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Assessment of SCoT and DAMD molecular markers in genetic diversity and species delimitation of three moss species grown in Iran

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Abstract

This study was conducted to assess the efficacy of SCoT and DAMD molecular markers in genetic diversity of three moss species and compare them with ISSR marker. Another objective was to evaluate the suitability of these DNA markers in species identification (delimitation) in three moss species, including *Neckera complanata*, *Homalothecium sericeum* and *Neckera crispa*. To prevent ecological impact on results, all samples were collected from one locality. PIC and MI in three markers showed closely ranged values. Our results revealed that the average values of Rp and the number of species-specific bands in SCoT primers were more than those of DAMD and ISSR. It showed considerable capability of SCoT marker in discriminating individuals. The highest value of genetic parameters Na (1.08), He (0.15) and I (0.23) were obtained with SCoT marker showing the power of this marker in genetic diversity analysis. Moreover, dendrograms produced from SCoT and DAMD data indicated similar results in the placement of closely related species. SCoT markers were shown to be more efficient than DAMD and ISSR markers based on the multiplex ratio (EMR), Rp, genetic diversity parameters (Na, He, I) and the number of species-specific bands. The findings demonstrated that the SCoT and DAMD markers could be applied for the estimation of genetic relationships and separation of two closely related genera. This is the first report of its type on the genetic diversity of mosses by application of SCoT and DAMD.

Key words: DAMD, Efficiency, Genetic diversity, Moss, SCoT.

INTRODUCTION

One of the main objectives in plant conservation programs is to comprehend the rate of genetic variation and conduct population studies among genotypes in plant genera. Levels of genetic diversity are influenced by gene flow and stochastic processes, as well as by natural selection and adaptation (Rosengren *et al*., 2013). Molecular genetic analyses of DNA represent a new challenge in biological conservation and provide high precision estimates for many critical parameters to conservation, such as population size, intraspecific genetic variation, gene flow, and genetic distance among populations (Terracciano *et al*., 2012). In this way, DNA molecular markers make it possible to assess the level of genetic diversity in different populations and evaluate the survival potential of species in threatened habitats (Terracciano *et al*., 2012).

The markers may show dominant/recessive or co-dominant inheritance as far as the nuclear genome is concerned. There are different DNA molecular markers like RAPD, ISSR, SRAP and RFLP for the estimating of genetic variation in plant species. One of the new and effective markers called Start Codon Targeted (SCoT) was applied based on the short conserved region flanking the ATG start codon in plant genes (Amirmoradi *et al.,* 2012).

SCoT markers as effective polymorphic markers produce more reproducible and reliable bands which show differences among individuals exceptionally well. Another reliable and reproducible technique is called directed amplification of minisatellite region DNA (DAMD) for directed amplification of minisatellite in PCR under high stringency conditions (Pakseresht *et al*., 2013).

Bryophytes were some of the first green plants to successfully colonize land 470–551 million years ago (mya) from aquatic algal ancestors. They represent a significant component of the ground vegetation with nearly 23,000 taxa distributed in the world. These are classified under three groups: the mosses (14,000 species), the liver-worts (6000 species), and the hornworts (300 species). Mosses are considered to have the most significant number of species among green plants, next to angiosperms (Budke *et al*., 2018). The potential for medical use in bryophytes is one of the reasons for exploring biological compounds. Bryophytes contain numerous potentially useful compounds, including, polysaccharides, sugar alcohols, fatty acids, aliphatic compounds, and aromatic and phenolic substances. Traditional herbalists list many uses for mosses, both the whole plants (sometimes used fresh, sometimes dried and ground) and the spores. In herbal medicine, moss is most commonly used as a diuretic or as a cure for coughs, depending on how the moss is processed and which moss is used. For example, *Neckera complanata* (Hedw.) Huebener*, Homalothecium sericeum* (Hedw.) Schimp and *Neckera crispa* Hedw. were used for antimicrobial activity and as a pesticide (Glime, 2017; Ozturk *et al*., 2018).

In recent years, molecular markers have been successfully applied for the classification and estimating genetic diversity in some species of bryophytes. The markers such as SSR, SCAR, RAPD, ISSR, ISJ, AFLP and ITS sequences primarily have used in some mosses. However, there is no report on the utilization of SCoT and DAMD techniques in bryophyte (Sawicki and Szczecińska, 2011).

