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Study of Genetic Variation in Three Species of Hypnea J.V. Lamouroux (Cystocloniaceae, Gigartinales) Species Using ISSR Markers

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A B S T R A C T

Hypnea species were found in southern coasts of Iran. This genus is an important red alga which comprises about 53 species worldwide and has a wide geographical distribution on the tropical shores around the world. In Iran, about 10 species of this genus have been reported from the subtidal zone of the Persian Gulf and Gulf of Oman coasts. The present study considers the assessment of genetic diversity of 10 populations of 3 species of Hypnea by using 6 ISSR primers. Genetic diversity parameters were determined among populations. The genetic divergence of the studied populations was checked by Neighbor-Joining (NJ) and Principal component analysis (PCA). Genetic differentiation of the studied species and populations was studied by AMOVA (Analysis of molecular variance) test. The Mantel test was performed to study the association between molecular distance and geographical distance of the studied populations. Grouping of the populations by NJ clustering separated the studied species in 2 distinct clusters. The most populations of H. musciformis formed a separate cluster and were placed far from the other species. H. ecklonii and H. cornuta showed some degree of relationship and were placed close to each other. AMOVA test showed significant genetic difference among populations. The Mantel test did not show a correlation between the genetic distance and geographical distance of these populations

Introduction

The genus *Hypnea* J.V. Lamouroux (1813) is an important red alga which comprises about 53 species worldwide (Guiry & al. 2006). *Hypnea* has a wide geographical distribution on the tropical shores around the world (Rodrigues Guimares 2011). Some species of *Hypnea* are used for the production of carrageenan (Mshigeni & Chapman 1994).

In Iran, the species of *Hypnea* were the most common species of red algae in the Persian Gulf and Oman sea coasts that were found in the subtidal zone of this area during late autumn to late spring but these species had a significant decrease in diversity and biomass in Iranian coasts in recent years.

Hypnea is characterized by uniaxial upright fronds composed of a distinct axial filament surrounded by a pseudo parenchymatous cellular medulla and a cortex. The life history is of the Polysiphonia type in which isomorphic, dioecious gametophytes and tetrasporophytes occur and a diploid carposporophyte develops on the female gametophyte. Spermatangia are produced in slightly or conspicuously swollen parts of terminal branchlets or proliferation or both and are cut off from outermost cortical cells in chains. Carpogonial branches are three-celled, are formed laterally on inner cortical cells, and are directed outward.

The taxonomy of the genus is very problematic and Hypnea species identification is complicated by a relatively simple morphology that is often influenced by the conditions of the habitat (Rodrigues Guimares 2011). For example, there are species such as *H. charoides* and *H. valentiae* which are almost similar to each other. Some introduced these species as complex *charoides-valentiae* (Yamagishi& Masuda, 1997; Lewmanomont, 1997)

Since the genus has a wide pantropical distribution and considerable morphological variation, there is a need for critical reassessment of species delimitation using extensive preserved collections, including those collected from the type localities (Masuda et al. 1997).

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In Iran, 10 species of *Hypnea* have been reported (Gharanjik & Rohani 2009, John & Al-Thani 2014). We collected 3 species of this genus from the Persian Gulf and Gulf of Oman coasts namely, *H. musciformis* (Wulfen) J. V. Lamouroux, *H. cornuta* (Kuetzing) J.Agardh and *H. ecklonii* Suhr.

A number of molecular markers have been used in red algal taxonomy including psaA, rbcL and cox1 sequences (Yang & Boo 2004; Freshwater et al. 1994; Saunders 2005; Robba et al. 2006), but inter-simple sequence repeats (ISSR) have not been used. The present study reports ISSR analysis of these algae specimens collected from 9 geographical regions of southern coasts of Iran for the first time. Genetic structure of the studied populations and the gene flow among them is also studied.

