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Isoenzyme Investigation and Morphometrics Study of *Neckera complanata* and *Neckera crispa*

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ABSTRACT

Bryophytes are small, non-vascular, and non-flowering plants. *Neckera complanata (*Hedw.*)* Huebener and *Neckera crispa* Hedew. are one of the most prominent species in the Hyrcanian wetland found in glossy pale or yellowish-green patches. There is no evidence for morphometry and isoenzymes biochemical markers (Peroxidase/Superoxide dismutase) works on this genus in Iran. The purpose of this study is to investigate the differences and similarities among *Neckera complanata* and *Neckera crispa* moss populations in the north of Iran using morphometry and isoenzymes biochemical markers (Peroxidase / Superoxide dismutase). For this purpose, 18 populations from three provinces including Golestan, Mazandaran, and Guilan were collected at the same altitudes in autumn 2017. The results of morphometry were shown leaf length and leaf apex width/length were the most effective traits for the separation of populations. The results of the morphometry method and the results of isozyme banding patterns were the same, although very minor differences were observed. The largest diversity of Shannon (Ĥ) belongs to the population of *Neckera crispa* from Hezarjarib while other populations have low genetic diversity. Because of the destruction of many habitats in the northern provinces of Iran and the increase in pollution in these areas, it can be said as a general result that perhaps the reason for low genetic diversity in *Neckera complanata* and *Neckera crispa* populations is the gradual extinction of these two species.

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Introduction

Iran is a country with a wide range of bryophyte, due to its vast climate. Many bryologists worked on the Iranian flora of mosses, such as Kürschner (1996), Frey and Kürschner (1977, 1983), Edw (1920), Jovet-Ast (1960), Störmer (1963), Kürschner (1996), and later Akhani and Kürschner in 2004 published 437 taxa (2 hornworts, 68 liverworts, 367 mosses). Mosses represent one of a variety of green plants and they are widely distributed from pole to pole. They occupy a broad range of habitats (Shirzadian, 2011). The mosses gametophytes are usually small, perennial plants and they are found in populations or colonies in characteristic growth forms, such as mats, cushions, turfs, or wefts (Wang, *et al*., 2005). Also, the sporophyte development takes place in the apices of short, lateral branches. Accordingly, Mosses are divided into 2 categories of acro-carp and pleuro-carp. In about 5000 species of mosses, the pleuro-carp mosses occur nearly in all ecosystems (Olsson *et al*., 2011). The Neckeraceae family belonging to the pleuro-carp mosses and consists of temperate and tropical taxa. Also, the Neckeraceae family is estimated with around 200 species. The *Neckera* Hedew.

genus is the largest, most widely distributed, and essentially temperate (Ji and Miao, 2009). The worldwide *Neckera* genus containing 50 species and the most diversity of this genus is in Asia (Enroth and Ji, 2007). *Neckera complanata* (Hedw.) Huebener is characterized by complanate leaves and numerous secondary, flagelliform branches, costa absent, and the apiculate leaf apex. The species occur on limestone rocks, as well as on the bark of deciduous trees (Kürschner *et al.,* 2012). Also, *Neckera crispa* Hedw. characterized by traits such as Leaves lingulate, shortly-pointed, strongly transversely undulate when dry; margin plane, serrate in the upper part of the leaf and costa faintly double and these are, strongly glossy, green or yellowish-green patches on tree trunks and rocks (Kürschner and Frey, 2011).

In the past, classification of Neckeraceae based on evolution has been discussed (Olsson *et al*., 2011) and it's been proven high variation among the population and considerable genetic diversity among species across epiphytic mosses. (Appelgren and Cronberg, 1999). Biosystematics studies can be used to overcome such problems (Enroth et al ., 2010).

Isoenzymes are a powerful tool for the investigation of gene variability between plant populations and more powerful than morphology. They are capable of solving questions of population biology, conservation biology, and ecology equally well. Isoenzyme electrophoresis has proved to be a reliable method in species identification (Moss *et al*., 1988). Isozyme electrophoretic analysis has been applied quite often to solve taxonomic relationships. So, especially where morphological characteristics overlap or assess intra- and inter-specific genetic variability. However, closely studies on bryophytes based on isozyme marker revealed unexpectedly high levels of genetic variation. It is unknown that genetic variability in bryophytes affects their evolutionary rate (Sabovljević *et al*., 2011). Isoenzyme data from *Trichoderma harzianum*, *T. aureoviride,* and *T. longibrachiatum* was showed a higher degree of relationship between *T. harzianum* and *T. aureoviride* than to *T. longibrachiatum*, also *T. harzianum* had a high level of genetic variation from others (Siddiquee et al., 2010). Melosic et al (2005) was shown that the isoenzyme system can separated *Sphagnum* species and also POD and EST have a high level of variation within and among *Sphagnum* species (Daniels, 1982). Szweykowski and Zielinski (1983) found four different peroxidase phenotypes in the *plagiothecium undulatum* population in polish. Plant peroxidases and superoxide dismutase have been used as biochemical markers for classifications and various types of biotic and abiotic stresses (Pan *et al*., 2006). The characterization also allows us to measure divergence within and among *Neckera* species.

This study aimed to measure possibilities to use allozymes (molecular characters) to study relationships between different populations of species *Neckera*, evaluate more morphology characters to identify mosses, and choose important characters for the future. The present study is the first report on morphometric and isozyme variation of mosses in the Hyrcanian forest.

