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INTRA-SPECIFIC DIVERSITY IN HYPNEA SPECIES (RHODOPHYTA, GIGARTINALES)

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The present study considers assessment of morphometry and genetic diversity of 24 populations of *Hypnea*. Statistical analyses indicated that characters such as algal branching in habit, size of alga and Position of tetrasporange sorus had the most important role in intera-specific variation. In both morphometry and genetic diversity analysis, grouping of the populations separated the studied species into four distinct clusters and groups but populations were spread in clusters.
Analysis revealed significant genetic difference among populations and some degree of genetic admixture and gene exchange among the studied populations.

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Key words: *Hypnea*; morphometry; genetic diversity; habit; tetrasporange; populations

مطالعه تنوع درون گونه ای در گونه های Hypnea (Rhodophyta, Gigartinales)

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مطالعه حاضر به بررسی مورفومتری و تنوع ژنتیکی ۲۴ جمعیت از Hypnea می‌پردازد. تجزیه و تحلیل‌های آماری نشان داد که صفاتی مانند روش پیشروی انشعابات در زیستگاه، اندازه و موقعیت تتراسپورانژها نقش مهمی در تنوع درون گونه ای داشته است. در هر دو تجزیه و تحلیل‌های مورفومتری و تنوع ژنتیکی، در گروه‌بندی جمعیت‌ها، گونه‌ها به خوبی از هم جدا شده‌اند اما جمعیت‌ها به خوبی از هم جدا نشده‌اند. تجزیه و تحلیل‌ها تنوع ژنتیکی و برخی درجات آمیختگی و تبادل ژنتیکی را بین جمعیت‌های مورد مطالعه نشان دادند.

INTRODUCTION

The genus *Hypnea* J.V. Lamouroux (1813) is an important red alga which has a wide geographical distribution on the tropical shores around the world (Rodrigues 2011). Some species of *Hypnea* are used for the production of carrageenan (Mshigeni & Chapman 1994). The genus comprises about 53 species world-wide (Guiry & al. 2006). In Iran, there are 9 species of *Hypnea* as listed in " Atlas of the sea algae of Persian Gulf and Oman sea coasts" (Gharanjik & Rohani-Ghadikolai 2009) of which, we collected 7 species from Persian Gulf and Oman sea coasts (these species listed in the table 1). In Iran, these species are the most common species of red algae in Persian Gulf and Oman sea coasts that are found in subtidal zone of this area during late autumn to late spring but these species have a significant decrease in diversity and biomass in Iranian coasts in recent years (Sargazi & al. 2016).

Hypnea is characterized by uniaxial upright fronds composed of a distinct axial filament surrounded by a pseudoparenchymatous 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 proliferations or both, and are cut off from outermost cortical cells in chains. Carpogonial branches are three celled, formed laterally on inner cortical cells, and are directed outward.

Agard (1852) divided this genus into three sections on the basis of their habits: the first known as *Spinuligerae* including *H. charoides* Lamouroux and *H. valentiae* (Turner) Montagne. These algae are attached to the substratum by a primary discoid holdfasts. 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. The second section was called *Virgatae* including *H. boergesenii* Tanaka. These algae with weakly developed creeping branches are not entangled and are called caespitose. *Pulvinatae* was the last section including *H. pannosa* J. Agardh. These species have branches coalesced to one another at points and form cushionlike algae.

The taxonomy of the genus is very problematic and identification of *Hypnea* species is complicated on the basis of a relatively simple morphology that is often influenced by the conditions of the habitat (Rodrigues 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).

A number of molecular markers has been used in red algal taxonomy including psaA, rbcL and cox1 sequences (Yang & Boo 2004; Freshwater & al. 1994; Saunders 2005; Robba & al. 2006), but inter-simple sequence repeats (ISSR) have not been used. ISSR markers are reliable, highly polymorphic, low cost and less laborious, need only a small amount of DNA and are very fast when compared to most other molecular markers. ISSR markers involve the amplification of DNA segments between two identical microsatellite repeat regions. "ISSRs have high reproducibility possibly due to the use of longer primers (16-25-mers) as compared to RAPD primers (10-mers), which permits the subsequent use of high annealing temperature (45-60°C) leading to higher stringency. This technique requires very small amount of template and is convenient in result recording and highly reproducible (Zietkiewicz & al. 1994)".