Material and methods

1. Subjects

Ten populations of 3 *Hypnea* species were analyzed by ISSR markers in the present investigation. These populations were collected from 9 localities in the Persian Gulf and Gulf of Oman coasts (Table 1 & Figs. 1).

Table 1- Populations of *Hypnea* and their localities

populations	Locality	Longitude	Latitude
H. ecklonii 2, H. musciformis 2	Qeshm: Shibderaz (Persian Gulf)	55º 55' 38.9" E	26º 41' 7.78" N
H. cornuta 1	Qeshm: Shahrdari (Persian Gulf)	56º 16' 41.77" E	26º 57' 45.2" N
H. ecklonii 1	Qeshm: Zeyton park (Persian Gulf)	56º 16' 9.34" E	26º 55' 55.2" N
H. cornuta 2	Bandar Lenge: Dowlat park (Persian Gulf)	54º51'37.2" E	26º32'4.58" N
H. musciformis 1	Bandar Abbas: Dowlat park (Persian Gulf)	56º 20'40.07" E	27º 11' 15.28" N
H. cornuta 3	Kong (Persian Gulf)	54º 57'11.95" E	26º 36' 4.77" N
H. musciformis 3	Tis (Gulf of Oman)	60º 38' 52"E	25º 16' 57"N
H. musciformis 4	Remin (Gulf of Oman)	60º 45' 6" E	25º 14' 8" N
H. musciformis 5	Kachu (Gulf of Oman)	60º 50' 51"E	25º 14' 32"N

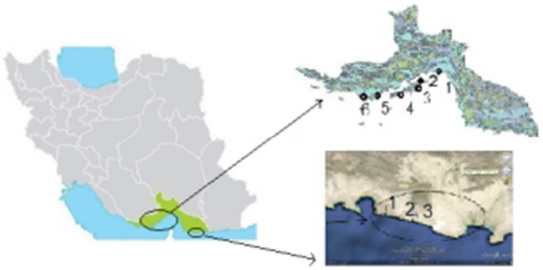

Fig. 1- Distribution map and localities of *Hypnea* populations studied from the Persian Gulf. Locality codes:1- Bandar Abbas, Dowlat park; 2- Qeshm, municipality; 3- Qeshm, Zeyton park; 4- Qeshm, Shibderaz; 5- Kong; 6-Bandar Lenge, Dowlat park. Gulf of Oman: Locality codes: 1- Tis; 2- Remin; 3- Kachu Distribution map and localities of *Hypnea* populations studied from Oman Sea. Locality codes: 1- Tis; 2- Remin; 3- Kachu

Samples from the field were transported live back to the laboratory in sterilized seawater, cleaned and sorted carefully under a dissecting microscope. Thalli were preserved in silica gel desiccant for DNA extraction. Materials for morphological observations were preserved in 4% formaldehyde-seawater.

2. DNA extraction and PCR

DNA was extracted from the thalli and dried in silica gel powder. The genomic DNA was extracted using CTAB-activated charcoal protocol (Krizman & al., 2006). The extraction procedure was based on activated charcoal and Polyvinyl Pyrrolidone (PVP) for binding of polyphenolics during extraction and on mild extraction and precipitation conditions. This promoted high-molecular-weight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 2% agarose gel.

Six ISSR primers were used in this study-namely-UBC810, 811, 834 designed by University of British Colombia and (GA)9T, (GA)9A, (GA)9C. Minimum of 6 randomly selected algae from each population was used for obtaining ISSR data.

PCR reactions were performed in a 23 µl volume containing 18.25 mM H_2o; 2.5 mM Tris- HCl buffer at pH 8; 0.35 mM $MgCl_2$; 0.5 mM of each dNTP (Bioron, Germany); 1 µM of a single primer; 20 ng genomic DNA and 0.4 mM of TaqDNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 45 s at 52°C and 2 min at 72°C. The reaction was completed by a final extension step of 10 min at 72°C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 1kb molecular size ladder (Fermentas, Germany).