Materials and Methods

Sampling procedure

In the current study, two species of *Neckera* genus (*N. complanata* and *N. crispa*) with nine populations in each species were collected from three regions (Guilan, Mazandaran, and Golestan) in the north of Iran in the autumn of 2017 (Table 1 and Fig. 1). The samples were transferred to the laboratory at 4˚ C. Some samples were used to study morphometric parameters and the fresh weight of the other samples measured and transported to -20˚ C for determination of peroxidase and superoxide dismutase isozymes banding patterns. Seventy-two individuals belonging to 18 populations (four replications for each population) were used for morphometric and isoenzyme analysis.

Morphometry

A total of 18 populations were collected to study the morphological variability. More than 40 quantitative and qualitative traits were investigated for the morphometric method.

Sixteen quantitative traits and 27 qualitative attributes were defined and measured for *Neckera* populations (*N. complanata* and *N. crispa*).

Table 1. Collection information (location, abbreviation, latitude, longitude, and altitude) of 18 populations of two species of *Neckera* genus (*N. complanata and N. crispa)* with nine populations in each in the north of Iran.

Species name*	City	Population name (abbreviation)	Latitude	Longitude	Altitude
N. complanata (Hedw.) Huebener	Guilan, Rezvanshahr	N.co.R	37°35'50.30"N	48°55'7.60"E	643
	Guilan, Masal	N.co.M	37°19'0.10"N	48°59'36.50"E	663
	Guilan, Siahkal	N.co.S	37° 0'20.90"N	49°51'51.10"E	520
	Mazandaran, Kelardasht	N.co.K	36°39'14.02"N	51° 7'17.83"E	613
	Mazandaran, Toskachal	N.co. T	36°30'44.10"N	36°30'44.10"N	1145
	Mazandaran, Dodangeh	N.co. Do	36°13'39.10"N	53°15'54.00"E	730
	Golestan, Derazno	N.co. D	36°43'2.50"N	54° 6'36.20"E	635
	Golestan, Aliabad	N.co. A	36°49'21.20"N	55° 0'3.80"E	620
	Golestan, Loveh	N.co. L	37°21'34.50"N	55°40'31.60"E	625
N. crispa Hedw	Mazandaran, Kelardasht	N. c. K	36°38'51.97"N	51° 7'3.80"E	453
	Mazandaran, Touskachal	N.c.T1	36°30'44.10"N	36°30'44.10"N	1145
	Mazandaran, Touskachal	N.c.T2	36°30'36.39"N	51°31'8.23"E	1175
	Mazandaran, Nowshahr	N.c. N	36°35'22.62"N	51°33'43.62"E	458
	Mazandaran, Royan	N.c. R	36°29'12.00"N	51°55'35.70"E	443
	Mazandaran, Ghaemshahr	N.c. Gh	36°13'40.41"N	52°53'2.34"E	754
	Mazandaran, Dodangeh	N.c. Do	36°13'39.10"N	53°15'54.00"E	730
	Mazandaran, Hezarjerib	N.c. H	36°32'31.40"N	53°25'29.80"E	570
	Mazandaran, Galogah	N.c. G	36°41'52.48"N	53°48'9.18"E	421

*Herbarium voucher number of all of the samples are 101

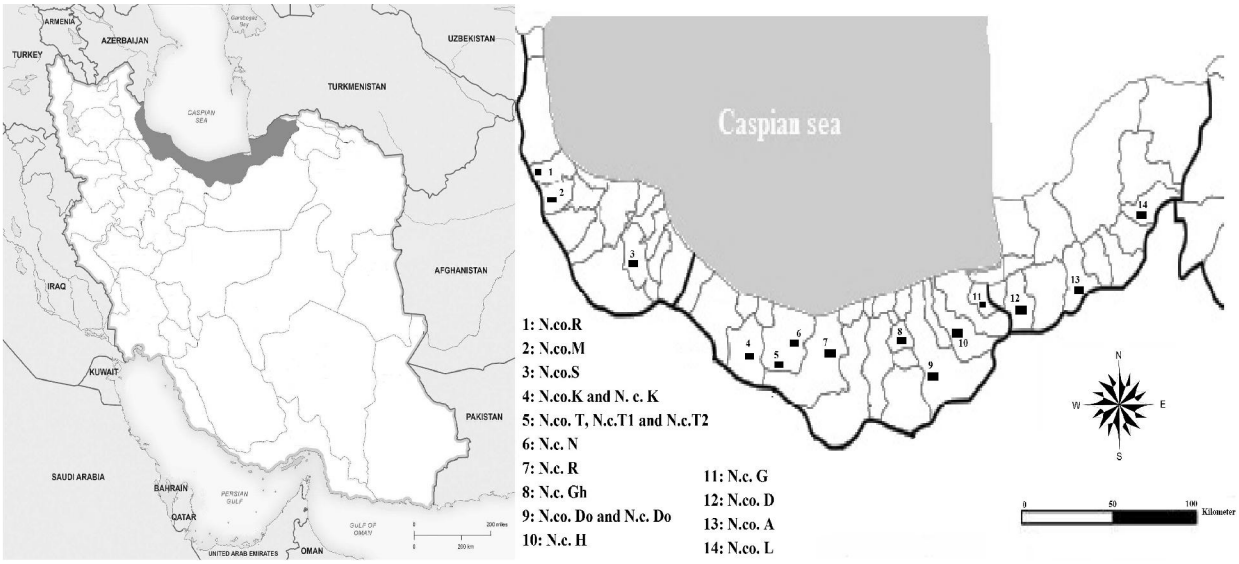

Fig. 1. Localities of populations of *N. complanata* and *N. crispa* in this study: The numbers refer to populations and species quitted in Table 1.