Hypnea species have been poorly studied taxonomically in Iran (Sargazi & al. 2016). Available information has usually been anecdotal or observational without the necessary testing of parameters of systematic that predict species diversity (Sargazi & al. 2016).

The present study reports morphometric and ISSR analysis of alga specimens collected from 11 geographical regions of Persian Gulf and Oman Sea.

Morphological variation and cox1 analysis of this genus can complete the identification of the species of this genus that have high morphological plasticity. ISSR analysis tries to reveal the genetic diversity of these populations for the first time in Iran. Genetic structure of the studied populations and the gene flow among them are also studied. These information produce data about gene pool of algae in the country that can be used for conservation studies. The potential of ISSR markers in the delimitation of *Hypnea* species and their relationship is discussed.

MATERIAL AND METHOD

Twenty-four populations of *Hypnea* species were analyzed by morphometry and ISSR markers (table 1) in the present investigation. This populations were collected from 11 localities in Persian Gulf and Oman Sea coasts (fig. 1).

Collection and preservation

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.

Morphometry

Minimum of 6 randomly selected algae from each population were used. In total, 18 morphological characters (quantitative and qualitative) were used for morphometry (table 2).

Inter-Simple Sequence assay

DNA was extracted from the thalli that was 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 Polyvenyl 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 were used for obtaining ISSR data.

Table 1. Populations of *Hypnea* and their localities.

populations	Locality
*H. pannosa*1, *H. boergesenii*1, *H. charoides*1, *H. valentiae*1	Qeshm: Shibderaz
*H. pannosa*2, *H. charoides*3	Bandar Lenge: Dowlat park
*H. pannosa*3, *H. boergesenii*2	Qeshm: municipality
*H. pannosa*4, *H. valentiae*2	Kong
*H. pannosa*5, *H. boergesenii*4, *H. charoides*5, *H. valentiae*3	Tis
*H. pannosa*6, *H. boergesenii*5, *H. valentiae*4	Remin
*H. boergesenii*3, *H. charoides*2,	Qeshm: Zeyton park
*H. boergesenii*6, *H. valentiae*5	Kachu
*H. charoides*4	Bandar Lenge: near wharf
*H. charoides*6, *H. valentiae*6	Beris

Table 2. Morphological characters and their coding.

no.	Character (Abbreviation for graphics)	Codes/units
1	algae color	1: purplish red, 2: greenish red, 3: dark red
2	algae size	1: larger than 50 cm,2: from 3-50 cm, 3: less than 3 cm
3	algae texture	1: cartilaginous, 2: membranous
4	algae habits	1: caespitose, 2: intricate-caespitose, 3: cushionlike
5	main axis clearness	1: percurrent, 2: not percurrent
6	abundance of lateral branches	1: profusely branched, 2: sparsely branched
7	special branches or branchlets	1: spines branches, and 2: none of them
8	direction of branching	1: wide angles- larger than 90º, 2: narrow angles- less than 90º
9	basal system	1: without discoid holdfast, 2: with discoid holdfast
10	small cells around axial cell	1: presence of these cells, 2: absence of these cells
11	lenticular thickenings	1: presence of these cells, 2: absence of these cells
12	size of medullary cells	1: isometric cells, 2: none isometric cells
13	width of main axes	1:larger than 1mm in diameter, 2: less than 1 mm in diameter
14	Number of cortical layers	1: 1 layer, 2: more than 1 layer
15	Width of branchlets	1:larger than 500 µm in diameter, 2: less than 500 µm in diameter
16	Position of tetrasporangesorus	1: apice of branchlets, 2: middle of branchlets 3: in angles of branchlets
17	Branchlets apex	1: acuminate apex, 2: not acuminate apex
18	Position of branchlets	1: all of branches, 2: only in middle of branches