3. Data analyses

The ISSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0).

Genetic diversity parameters were determined in each population. These parameters were Nei's

genetic diversity (He), Shannon information index (I) (Weising 2005, Freeland & al., 2011), number of effective alleles and percentage of polymorphism and were performed in GenAlex 6.4 (Peakall&Smouse, 2006).

The genetic divergence of the studied populations was checked by the Neighbor-Joining (NJ) tree (PAST ver. 2.17, Hamer et al., and 2012). Principal component analysis (PCA) was used for these analyses and was conducted with GenAlex 6.4.

Genetic differentiation of the studied species and populations was studied by AMOVA (Analysis of molecular variance) test (with 1000 permutations) as performed in GenAlex 6.4 (Peakall & Smouse, 2006) and Hickory (ver. 1.0) (Holsinger & al., 2003), a Bayesian program that calculates the θ B value (Holsinger & al., 2003). The Mantel test (Podani, 2000) was performed to study the association between molecular distance and geographical distance of the studied populations.

Results

The primers produced 45 polymorphic and reproducible bands. GA (9T) primer produced the highest number of bands (13 bands). The highest number of specific bands were produced with UBC-834 primers. Specific bands were seen in Some of the populations for example, *H. cornuta* showed highest number of specific bands with a single specific band of the UBC-834 (1200 kb molecular weight), UBC-811(1000 kb and 1200 kb molecular weight), GA (9C) (1000 kb molecular weight), and GA (9T) (350 kb molecular weight). The highest number of total bands was observed in Qeshm-Shahrdari population of *H. cornuta* with 32 bands.

Genetic diversity parameters of populations are provided in Table 2. The number of effective alleles ranged from 1.027 in Lenge-Dowlat Park population of *H. cornuta* to 1.242 in Qeshm-Zeyton Park population of *H. eklonii*. Shannon index varied from 0.023 in Lenge-Dowlat Park population of *H. cornuta* to 0.202 in Qeshm-Zeyton Park population of *H.eklonii*. The highest value of gene diversity occurred in Lenge-Dowlat Park population of *H. cornuta*, was 0.153.

Table 2- Genetic diversity parameters of *Hypnea* populations

Populations	Na	Ne	I	He	UHe	%P
*H.e*1,Qesm, Park Zeyton	0.933	1.242	0.202	0.137	0.153	35.56
*H.e*2,Qeshm, Shibderaz	0.756	1.157	0.142	0.094	0.104	26.67
*H.c*1,Qeshm, Shahrdari	0.911	1.134	0.110	0.075	0.083	20.00
*H.c*2, Bandar Lengeh, Dolat park	0.733	1.027	0.023	0.015	0.017	4.44
*H.c*3, Kong	0.844	1.094	0.077	0.053	0.059	13.33
*H.m*1, Bandar Abass, Dolat park	0.578	1.082	0.065	0.045	0.050	11.11
*H.m*2, Qeshm, Shibderaz	0.667	1.066	0.064	0.041	0.046	13.33
*H.m*3,Tis	0.578	1.066	0.057	0.038	0.042	11.11
*H.m*4, Remin	0.556	1.066	0.038	0.038	0.042	11.11
*H.m*5, Kachu	0.578	1.077	0.064	0.044	0.048	11.11

Na: No. of different alleles; Ne: No. of effective alleles; P%: percentage of polymorphism; uHe = Unbiased gene diversity; I: Shannon index; He: gene diversity; P%: Polymorphic Information.