They include the shape of the gametophyte and Sporophyte, form of leaf, shape of the leaves margins, presence or absence of costa, form of costa, double costa or single costa, form of peristome, plant color, habitat, leaves on the stem, leaf tip shape, stem form, presence or absence of secondary stem, laminal cell placement, fruiting body placement, shape of the leaves margins, secondary stem placement, plant length, leaf length, upper leaf width, middle leaf width, end of leaf width, leaf tip length, leaf tip width, the number of leaf apex cells, costa length, costa width, the number of costa cells, upper laminal cell width, middle laminal cell length, middle laminal cell width, marginal cell width, ratio of upper marginal laminal cell length, the number of marginal laminal cell, costa distance to marginal cell, allarcell length, allarcell width, length of the first branch, number of teeth in each side, teeth length, Ratio of upper

marginal laminal cell width, basal marginal laminal cell length, ratio of middle marginal laminal cell length, number of cells per tooth, distance between the teeth and teeth width. We performed analyses using only quantitative characters. After the measurement of traits and coding them, they were analyzed and their hierarchical clustering chart was studied by using the WARD method (IBM SPSS statistics 25).

Electrophoretic analysis

POD and isoenzymes extraction

The plant samples were carefully cleaned and washed three times in de-ionized water. To extract the enzyme, 10 ml of potassium phosphate buffer (0.1 M) was used. The extraction solution was centrifuged for 25 minutes at 4°C and 16000 g. The upper phase containing enzymes was transferred to new test tubes and kept on -80 ˚C until electrophoresis (Abeles and Biles (1991).

Isozymes determination

Peroxidase isoenzymes were determined using the Abeles and Biles (1991) method. To determine the peroxidase isozymes, 15% polyacrylamide gel was prepared. The gel was flushed with 200 ml sodium acetate buffer containing Benzidine 2 mM and H_2O_2.

SOD extraction and isoenzymes extraction

Similar to POD extraction, the plants were carefully cleaned under a dissecting microscope. Then sample was extracted (3 ml /g) with Tris-HCl buffer (50 mM, pH 7.5), containing 1mM DTT, 2 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w/v) polyvinylpyrrolidone (PVP). After extraction, the samples were centrifuged at 4°C for 15 min at 10,000 rpm. The upper phase containing proteins was used to perform SOD isoenzymes (Sabovljević *et al*. 2011).

Isozymes determination

To determine the SOD isoenzymes, sodium dodecyl sulfate-polyacrylamide gels electrophoresis by the method of (Beauchamp and Fridovich, 1971) was performed. he sodium dodecyl sulfate-polyacrylamide gels (10%) were incubated for 60 minutes in dark in a staining buffer (0.24 mM NBT, 28 µM riboflavin, 28 mM TEMED, 0.5 M EDTA, and 50 mM Monosodium phosphate). Then the gel was illuminated by two fluorescent lamps (20W each) to promote the photoreactive staining and continued until the bands became visible.

Data analysis

Band frequencies were calculated for each population and each enzyme system (Table 3). The intra-population variation was estimated by the Shannon diversity index, H′ (Shannon, 1948), and is presented in Table 4. Based on the band frequencies matrix the affinities and divergence among populations were examined by cluster analysis and Ward method operating the software SPSS version 25 and also UPGMA method using the software NTSYS-pc version 2.02k which was utilized based on Euclidean distances (Table 5). The results were similar in both methods.

Results

Morphometric analysis

The dendrogram of 18 specimens revealed: The main branch at the 25th level is divided into two branches (1 and 2), which covers about 75 percent similarity of traits. Branch1 consists of two subbranches: 1-1 (N.co.Do, N.co.A, N.co.K, N.co.D, N.co.S and N.co.L) and 1-2 (N.co.R, N.co.T and N.co.M). Branch 2 consists of two subbranches: 2-1 (N.c.N, N.c.H and N.c.R) and 2-2 (N.c.K, N.c.G, N.c.T1, N.c.T2, N.c.Do and N.c.Gh). According to this chart, two species of *N. crispa* and *N. complanata* are well-differentiated (Fig. 2).

PCA reveals that 75.96 % of the observed total variance is explained by four components (49.844, 10.346, 9.766, and 6.012%, respectively). In this study, the first four components consist of the most amount of total variance (Fig. 3). All traits which were used for this analysis are plant length, leaf length, upper leaf width, middle leaf width, end of leaf width, leaf tip length, leaf tip width, the number of leaf apex cells, costa length, costa width, the number of costa cells, upper laminal cell width, middle laminal cell length, middle laminal cell width, marginal cell width, the ratio of upper marginal laminal cell length, the number of Marginal laminal cell, costa distance to the marginal cell,