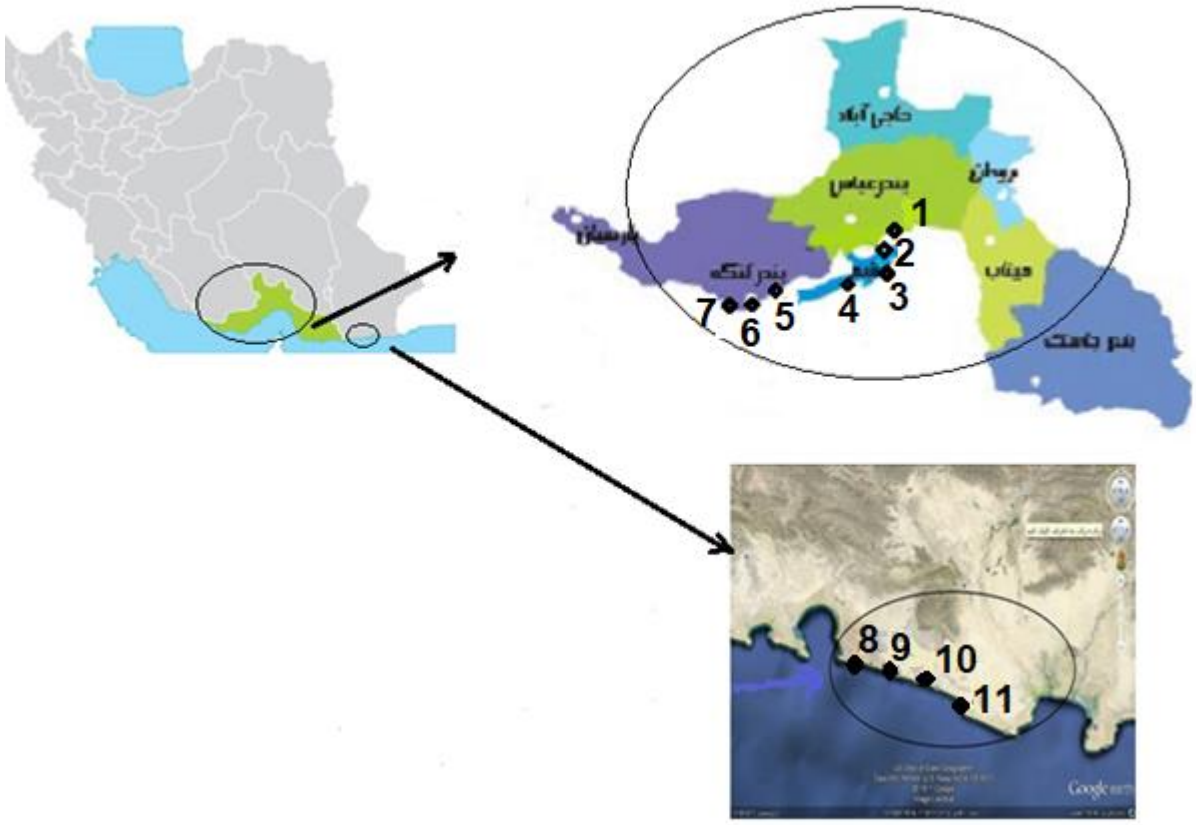

Fig. 1. Distribution map and localities of *Hypnea* populations studied from Persian Gulf and Oman Sea. Locality codes:1- Bandar Abbas, Dowlat park; 2- Qeshm, municipality; 3- Qeshm, Zeyton park; 4- Qeshm, Shibderaz; 5- Kong; 6-Bandar Lenge, Dowlat park; 7- Bandar Lenge,near wharf; 8- Tis; 9- Remin; 10- Kachu 11-Beris

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 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).

Data analyses

Morphometry

The quantitative morphological characters were divided into discrete groups and along with qualitative characters were coded as binary and multistate characters. UPGMA (Unweighted Paired Group using Average mean) and NJ (Neighbor Joining) methods with 100 times bootstrapping as well as principal components analysis (PCA), principal coordinate analysis (PCoA) were performed to group the plants specimens based on morphological characters. The Gower distance were used for clustering methods. Cophenetic correlation was determined to check the fit of dendrograms to the original distance matrix (Podani, 2000). Data analyses were performed by using PAST ver. 2.17 (Hamer & al., 2012).