The present survey was aimed to study the efficiency of these methods on DNA polymorphism and other genetic diversity parameters in mosses. Another objective of the present study was to evaluate the suitability of three types of DNA markers for species identification (delimitation) in three moss species *Neckera complanata, Neckera crispa* (Neckeracae) *and Homalothecium sericeum* (Brachytheciaceae). There are morphological similarities between *N. crispa* and *N.complanata*. *N. crispa* has glossy leaves which are undulate both when moist and dry while, the undulate leaves have never been seen in the moist status of *N. complanata*. Dry *N. complanata* may appear slightly undulate, but it never has leaves with a markedly undulate surface like those of *N. crispa* (Atherton *et al.,* 2010). Considering these morphological difficulties, we employed this genus as a suitable subject to determine the efficiency of the mentioned markers in mosses. In this way, comparing this genus to other genus belonging to a different family (*Homalothecium*, Brachytheciaceae) could be helpful. Although the importance of these two families is postulated because of the antimicrobial activity (Oztopcu-Vatan *et al.,* 2011).

MATERIALS AND METHODS

Plant materials and DNA extraction

Nine moss samples from three species, *Neckera complanata, Homalothecium sericeum,* and *Neckera crispa* were gathered. To prevent ecological impact on results, we collected all samples from one locality in Sisangan forest, Noor, Mazandaran province in the north of Iran. Fresh leaves were collected randomly from 3 plants in each of the studied populations and dried using silica gel powder. A CTAB modified protocol was used to extract genomic DNA (Ghasemzade Baraki *et al.*, 2018). The quality of extracted DNA was examined by running samples on a 0.8% agarose gel.

PCR amplification and electrophoresis

Three ISSR primers, three SCOT primers, and three DAMD primers were used. PCR reactions were carried out in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Cinna Gen Co, Iran), 0.2 µM of a single primer, 20 ng genomic DNA, and 3 U of Taq DNA polymerase (Cinna Gen Co, Iran). The amplification reactions were performed with a T100 thermocycler (BIORAD, USA) with the following program: 5 min initial denaturation step at 94 °C, followed by 40 cycles of 1 min at 94 °C, annealing at 55 °C (ISSR) , 57 °C (SCOT) and 59 °C (DAMD) for 1 min and extension at 72 °C for 2 min. The reaction was completed by a final extension step of 10 min at 72 °C. The amplification products were observed by running samples on a 1% agarose gel. Gels were photographed under UV light. PCR fragment sizes were estimated using a 100 bp DNA ladder (Fermentas, Germany).

Data analyses

Reproducible bands of each locus were scored as binary present (1) or absent (0) and data matrices of SCOT,

DAMD and ISSR loci were assembled for further analysis. Parameters including Nei's gene diversity (H), Shannon information index (I), the number of effective alleles (Ne), and percentage of polymorphism (P%) were determined (Weising, 2005; Freeland *et al*., 2011).

Dice genetic distance among populations was used for Neighbor-Joining (NJ) clustering. These analyses were conducted with PAST ver. 2.17 software (Hammer *et al*., 2012).

Analysis of molecular variance (AMOVA) test (with 1000 permutations) was done by GenAlex 6.4 software (Peakall and Smouse, 2006). We calculated polymorphism information content (PIC), Marker index (MI), Effective multiplex ratio (EMR), Discriminating power (D) and Resolving power (Rp) for each primer using PICcalc (Nagy *et al*., 2012).

RESULTS

Efficiency of markers in determining DNA Polymorphism

In early experiments, three SCoT primers, three ISSR primers, and three DAMD primers were applied to investigate nine samples of three moss species. All of the primers displaying separate and reliable band patterns were employed for band scoring, following genetic similarity analysis and cluster analysis. Figures 1-3 showed patterns of banding belonging to some of SCoT, DAMD and ISSR primers.