allarcell length, allarcell width, length of the first branch, number of teeth in each side, teeth length, Ratio of upper marginal laminal cell width, basal marginal laminal cell length, the ratio of middle marginal laminal cell length, number of cells per tooth, the distance between the teeth and teeth width. The characters that contribute considerably to the four first PCA axes are as follows: component 1includs: Plant length, Leaf length, Upper leaf width, Middle leaf width, End of leaf width, Leaf tip length, Leaf tip width, the number of leaf apex cells, costa length, costa width, the number of costa cells, upper laminal cell width, middle laminal cell length, the ratio of upper marginal laminal cell length, basal marginal laminal cell length, the ratio of middle marginal laminal cell length, the ratio of middle marginal laminal cell width, costa distance to the marginal cell, allarcell length, allarcell width, length of the first branch, number of teeth in each side, teeth length, teeth width, the distance between the teeth and distance between the costa. Component 2 includes leaf length, upper leaf width, middle leaf width, end of leaf width, costa length, costa width, the number of costa cells, middle laminal cell length, marginal cell width, ratio of upper marginal laminal cell length, the ratio of upper marginal laminal cell width, the ratio of middle marginal laminal cell width, the number of marginal laminal cell, costa distance to the marginal cell, the distance between the teeth and distance between the costa.

Component 3 includes leaf length, upper leaf width, middle leaf width, end of leaf width, leaf tip length, leaf tip width, costa length, upper laminal cell width, middle laminal cell width, marginal cell width, the ratio of upper marginal laminal cell length, basal marginal laminal cell length, the ratio of middle marginal laminal cell length, the ratio of middle marginal laminal cell width, allarcell width, teeth width and distance between the costa. Component 4 includes leaf length, upper leaf width, middle leaf width, end of leaf width, leaf tip length, leaf tip width, costa length, costa width, The number of costa cells, marginal cell width, ratio of upper marginal laminal cell width, the ratio of middle marginal laminal cell width, The number of marginal laminal cell, costa distance to the marginal cell, allarcell length, allarcell width and length of the first branch.

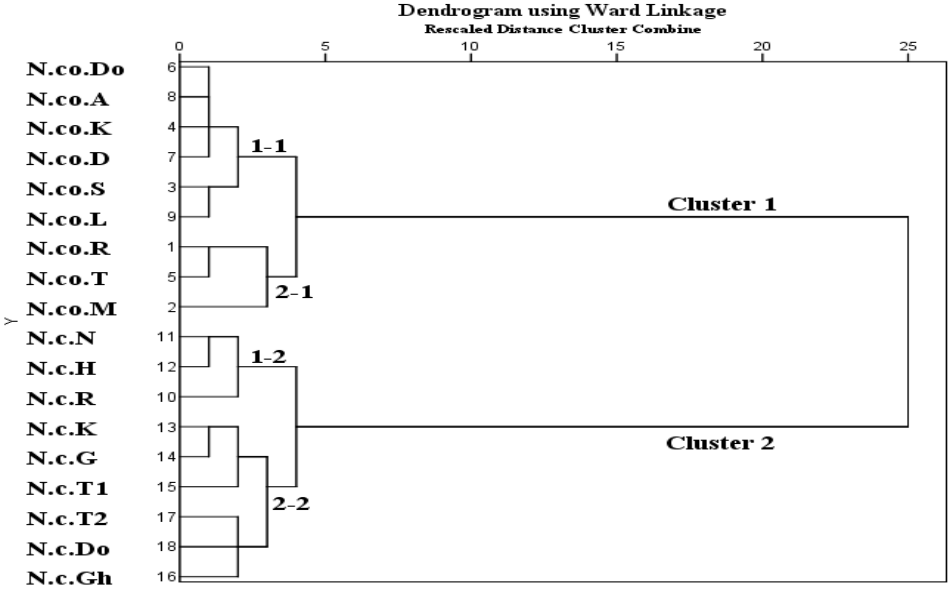

Fig. 2. Morphometry relationship among *Neckera* genus: Numbers above branches are wards support values as estimated by the morphological cladistic analysis. This simplified strict consensus tree depicts the position of *N. complanata* and *N. crispa* as separate lineages.