ISSR analysis

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 Neighbor Joining (NJ) tree followed by 100 times bootstrapping, principal coordinate analysis (PCoA) after 999 permutations and multidimentional scaling (MDS). PAST ver. 2.17 (Hamer & al., 2012) and DARwin ver. 5 (2012) were used for these analyses**.**

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 association between molecular distance and geographical distance of the studied populations. The occurrence of gene flow among populations was checked by different methods. First we performed indirect Nm analysis of POPGENE ver. 2 for ISSR loci studied according to the following formula, Nm = estimate of gene flow from Gst, Nm = 0.5(1 - Gst)/ Gst.

Then the genetic divergence of the studied populations was checked by principal coordinate analysis provided and after 999 permutations. Model-based clustering as performed by STRUCTURE software ver. 2.3 (Pritchard & al. 2000), and the studied populations were grouped based on genetic affinity. 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 performed 20 times for each value of K (2–5) 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 the analysis followed the method suggested by Falush & al. (2007).

STRUCTURE Harvester web site (Earl & von Holdt 2012) was used to visualize the STRUCTURE results and also to perform Evanno method to identify the proper number of K (Evanno & al., 2005). The choice of the most likely number of clusters (K) was carried out comparing log probabilities of data [Pr (X|K)] for each value of K (Pritchard & al., 2000), as well as by calculating an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values, as described by Evanno & al. (2005). AMOVA test (with 1000 permutations) as performed in GenAlex 6.4 (Peakall & Smouse 2006), was used to show the molecular difference among the studied geographical populations. The Mantel test (Podani 2000) was performed to study association between the molecular distance and geographical distance of the studied populations.

RESULTS

Morphometry

UPGMA and NJ clustering methods produced almost similar results and therefore UPGMA's tree is only discussed. In general, two major clusters were formed in UPGMA's tree with 100% bootstrap value. The first major cluster comprised of *H. boergesenii*. Second major cluster contains two subclusters. First subcluster is comprised of *H. charoides*. The second subcluster is comprised of *H. pannosa* and *H. valentiae* (fig. 2). The mentioned tree showed that species of this genus are located in separate clusters but populations cannot be separated clearly.

PCA and PCoA plots obtained also produced similar results. Therefore, only PCA plot is presented and discussed here. PCA biplot (figure not given) and PCA loadings were obtained to identify the most important morphological characters differentiating the studied populations. PCA analysis showed that, the first 3 components comprised about 81.63 % of total variation. Algal brancing in habit as the first factor (with about 34.04 % of total variance) possessed the highest positive correlation. Size of the alga as the second factor (with about 32.96 % of total variance) and position of tetrasporange sorus as the third factor (with 14.62% of total variance) had the next highest positive correlation (table 3).

Table 3.Contribution of the variables to components 1 (PC1), 2 (PC2) and 3 (PC3).

Variables	PC1	PC2	PC3
algae color	0.12036	0.064911	0.04506
algae size	-0.14212	0.62417	0.1046
algae texture	0.078148	0.34571	-0.02947
Algal branching in habits	0.5996	0.40645	-0.14799
main axis clearness	0.103	-0.15782	-0.29703
main axis shape	0.023331	-0.13276	-0.26496
abundance of lateral branches	-0.2433	0.28496	0.08906
special branches or branchlets	-0.10689	0.17467	0.30186
direction of branching	-0.32079	-0.00025	0.10111
basal system	0.32145	0.060749	-0.11853
small cells around axial cell	0.31597	0.067264	-0.12686
lenticular thickenings	0.036383	0.010919	-0.00428
size of medullary cells	-0.00744	-0.09304	-0.21355
width of main axes	-0.32145	-0.06075	0.11853
Number of cortical layers	-0.00744	-0.09304	-0.21355
Width of branchlets	0.2654	-0.2079	0.73869
Position of tetrasporangesorus	0.056787	0.30277	-0.04928
Apice of branchlets	-0.29511	-0.05491	0.11419
Position of branchlets	0.12036	0.064911	0.04506

