropriate time (Tables 2 and 3).

Material	The final concentration is 25 µl	The amount of material in the volume of 25 microns
Water twice distilled sterilized	_	µl 9/5
PCR base solution	X1	µl 12/5
Forward initiator	40 ng	1 µl
Backward initiator	40 ng	1 µl
DNA pattern	20 ng	1 µl
Final volume	_	25 µl

Table 2. *Materials used in the preparation of the mixture for polymerase chain reaction.*

Number of cycles	Stages	Time	Temperature (ºC)
1	First return	5 min	95
35	Second return	1 min	94
	Connection	50 min	57
	Development	min	72
1	Final development	10 min	72

Table 3. *Time and temperature required for different stages of PCR in PCR-RFLP technique.*

Results

Determination of DNA quality extracted from samples

DNA samples were first loaded in agarose gel 1% to confirm the correct extraction procedure. The observed bands showed good DNA extraction in the samples (Figure 1) [16].

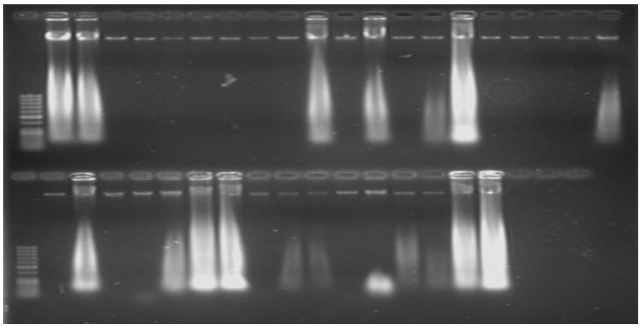

Figure 1. *Genomic DNA electrophoresis on 1% agarose gel. The first wells in each row are related to DNA Ladder 50 bp and the rest of the wells (36) are related to the genomic DNA of the samples studied in this study.*

Genetic diversity observed based on PCR-RFLP technique

The polymerase chain reaction for DNA samples extracted from the *Donalilla* strains (36-strains) at the northwestern and west agricultural biotechnology research institute, was performed using ITS-initiated primers of the *Donalilla* genus, called ITSF and ITSR primers and the band pattern was observed on about 700 pairs of agarose gels (Figure 2) [17].

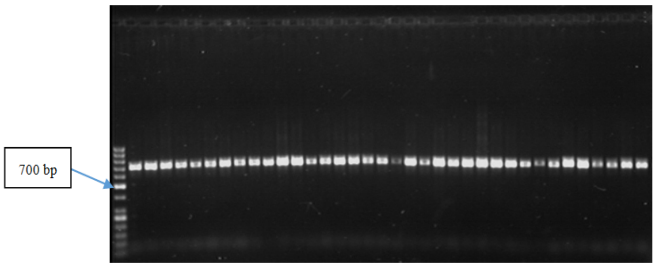

Figure 2. *The first well for the Ladder 50 bp, the wells 2 to 37 for the PCR products of the strains of different regions, multiplied by the initials of the Donalilla its identifier.*

PCR-RFLP polymerase chain reaction results

ITS refers to the part of the non-functional RNA located on the primary transcript between functional ribosomal RNAs (rRNA) called from '5' to '3'. Primary and polycystic rRNA transcripts include Externally Transcribed Sequences from (ETS)5', 18s rRNA, ITS1, 5/8 s rRNA, ITS2, 28s rRNA and finally ETS at the end of 3'. During puberty, the initial rRNA transcripts of the ITS and ETS sequences are removed and later destroyed as immature mature products. Ribosomal RNA encoding genes and spacers are duplicated and sequentially located on genomic DNA, thousands of copies of which are on the genome which has been isolated by non-transcriptional DNA regions called IGS and NTS internal non-transcriptional spacers [18].