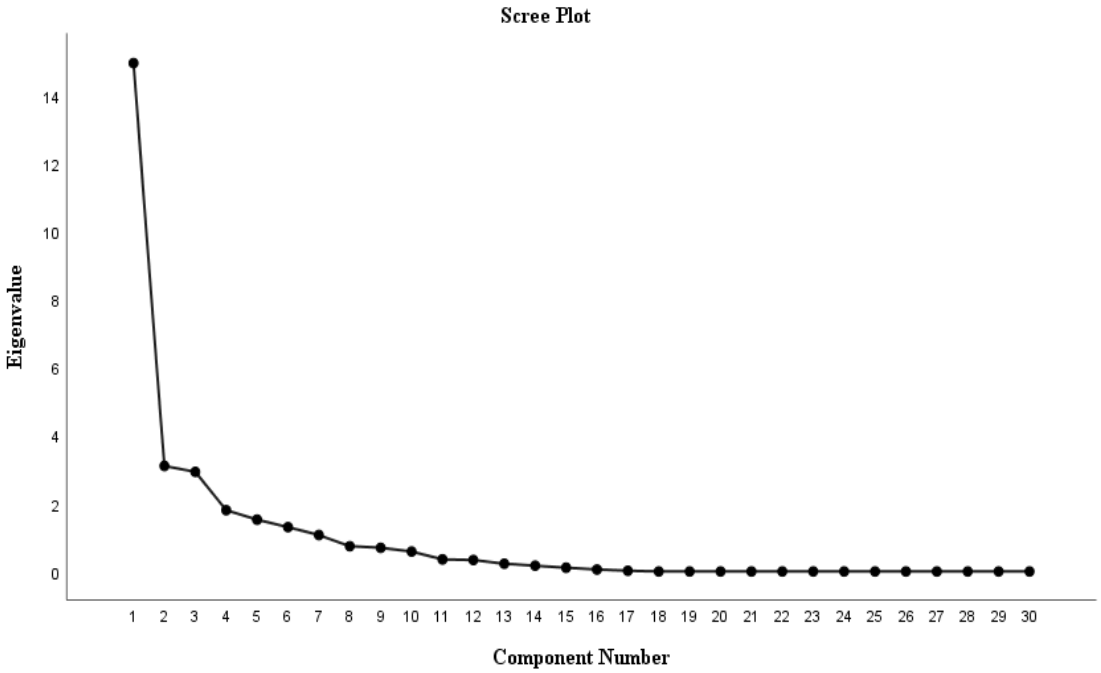

Fig. 3. Scree plot of morphometric analysis: This analysis between *N. complanata* and *N. crispa*; Scree plot that showed first components with the most variance; The four first components in this graph represent the highest variance of the total variance; Four first component has a 75.968 % variance in this chart.

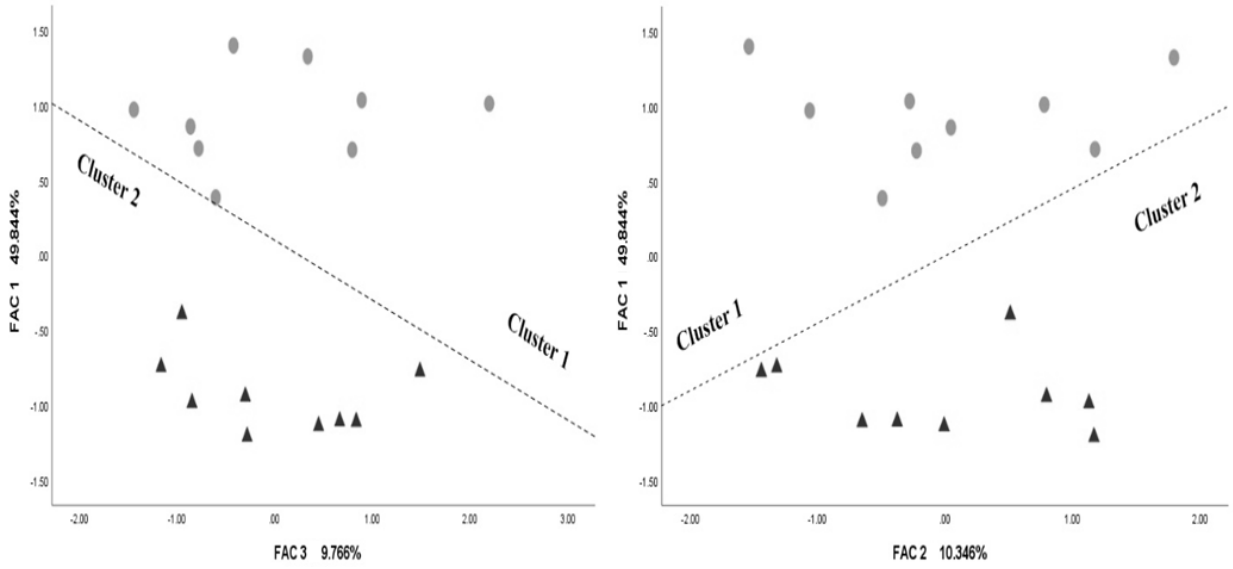

Fig. 4. PCA (fac1: fac2 and fac1: fac3) in 18 populations of *N. complanata* and *N.* crispa between clusters one and two based on quantitative character. This result separated two species into two clusters. The circle forms are related to *N. crispa* populations and square shapes related to *N. complanata* populations.

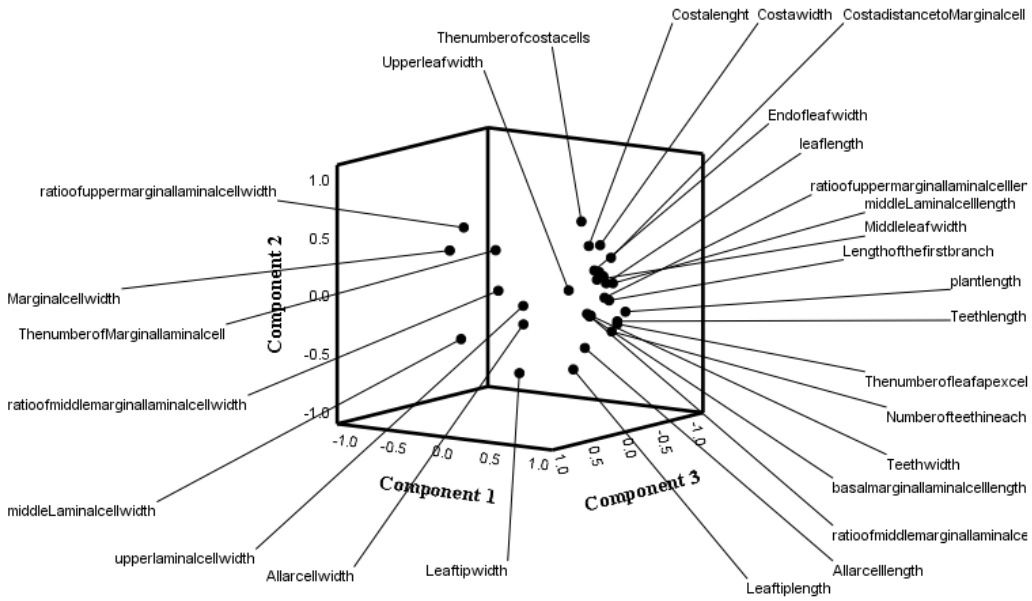

Fig. 5. The component plot in rotated space for the 28 examined characters.