Grouping of the populations based on Nei's genetic distance by NJ tree produced the following results (Figs. 2). In general two major clusters were formed and the studied species were placed almost in distinct clusters/groups. *H. cornuta* and *H. ecklonii* formed the first main cluster, while *H. musciformis* is the second main cluster. In the first main cluster, *H. cornuta* and *H. ecklonii* were placed in two separate sub clusters. Of course, it is noteworthy that 4 individuals of *H. musciformis* are located in the first cluster, but below the separate sub cluster and next to other individuals of *H. musciformis.*

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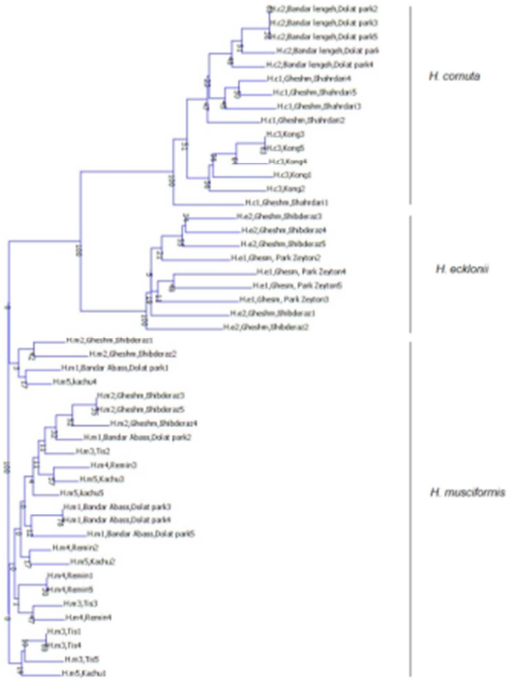

Fig. 2- NJ tree showing the genetic affinity of the studied *Hypnea* species.

In the PCA plot, these 3 species are well separated (Fig. 3).

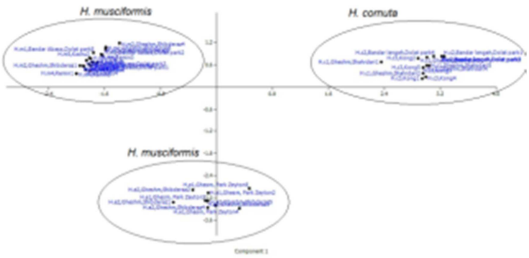

Fig. 3- PCA plot of Hypnea species

AMOVA test produced significant genetic difference among the studied species (PhiPT value = 0.763, P = 0.01). The test also revealed that 76% of total variation is attributed to among-population differences and 24% due to within-population variation. However inter-population differences are higher than intra-population differences. Therefore, these results revealed the genetic distinctness of the studied species (figure 4).

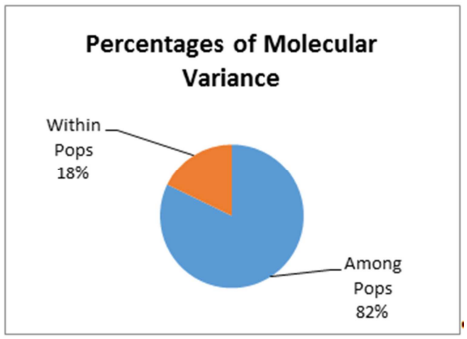

Fig. 4- Results of Analysis of Molecular Variance

T6he Mantel test was not significant and geographical distance didn't impact on genetic diversity (R2 = 0.035, P = 0.20).

Discussion

Many phylogenetic studies have been done about *Hypnea* around the world but the study of the diversity of these 3 species has never been done. The study of three other species of this Genus was performed previously (Sargazi & Riahi, 2018). The results of this study are almost in agreement with the other phylogenic studies. Some scientists explore phylogeny within the genus; they studied on rbcL, cox1 and psaA sequences (Geraldino et al. 2006, 2010; Rodrigues 2011; Freshwater et al. 1994; Robba et al. 2006, …). These 3 species show similarities because of being in one section. Agard (1852) divided this genus into three sections on the basis of their habits: the first known as spinuligerae including *H. charoides*, *H. valentiae*, *H. ecklonii*, *H. musciformis* and *H. cornuta*. The second section was called virgatae including *H. boergesenii*. Pulvinatae was the last section including *H. pannosa*. (Just listed 7 collected species from Iran). As you can see, these 3 species are in the same section due to the same habit. These algae with developed creeping branches are conspicuously entangled at the basal part and are called entangling (intricate-caespitose) tufts because each alga has several axea. In most phylogenetic studies, only *H. musciformis* and *H. cornuta* have been studied and are located together in one clade in the phylogenetic trees (Geraldino et al. 2006, 2010; Rodrigues 2011). Most studies on *H. ecklonii* are morphology and in phylogenetic studies, there are not many studies (Abbot, 1995; Abbot et al. 1976; Hewitt, 1960; Masuda et al. 1997; Mshigeni, 1978, ...). As we expected, the populations of these 3 species to be well separated in subclusters. In addition, these species are put together in the same cluster due to a series of similarities.