Comparison of ITS sequencing, due to easy proliferation even of small amounts of DNA (due to high number of copies of rRNA genes) as well as the high diversity seen in this region even in closely related species, is largely used in taxonomic and molecular phylogeny studies. In this study, PCR-RFLP technique was used to study the diversity of ITS region of *Donalilla* strains since the sequence of strains is not available and considering that the diversity of this region between strains is about some nucleotides and the study of this level of variety in Agarose gel is impossible [19].

ITS initiators are designed at the beginning and the end of the ITS1+5/8 s rDNA+ITS2 sequence of *Donalilla*. This sequence has about 700 base pairs and the existence of 700 bands of base pairs in all samples indicates that this sequence has been maintained and exists in all samples. These initiatives were well reproduced in all specimens, indicating that their connection between the *Donalilla* species has been well preserved. These initiatives reproduced the full ITS component in all of these areas [20].

Enzymatic cutting

After observing the 700-pair base bands related to the ITS piece on all sides, PCR-RFLP sequence was performed on PCR products in order to more accurately identify the strains, using effective shear enzymes. Gene Doc software has been used to find the effective shear enzymes that affect the ITS sequence of the *Donalilla* algae. Using this software, the effect of a number of shear enzymes on ITS sequences of a number of Iranian strains of *Donalilla* algae registered in the NCBI gene bank was investigated and effective enzymes were identified. Using the results of this software, 3 restricted enzymes were selected that had a visible and different cutting position (on the gel) on the ITS sequences. Selected enzymes include Bsp143I, NcoI and MseI, which showed a shear pattern similar to the software results. Enzymatic treatment was performed after PCR and replication of ITS fragment belonging to 36 Donalila strains belonging to Chichest, Sharafkhaneh, Qom, Gavkhoni and Maharloo areas. PCR products were treated for 12 hours in the specific conditions of each enzyme, in terms of temperature and enzymatic buffer and the results below were obtained (Figures 3-5).

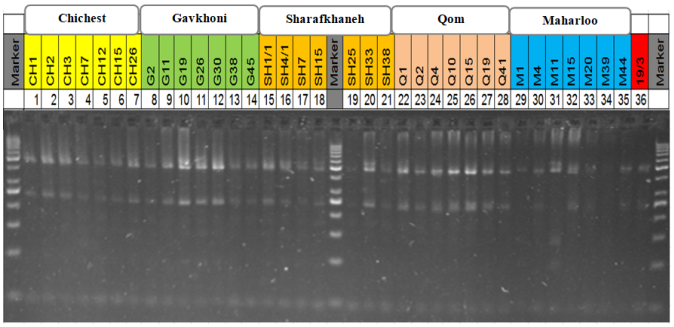

Figure 3. *Gel for digestion of ITS part (PCR product 36 of Donalila strains) by enzyme Bsp143I, Ladder 50 bp.*

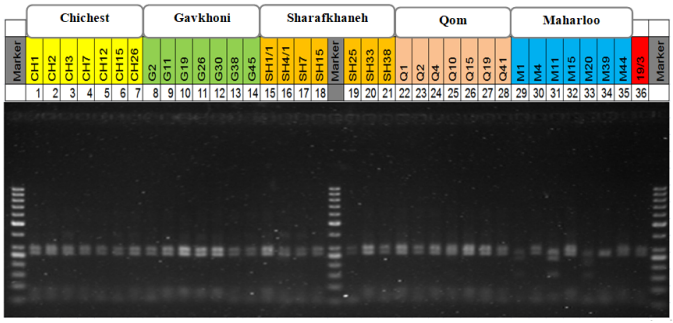

Figure 4. *Gel for digestion of ITS part (PCR product 36 of Donalila strains) by MseI enzyme, Ladder 50 bp.*

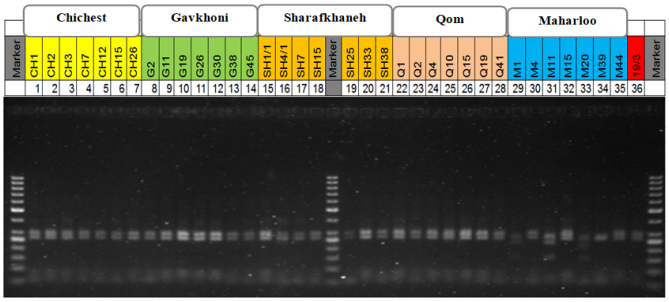

Figure 5. *Gel for digestion of ITS fragment (PCR product 36 of Donalila strains) by NcoI enzyme, Ladder 50 bp.*

The comparison of the results from the software and the photos of the gels shows that the enzymes Bsp143I, NcoI and MseI showed the same shear pattern as the software.