A set of three SCoT primers were used to fingerprint nine samples of mosses. SCoT primers amplified a total of 80 fragments, with a mean of 26.6 bands (range of 23-29) per primer. Of the 80 bands, 78 (97.5%) were polymorphic. Polymorphism percentage ranged from 93.1% to as high as 100% with an average

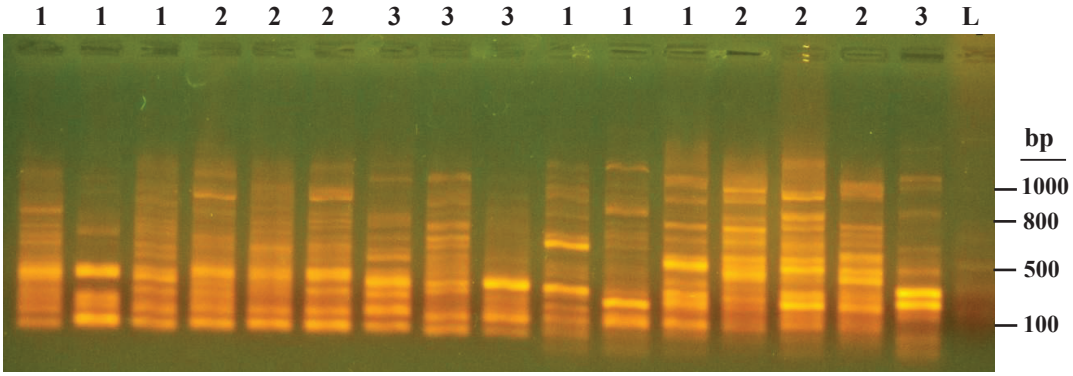

Figure 1. Gel photograph of SCoT amplified products using S 1 and S 7 primers (L. 100 bp molecular- size marker, fermentas). The numbers are in accordance with Table 2.

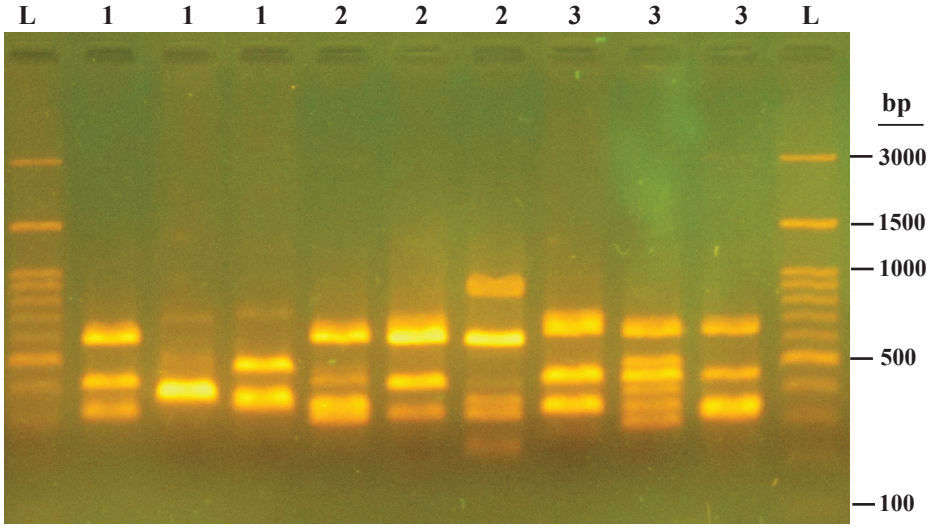

Figure 2. Gel photograph of DAMD amplified products using primer URP 38F (L. 100 bp molecular- size marker, fermentas). The numbers are in accordance with Table 2.