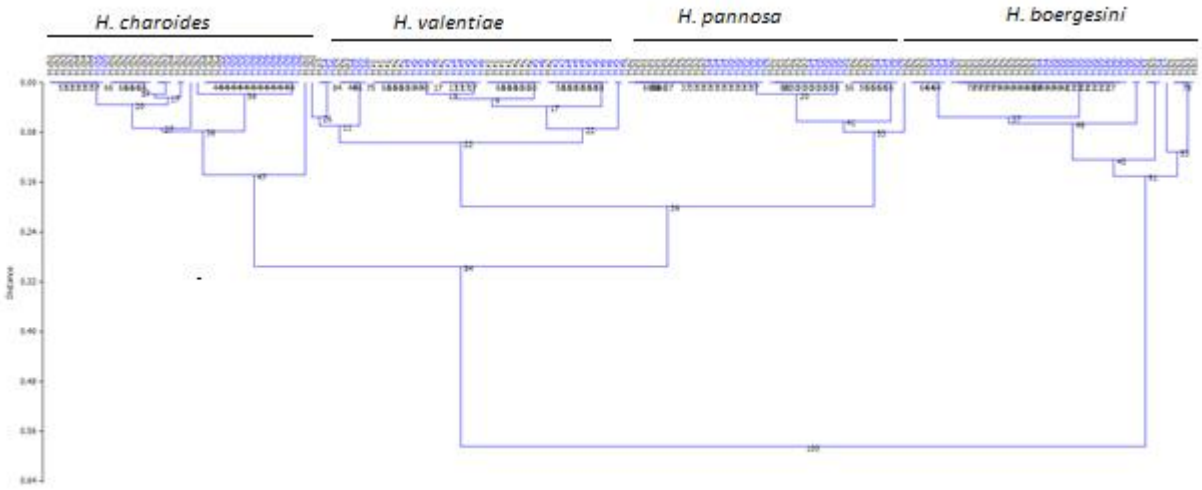

Fig. 2- UPGMA tree of morphometric data of 34 populations of *Hypnea*.

There are not similar studies to compare the results of this paper in regard to previously published literatures but some scientists explored morphology within the genus (Agardh1852, Lewmanomont 1997, Masuda & al., 1997, Mshigeni & Chapman 1994). We tried to use the same characters that other scientists also used and the species can easily be distinguished using these characters. The results of UPGMA tree are consistent with three sections that Agardh (1852) introduced. *Hypnea boergesinii* and *H. pannosa* were separated from other species in clusters. In addition, despite the high morphological similarities of *H. charoides* and *H. valentiae*, we were able to distinguish between these species on the basis of three characters (main axis clearness, abundance of lateral branches and direction of branching). *Hypnea charoides* has not clear main axis, the lateral branches are abundance and the angles of branching are smaller than *H. valentiae*. These two species were relatively well separated in UPGMA tree.

ISSR assay

Out of the 6 ISSR primers used, 6 primers produced 50 polymorphic and reproducible bands. GA (9T) primer and *H. pannosa* produced the highest number of bands (13 & 10 bands, respectively). Specific bands were produced with UBC-811 primers showing the highest number. Some of the populations showed presence of specific bands. For example, *H. pannosa* showed highest number of specific bands with a single specific band of the UBC-834 (100 kb and 1000 kb molecular weight), UBC-811(1000 kb, 1100 kb and 1200 kb molecular weight), UBC-810 (200 kb and 1100 kb molecular weight), GA (9C) (350 kb molecular weight), GA (9A) (350 kb and 1100 kb molecular weight) and GA (9T) (150 kb and 1000 kb molecular weight). Similarly, *H. boergesenii* had single specific bands with 1200 kb, 900 kb and 200 kb molecular weight produced by primer GA (9C), GA (9T) and UBC-810. The highest number of total bands was observed in Bandar Lenge-near wharf population of *H. pannosa* with 37 bands.

Genetic diversity parameters of populations are provided in table 4. The number of effective alleles ranged from 1.042 in *H.*valentiae2 to 1.206 in *H.charoides*1. Shannon index varied from 0.042 in *H.*valentiae2 to 0.180 in *H.charoides*1. The highest value of gene diversity occurred in *H. charoides* 6, was 0.147.

Grouping of the populations based on Nei's genetic distance by NJ tree and MDS plot produced similar results (figs. 3, 4). In general four major clusters were formed and the studied species were placed almost in distinct clusters/groups. *Hypnea panossa* formed the first main cluster, while *H. boergesenii* is the second main cluster and *H. charoides* comprised the third main cluster.