Evaluation of reproductive strips (tapes) and cluster analysis

Valuation of the resulting tapes was done through double valuation. The value of 1 was applied to the existence of the tape and the value of zero was applied to the absence of the tape. The data were statistically analyzed using NTSYSpc 2.02 software. Dendrogram of individuals' genetic association was obtained using the UPGMA classification method and based on Simple Matching similarity coefficient due to the appropriateness of this similarity coefficient for the dominant markers (Figure 6).

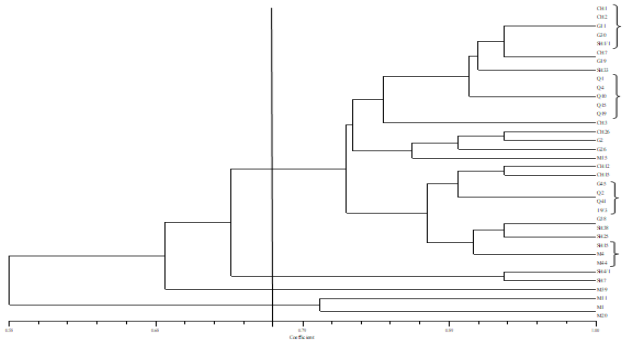

Figure 6. *Dendrogram of the data of 3 enzyme-restricted enzymes in PCR-RFLP technique using UPGMA classification method and Simple Matching similarity coefficient.*

The dendrogram obtained from the data, categorized the study population into four general groups. The first group includes strains CH1, CH2, G11, G30, SH1/1, SH33, Q1, Q4, Q10, Q15, Q19, CH3, CH7, G19, G2, G26, CH12, CH15, G38, SH38, G45, Q2, Q41, 19/3, SH25, SH26, M15, SH15 and M4. The second group includes SH7 and SH4/1 strains, both of which belong to Urmia Lake (Sharafkhaneh Port). The third group includes only the M39 strait, which belongs to Maharloo Lake. The fourth group includes the three sides of M1, M11 and M20, all three of which belong to Maharloo Lake. Placing the strains of an area in a group indicates the existence of genetic homogeneity. The fourth group is somewhat heterogeneous compared to the previous two groups and shows the sides of different regions together. It seems that more and stronger indicators should be used for more accurate segregation and classification of this group. The strains of an area are always genetically related and there is no unbreakable boundary between them. However, determining more precise communication between the strains requires more powerful indicators [21].

G45, Q1, Q41 and standard *D. salina* (3.19) strains are 100% similar. Due to the role of ITS sequence in species identification, these specimens can also be classified as D. Salina. On the other hand, the sequence of M1, M11 and M20 strains has the longest distance and the least similarity to then its sequence of 19.3 strain. Based on the results, it can be considered that these samples are not classified as *D. salina* (Figure 7).