Isoenzymes analysis

The results showed in 2 enzymes attributed to 4 putative isoenzymes: PRX-1, PRX-2, SOD-1, and SOD-2 (Table 3). The maximum number of isozyme bands (six bands) belonged to the populations of N.co.R, N.co.T, N.co.L, N.c.R and N.c.T2 and the minimum number of the band belonged to populations N.c.K and N.c.G with two bands. According to the results obtained from isoenzyme bands (Fig. 11), four bands were observed in each population. Band number one and six was detected in all populations of either *N. complanata or N. crispa*. The bund number two and five was observed in populations of N.c.K, N.c.T1, N.c.T2, N.c.N, N.c.R, N.c.Gh, N.c.Do, N.c.H N.c.G. The band number three and four were observed in populations of N.co.R, N.co.M, N.co.S, N.co.K, N.co.T, N.co.Do, N.co.D, N.co.A and N.co.L. All populations of each species have four types of band.

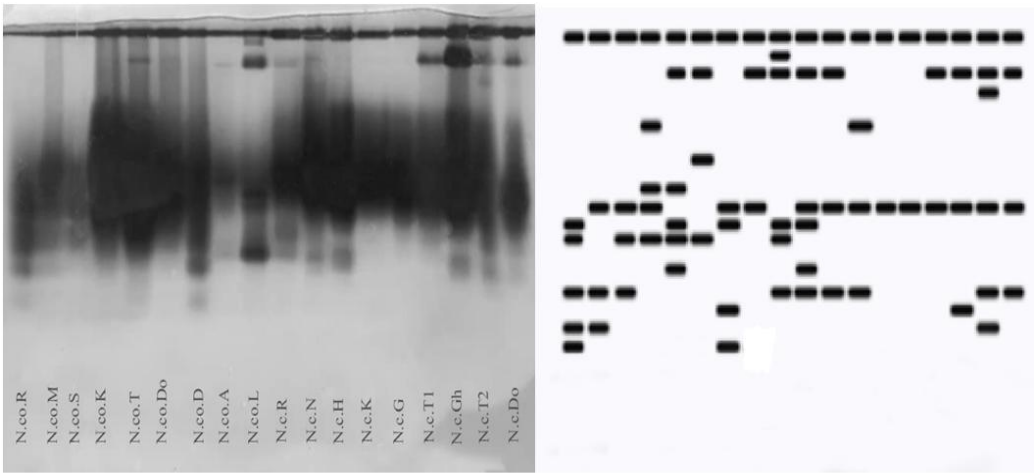

Fig. 6. Electrophoretic patterns of peroxidase isoenzyme bands in different populations of *N. complanata* and *N. crispa*.

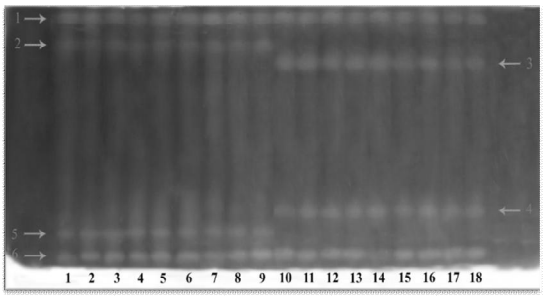

Fig. 7. Electrophoretic patterns of superoxide dismutase isoenzyme bands in different populations of *N. complanata* and *N. crispa*.

The frequency values ranged from 0 to 1. The mean number of allozymes for each isozyme varied from 1.00 to 2.50. The average value of the Shannon diversity index (H) calculated for each population ranged from 0.665, in N.c. N and N.c. R, to 1.645 in N.co. R (Table 4). The average Ĥ for each enzyme system ranged from 1.285 to 1.364. The average taxonomic distance, Euclidean distance was calculated for 18 populations. The minimum Euclidean distance is seen between the population 'N.c. K' and 'N.c. G', 'N.c. Do' and 'N.c. N' equaling 0.000 and the maximum distance was recognized among the populations N.co. R and N.c. Gh equals 3.464. Based on the dendrogram produced by the Ward method (Fig. 8) each cluster is divided into two sub-cluster. The dendrogram of isoenzyme revealed that the main branch at the 25th level is divided into two clusters (1 and 2), which covers about 75 percent similarity of traits. cluster 1 consists of two sub-cluster (1-1 and 1-2). sub-cluster 1-1 divided to subbranch 1.1.1 (N.c.N and N.c.Do), subbranch 1.1.2 (N.c.T2) and subbranch 1.1.3 (N.c.R). sub-cluster 1.2 including of subbranch 1.2.1 (N.c.T1and N.c.Gh), subbranch 1.2.2 (N.c.K, N.c.G) and subbranch 1.2.3 (N.c.H). The cluster 2 consists of two subbranches 2.1(N.co.Do, N.co.A, N.co.M, N.co.S, and N.co.K) and 2.2 (N.co.T, N.co.L, N.co.R and N.co.D).