The studied populations of *H. charoides, H. boergesenii* and *H. pannosa* showed close genetic affinity and formed one cluster. The populations of *H. valentiae* showed more genetic affinity to the last tree species and joined them with some distance forming the fifth cluster.

MDS plot also revealed genetic differences of the studied species and formed 4 distinct groups. It revealed that *H. panossa* specimens were placed far from the other studied species, as it was true for members of *H. boergesenii*.

Table 4. Genetic diversity parameters of *Hypnea* populations.

Populations	Na	Ne	I	He	UHe	%P
H. pannosa1	0.757	1.16	0.12	0.089	0.095	22.97%
H. pannosa2	0.838	1.185	0.167	0.111	0.118	31.08%
H. pannosa3	0.703	1.149	0.125	0.084	0.089	22.97%
H. pannosa4	0.541	1.052	0.037	0.026	0.028	5.41%
H. pannosa5	0.608	1.118	0.083	0.060	0.064	12.16%
H. pannosa6	0.554	1.060	0.035	0.042	0.044	6.76%
H. boergesenii1	0.459	1.064	0.062	0.040	0.044	12.16%
H. boergesenii2	0.446	1.062	0.053	0.036	0.039	9.46%
H. boergesenii3	0.284	1.070	0.052	0.037	0.039	8.11%
H. boergesenii4	0.351	1.078	0.055	0.040	0.043	8.11%
H. boergesenii5	0.378	1.060	0.048	0.033	0.035	8.11%
H. boergesenii6	0.649	1.148	0.137	0.090	0.098	27.03%
H. charoides1	0.838	1.206	0.180	0.122	0.131	31.08%
H. charoides2	0.514	1.064	0.046	0.033	0.036	6.76%
H. charoides3	0.581	1.075	0.063	0.043	0.046	10.81%
H. charoides4	0.541	1.092	0.074	0.051	0.055	12.16%
H. charoides5	0.838	1.218	0.186	0.127	0.139	31.08%
H. charoides6	0.946	1.256	0.217	0.147	0.157	37.84%
H. valentiae1	0.500	1.089	0.072	0.049	0.053	12.16%
H. valentiae2	0.405	1.042	0.044	0.028	0.030	9.46%
H. valentiae3	0.595	1.142	0.122	0.083	0.099	21.62%
H. valentiae4	0.595	1.153	0.132	0.089	0.098	22.97%
H. valentiae5	0.351	1.072	0.058	0.040	0.043	9.46%
H. valentiae6	0.432	1.100	0.085	0.058	0.062	14.86%

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.