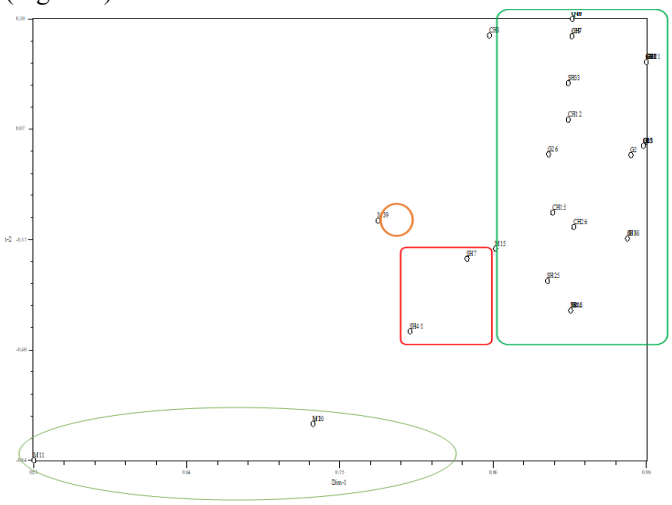

Figure 7. *Two-dimensional pattern of distribution of the studied samples based on two main factors resulting from PCR-RFLP indicator data analysis.*

As can be seen in the scatter plot, the M1, M11 and M20 strains are closely placed in one group and the M39 is a single-strain creating a group with a relatively small distance to the fourth group and the distribution of other aspects has itself been an emphasis on the classification.

Upon further examination of the dendrogram and data from the PCR-RFLP technique, it is observed that the strains belonging to each region have many similarities, which is a sign of the great similarity of the ITS of the strains of a region. Of course, the situation is somewhat different between the strains of the regions of Maharloo. According to the dendrogram information, there is a perfect similarity between M4 and M44 strains and also between M1 and M20 strains [22].

However, there are few similarities between the M1, M11 and M20 strains with other strains in the Maharloo region, indicating the existence of an intra-species diversity in the Maharloo region.

Discussion

Donalila is a single-celled green algae that is responsible for the production of raw materials in super-saturated environments around the world. *Donalilla* is a saline green single-celled alga that lacks a strong cell wall and the shape of the cell is completely variable due to the absence of a hard cell wall. Cell size can vary from 2 micrometers to 24 micrometers with different culture media.

The production of high amounts of glycerol and beta-carotene by this microalga has provided the motivation and driving force for further use of this micro-algae. Solar energy can be trapped with the help of this algae and some biochemical products with important industrial uses, can be produced. In recent years, the cultivation of this algae have been increased due to its carotenoid production.

DNA-based molecular methods and techniques have provided new possibilities for obtaining information about the genetic diversity of organisms. The information obtained through such studies is very important and necessary in many areas of biology. The genetic diversity of 36 Iranian *Donalila* species was investigated using the CAPS marker. Using the CAPS technique, the ITS diversity of each region was investigated that the strains of each region were more similar, except for the strains of the Maharloo region that, in addition to being very different from the strains of other regions, also they had a diversity within the population of this area. M1, M11 and M20 strains differed most from the other strains in the ITS sequence [23].

Examination of data from RAPD markers shows that the strains belonging to one area still bear the most resemblance. Of course, sometimes there are similarities between the strains of different regions, which can be justified to some extent due to the proximity of the regions and the exchange of gene pool between these strains, but having similarities is surprising in the case of strains of distant regions and these need to be further explored with more powerful indicators.

Conclusion

On the other hand, it was observed that the situation is a little different in Maharloo area. The M1 and M20 strains of the Maharloo region in the ITS region are one hundred percent similar and fall into the same category using the PCR-RFLP technique. The results from the RAPD indicators show that M20 strain of Maharloo region has the greatest difference and the least similarity with other strains of the studied population, even the strains of the Maharloo region. The results obtained from the technical data confirm the existence of an intra-species diversity among the studied samples of Maharloo region, which indicates a completely different evolutionary path of the strains of this region with the strains of other regions. In fact, at the beginning of the earth's formation, when the earth's surface was covered with water, Maharloo Lake was part of the Persian Gulf, but Lake Urmia, Qom and Gavkhoni were part of the Caspian Sea, which modern lakes have emerged over the years with decreasing rainfall and drought. Today, according to the different origins of these lakes and wetlands, different evolutionary routes of these microalgae can be justifiable.

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*Correspondence to

Dr. Azam Panahpour

Department of Biology

Azarbayjan University of Shahid madani

Tabriz

Iran

Email: azampanahpour1@gmail.com