Discussion

Numerical taxonomy and isoenzyme investigation are two effective methods that are used in the separation of populations. In the past, the traditional classification of *N. complanata* and *N. crispa* is based on morphological characters (Anderson *et al* 1990; Hedenäs, 1992; Guo et al., 2006). Therefore, we used a morphometric method to identify and classify species accurately, because in morphometry more characters are investigated and classification is more accurate than classical taxonomy. We used quantitative and qualitative traits but, the qualitative attributes were not effective for the separation of populations. For statistical analysis, quantitative traits were used. Smith, 2004; Cano *et al*., 2004 and 2005 and Mubo *et al*. 2004, also used only quantitative traits for similar studies.

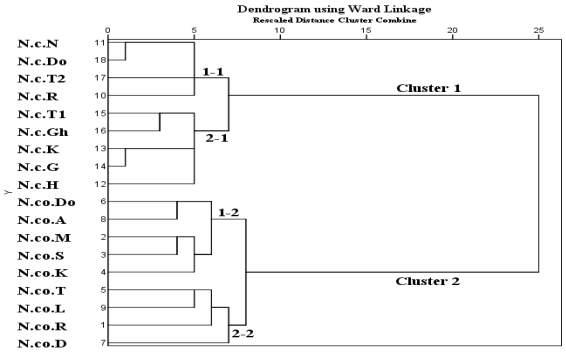

Fig. 8. Cluster dendrogram for isoenzymes analysis of 18 samples of *N. complanata* and *N. crispa* based on the Euclidean distances have been shown in Table 5.

The Scree plot diagram (Fig. 3) based on PCA analysis was showed four main components, which have the highest amount of total variance (75.96 %). Other components including the little amount of total variance. Morphometric analysis of *N. oligocarpa* revealed differences in seta length, angle of leaf apex, and leaf length which are logical reasons for separating of populations (Appelgren and Cronberg, 1999). In the present research, leaf length and Leaf apex width/ length were the most effective traits for the separation of populations. Therefore, leaf apex and leaf length seem are the most important factors for the separation of species in the *Neckera* genus. According to the obtained Cluster analysis diagram, two species with 75% similarity were separated into two different clusters. In the present study, the results of dendrograms revealed the close relationships between the populations N.co.S and N.co.L in the same sub-branches, but the N.co.R population was separated into different subbranches. While two populations of N.co.S and N.co.L maintains an extraordinary geographical distance

Table 2 Band frequencies for two enzyme systems among 18 populations of *N. complanata* and *N.* crispa in the north of Iran. For abbreviation of the populations see Table 1.

Population		N.co.R	N.co.M	N.co.S	N.co.K	N.co.T	N.co. Do	N.co. D	N.co. A	N.co. L	N. c. K	N.c.T1	N.c.T2	N.c. N	N.c. R	N.c. Gh	N.c. Do	N.c. H	N.c. G
Isozymes	Allozymes																		
POD	A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	C	0.00	0.00	0.00	0.00	0.50	0.50	0.00	0.50	0.75	0.50	1.00	0.50	0.00	0.00	0.75	0.75	0.75	0.75
	D	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.00
	E	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00
	F	0.00	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	G	0.00	0.00	0.00	0.50	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	H	0.75	1.00	1.00	1.00	0.75	0.75	1.00	0.75	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	I	1.00	0.25	0.25	0.50	0.75	0.25	1.00	0.25	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.25	0.00
	J	1.00	0.25	1.00	1.00	1.00	1.00	0.25	0.25	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	K	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	L	1.00	1.00	1.00	0.25	0.00	0.25	0.00	0.00	0.50	1.00	1.00	1.00	0.00	0.00	0.00	0.00	1.00	0.75
	M	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
	N	0.75	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00
	O	1.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SOD	A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	B	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.50	0.50	0.50	0.50	1.00	1.00	0.75	1.00
	D	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	1.00	0.50	0.50	0.50	1.00	1.00	0.75
	E	0.50	0.50	0.50	0.75	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	F	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table 3 The Shannon (H) diversity index for two enzyme systems among 18 populations of *N. complanata* and *N. crispa*.

Population	N.co.R	N.co.M	N.co.S	N.co.K	N.co.T	N.co. Do	N.co. D	N.co. A	N.co. L	N. c. K	N.c.T1	N.c.T2	N.c. N	N.c. R	N.c. Gh	N.c. Do	N.c. H	N.c. G	Mean
POD	1.938	1.660	1.528	1.860	1.903	1.688	1.430	1.468	1.613	1.263	1.098	1.351	0.000	0.000	0.682	0.714	1.872	1.088	1.286
SOD	1.351	1.351	1.351	1.379	1.386	1.386	1.386	1.386	1.386	1.386	1.351	1.351	1.329	1.329	1.351	1.386	1.379	1.379	1.364
Mean	1.645	1.506	1.440	1.620	1.645	1.537	1.408	1.427	1.500	1.325	1.225	1.351	0.665	0.665	1.017	1.050	1.626	1.234	1.354