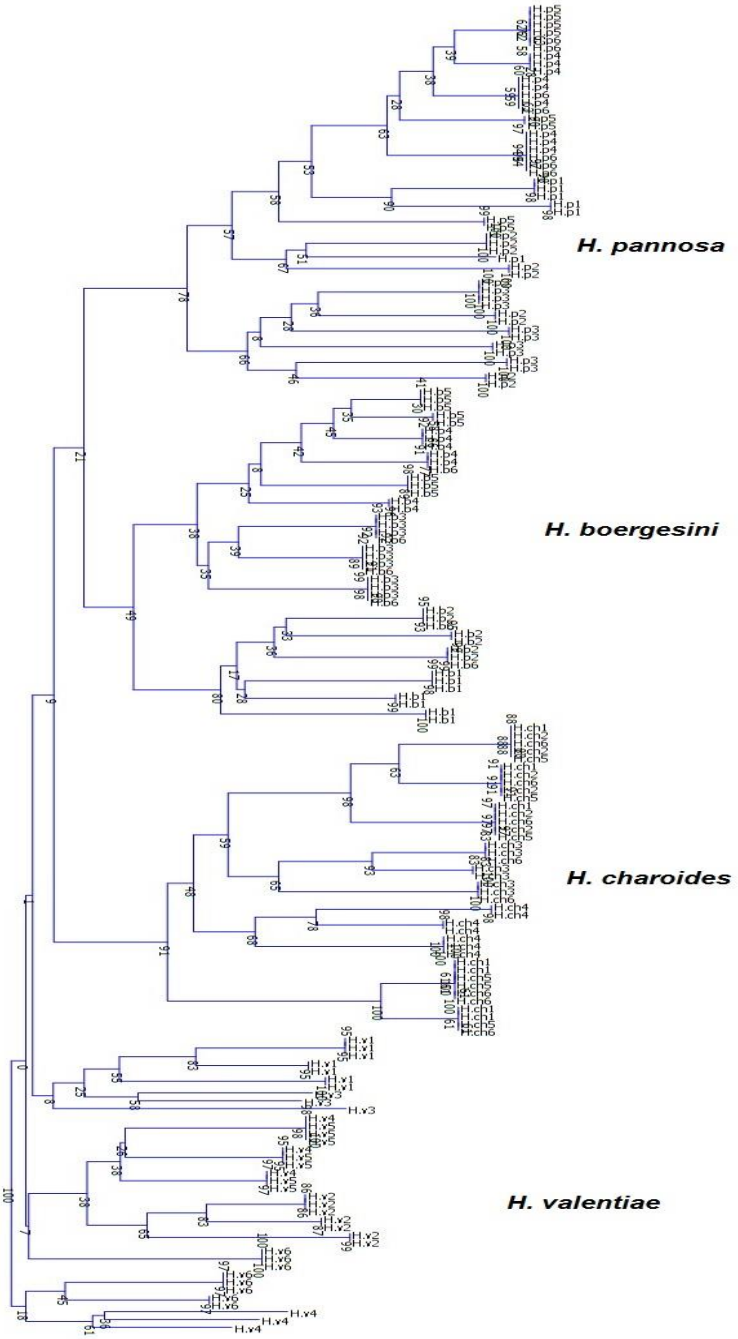

Fig. 3. NJ tree showing the genetic affinity of the studied *Hypnea* species.

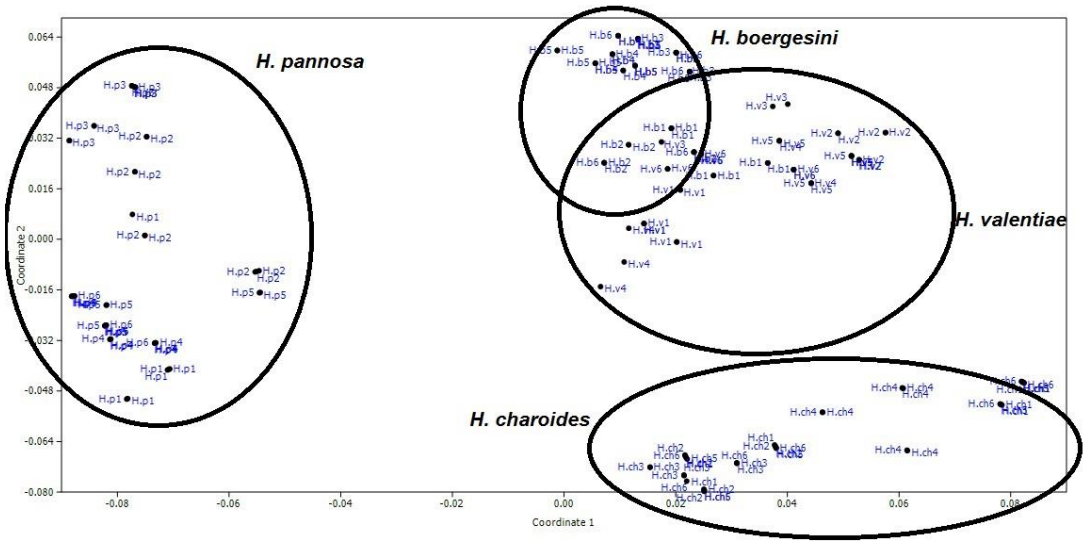

Fig. 4. MDS plot of *Hypnea* species.

AMOVA test produced significant genetic difference among the studied species (PhiPT value = 0.734, P = 0.01). The test also revealed that 73% of total variation is attributed to among population differences and 27% due to within population variation. However inter-population differences are higher than intra-population differences. Significant genetic difference was also indicated by Gst value (0.83, P = 0.01), Dest value (0.299, P = 0.01) and Hickory test (θ B value = 0.3). Therefore, these results revealed genetic distinctness of the studied species. The Mantel test was not significant and geographical distance didn't impact on genetic diversity (R^2 = 0.03, P = 0.20).