The presence of ISSR polymorphic bands in the populations of *Hypnea* indicates the presence of a genetic polymorphism in these populations.

However, we observed some private ISSR bands in *H. cornuta* that were not common with the other studied species. Also, the highest diversity was observed in *H. ecklonii*. The lowest variation was observed in *H. cornuta* populations (Based on diversity parameters). The reason for this diversity can be related to reproduction type in these species. Of course, in sampling in the various seasons and years, no algae with sexual reproduction were sampled.

In NJ tree, all species were located in separate clusters and subclusters but populations were spread in clusters and subclusters and were not well separated. In the PCA plot, these 3 species are well separated. So that even the Persian Gulf and Gulf of Oman populations did not show much difference according to ISSR marker genes. of course, this result is in agreement with the Mantel test that geographical distance didn't impact on genetic diversity.

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مطالعه تنوع ژنتیکی در سه گونه از جنس (Rhodophyta, Gigartinales) Hypnea با استفاده از مارکرهای ISSR

فاطمه سرگزی[1*]

چکیده

گونه‌های هیپنهآ در سواحل جنوبی ایران یافت می شوند. این جنس شامل حدود ۵۳ گونه می‌باشد که توزیع جغرافیایی وسیعی در سواحل گرمسیری سراسر جهان دارد. در ایران حدود ۱۰ گونه از این جنس از مناطق زیر جذرومدی سواحل دریای عمان و خلیج فارس گزارش شده است. مطالعه حاضر به بررسی تنوع ژنتیکی ۱۰ جمعیت از ۳ گونه هیپنهآ با استفاده از ۶ پرایمر ISSR می‌پردازد. پارامترهای تنوع ژنتیکی در بین جمعیت‌ها تعیین شد. واگرایی ژنتیکی جمعیت‌های مورد مطالعه توسط روش اتصال به نزدیک‌ترین همسایه (NJ) و تجزیه به اجزای اصلی (PCA) بررسی شد. تمایز ژنتیکی گونه ها و جمعیت‌های مورد مطالعه توسط AMOVA (آنالیز واریانس مولکولی) مورد بررسی قرار گرفت. تست مانتل برای بررسی رابطه بین فاصله ملکولی و فاصله جغرافیایی جمعیت‌های مورد مطالعه انجام شد. گروه‌بندی جمعیت‌ها توسط روش خوشه بندی NJ گونه‌های مورد مطالعه را در دو خوشه جدا قرار داد. بیشتر جمعیت‌های H. musciformis یک خوشه جداگانه را تشکیل دادند و دور از ۲ گونه دیگر قرار گرفتند. H. ecklonii و H. cornuta تفاوت ژنتیکی معناداری را در بین جمعیت‌هایشان نشان دادند و نزدیک به همدیگر قرار گرفتند. روش AMOVA تفاوت ژنتیکی معناداری را بین جمعیت‌ها نشان داد. روش Mantel همبستگی بین فاصله جغرافیایی و ژنتیکی را در این جمعیت‌ها نشان نداد.

واژگان کلیدی: ISSR، سواحل جنوبی، تست مانتل، تنوع ژنتیکی، هیپنهآ.

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