Table 4 Euclidean distances; pair-wise comparisons of *N. complanata* and *N. crispa* populations based on isoenzyme diversities.*

	N.co. R	**N.co.M**	**N.co. S**	**N.co. K**	**N.co. T**	**N.co. Do**	**N.co. D**	**N.co. A**	**N.co. L**	**N.c. R**	**N.c. N**	**N.c. H**	**N.c. K**	**N.c. G**	**N.c. T1**	**N.c. Gh**	**N.c. T2**	**N.c. Do**
N.co. R	0.000																	
N.co.M	2.000	0.000																
N.co. S	2.000	1.414	0.000															
N.co. K	2.646	2.236	1.732	0.000														
N.co. T	2.449	2.828	2.449	2.236	0.000													
N.co. Do	2.449	2.449	2.000	2.236	2.000	0.000												
N.co. D	2.449	2.449	2.449	2.646	2.449	2.828	0.000											
N.co. A	2.646	1.732	1.732	2.000	2.236	1.732	2.236	0.000										
N.co. L	2.000	2.449	2.000	2.646	2.000	2.000	2.828	2.236	0.000									
N.c. R	3.162	2.828	2.828	3.317	2.828	3.162	2.828	2.646	2.828	0.000								
N.c. N	3.162	2.449	2.449	3.000	3.162	2.828	3.162	2.236	2.828	1.414	0.000							
N.c. H	3.162	2.449	2.449	2.646	3.464	3.162	3.162	2.646	3.162	2.000	1.414	0.000						
N.c. K	3.162	2.449	2.449	2.646	3.162	2.828	2.828	2.236	3.162	2.000	1.414	1.414	0.000					
N.c. G	3.162	2.449	2.449	2.646	3.162	2.828	2.828	2.236	3.162	2.000	1.414	1.414	0.000	0.000				
N.c. T1	3.317	2.646	2.646	2.828	3.000	2.646	3.000	2.000	3.000	1.732	1.000	1.732	1.000	1.000	0.000			
N.c. Gh	3.464	2.828	2.828	3.000	3.162	2.828	2.828	2.236	3.162	2.000	1.414	2.000	1.414	1.414	1.000	0.000		
N.c. T2	3.162	2.449	2.828	3.317	3.464	3.162	3.464	2.646	3.162	2.000	1.414	2.000	2.000	2.000	1.732	2.000	0.000	
N.c. Do	3.162	2.449	2.449	3.000	3.162	2.828	3.162	2.236	2.828	1.414	0.000	1.414	1.414	1.414	1.000	1.414	1.414	0.000

*This is a dissimilarity matrix

The isoenzymes method has provided conditions to be successful in supporting classification (Cheniany *et al*., 2007). However, no studies are dealing with isoenzyme variation on populations of *N. crispa* and *N. complanata*. So, we used an isoenzyme marker for the classification of species and populations. The results of the morphometry method and the results of isozyme banding patterns were the same, although very minor differences were observed. Although, in some cases, the morphological analysis of plants and isoenzyme studies gave conflicting results (Melosik *et al*., 2005). Several reasons have been discussed for low agreement between the isoenzyme banding patterns and morphometry methods. The difference in the genetic regulation of the markers and gene expression of morphological traits is due to the environmental conditions in which it grows, for the most important reasons (Royo *et al*., 2004). Appelgren and Cronberg (1999) based on isoenzyme studies, stated that there are two different genotypes with similar morphology in *N. pennata* that are genetically distinct but have a similar morphology. It seems that the merging of N.co.L and N.co.R belonging to the *N.complanata* species may be due to the similar genetic differentiation of the plant with preserved morphology. Mosses are due to the prevalence of asexual reproduction and also because of dominance at gametophytic generation containing a little genetic variability between and within the populations. But in epiphytic mosses the spores are released from the ground at a higher level, so the potential for long-distance dispersal of spores causes increased sexual reproduction and genetic variation (Rosengren *et al*., 2014). The most amounts of Shannon (Ĥ) diversity as 1.645 (Table 4) for population N.co. R in comparison with other populations, suggesting a high variation in this region. This variation can be explained by the occasional establishment of spores as well as frequent cross-fertilization in the unisexual species (Akiyama, 1994; Appelgren and Cronberg, 1999). The results of Melick *et al*., 1994 showed clear genetic differences between the *Grimmia antarctici*, *Bryum pseudotriquetrum,* and *Ceratodon purpureus*, also a little intraspecific diversity was observed. The results of Melosik *et al*., 2005 showed that diversity of isoenzyme bands can be used to differentiate Sphagnum moss species and also suggested that peroxidase and acid phosphatase isoenzymes were more effective in identifying *Sphagnum* subsecundum than other enzymes studied. The results of this study also found that isoenzymes POD is effective for the differentiation of the studied populations. Therefore, it can be said that peroxidase is an effective enzyme in isolation of other moss species. Atmospheric pollution and endangered habitats for epiphytic mosses have reduced genetic diversity (Cronberg, 2000). Because of the destruction of many habitats in the northern provinces of Iran and the increase in pollution in these areas, it can be said as a general result that perhaps the reason for low genetic diversity in *N. complanata* and *N. crispa* populations is the gradual extinction of these two species. As before *N. crispa* was reported in Golestan and Mazandaran provinces but we found it just Mazandaran province, it can be a reason for the destruction.

Conflicts of interest

We have no conflict of interest.

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