K-Means clustering and Evanno test revealed the presence of 4 genetic groups in the studied species and populations. K-Means clustering produced the highest BIC value (230.201) for k = 4, and the lowest value of pseudo-F (18.88) for k =11. Similarly, Evanoo test produced the highest delta k value for k = 4 (fig. 5). Therefore, both results revealed genetic divergence of the studied species. This is clearly observed in STRUCTURE plot obtained based on k = 4 (fig. 5). The members of four studied species contained different allelic forms (different colors) which suggested extensive genetic divergence among these species. Some degree of genetic admixture was observed among the studied species.

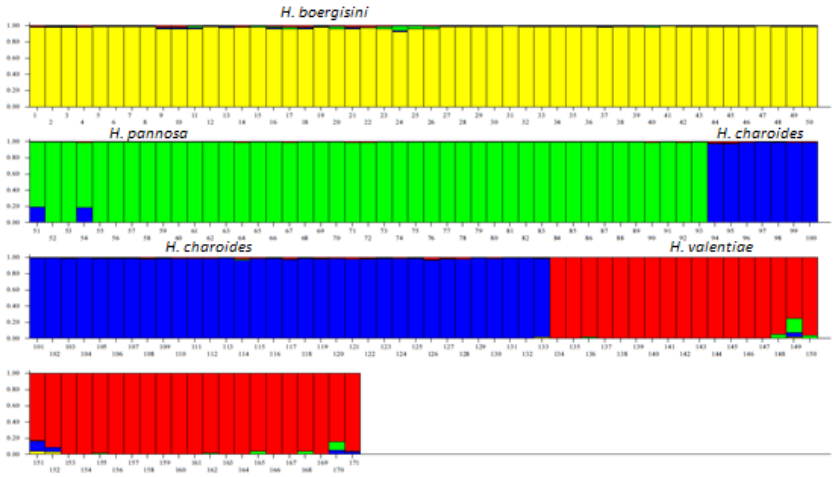

Fig. 5. STRUCTURE plot of *Hypnea* species based on k = 4.

DISCUSSION

As far to our knowledge, there are no previous studies for comparison of the species of *Hypnea* on the basis of genetic diversity and morphometry. However, some scientists explored phylogenetic studies within the genus, and they studied the relationship of the species according to rbcL, cox1 and psaA sequences (Geraldino & al. 2006, 2010; Rodrigues 2011). The present ISSR analyses of *Hypnea* species is almost in agreement with the other phylogenic and morphological studies performed for the genus. For example, results of grouping by UPGMA are identical with grouping of these species in three sections that was performed by Agardh (1852). Separation of *H. pannosa* and *H. boergesenii* from the other species is well supported in both studies.

In the present morphometric study, *H. charoides* and *H. valentiae* were highly similar species so that morphological data showed high similarity of these species. Despite to the high morphological similarity of *H. charoides* and *H. valentiae*, genetic diversity was also observed.

We attempted to select characters that are more diverse among species and less affected by habitat conditions to have a good selection of morphological characters. These characters are introduced in the identification keys by most researchers. Hence, morphometric results are consistent with different identification keys. Some morphological characters such as color and main axes clearness were different in populations of each species because these characters were affected by habitat conditions.

In both morphometry and ISSR trees, all species were located in separate clusters but populations were spread in different clusters and were not well separated. So that even the Persian Gulf and Oman Sea populations did not show much difference according to morphology and ISSR marker genes.

The presence of ISSR polymorphic bands in the populations of *Hypnea* indicates the presence of genetic polymorphism in these populations. However, we observed some private ISSR bands in *H. pannosa* that were not common with the other studied species.

Since even single base change at the primer annealing site is manifested as appearance or disappearance of ISSR bands, these bands may indicate the occurrence of genetic changes in these species.
We observed high rate of diversity in populations of *H. charoides, H.valentiae and H. pannosa*.

STRUCTURE analysis and K-Means clustering results showed genetic distinctness of the studied species. The absence of high degree of gene flow among these species has led to genetic isolation of the studied species from each other.

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