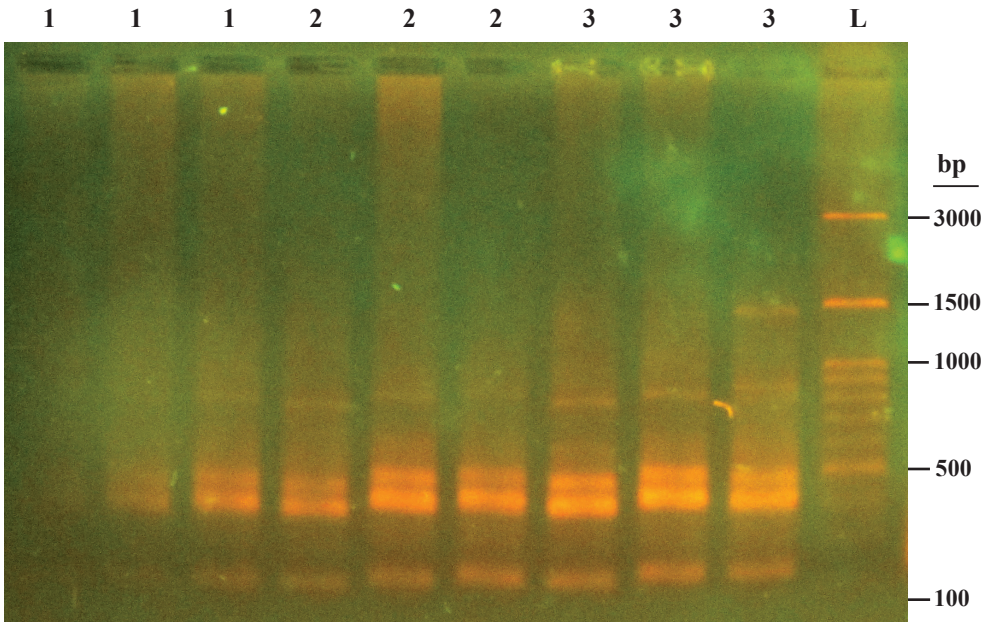

Figure 3. Gel photograph of ISSR amplified products using primer AGC5GC. (L. 100 bp molecular size marker, fermentas). The numbers are in accordance with Table 2.

Table 1. Marker parameters calculated for each SCOT, DAMD and ISSR primers.

Primer		Sequence 5' -3'	Bands detected (%)			PIC	MI	RP	EMR	D
			TB	PB	PPB					
ScoT	S1	CAACAATGGCTACCACCA	29	27	93.1	0.3750	0.025	18	13.33	0.789
	S7	CAACAATGGTCACCACGG	28	27	96.42	0.3753	0.025	18.66	12.77	0.792
	S18	ACCATGGCTACCACCGCC	23	22	95.65	0.3735	0.028	13.77	11.66	0.743
DAMD	URP 9F	ATGTGTGCGATCAGTTGCTG	16	14	87.5	0.3782	0.031	9.33	9.11	0.677
	URP 38F	AAGAGGCATTCTACCACCAC	17	17	100	0.3861	0.020	9.11	6.55	0.852
	OGR B01	AGGGCTGGAGGAGGGC	15	13	86.66	0.3745	0.025	8.44	7	0.784
ISSR	GA9A	GAGAGAGAGAGAGAGAGAA	14	9	64.28	0.4016	0.030	4.66	10.66	0.42
	AGC5GG	AGCAGCAGCAGCAGCGG	13	11	84.61	0.3433	0.029	6.88	6.88	0.72
	AGC5GA	AGCAGCAGCAGCAGCGA	10	7	70	0.3468	0.030	4	5.66	0.68

TB: Total band, PB: Polymorphic band, PPB: Percentage of polymorphic band, PIC: Polymorphic information content, EMR: Effective multiplex ratio, MI: Marker index, RP: Resolving power of primer, D: Discriminating power.

polymorphism of 96.50% across all samples. PIC values ranged from 0.512 (S1) to 0.519 (S18), with an average value of 0.517 per primer (Table 1). Genetic diversity parameters were determined for the studied populations and primers. The highest values for Ne (1.088), He (0.159), I (0.231) and H (1.284) were obtained in *Neckrea complanata* population while the lowest values were obtained in *Neckera crispa* (Table 2). Rp values ranged from 13.77 to18.66 using SCoT marker. The EMR values for this marker indicated ranges of 11.66-13.33. Also, MI values were about 0.025-0.028.

Three DAMD primers were used to assess diversity in nine moss samples. DAMD primers generated 48 fragments with a mean of 16. Of the 48 bands, 44 (91.66%) were polymorphic, 13 in URPOGR to 17 in URP 38F polymorphic bands were amplified by primers, with an average of 14.6 polymorphic bands per primer. The polymorphism per primer among the tested species ranged from 86.66% in URPOGRto 100% URP 3F, with an average of 91.38%. The polymorphic index ranged from 0.159 in URPOGR to 0.612 in URP 38F, with an average of 0.13. The PIC varied from 0.524 in 9F to 0.540

in OGR, with an average of 0.531. (Table 1) Genetic diversity parameters were determined for the studied populations and primers. The highest values for Ne (0.91), He (0.105), I (0.15) and H (1.182) were obtained in population *Homalothecium sericeum* while the lowest values of them occurred in *Neckera crispa* (Table 2). Rp values ranged from 8.44 to 9.33 using DAMD marker. The EMR values for this marker ranged between 6.55 to 9.11. Also, MI values were about 0.02-0.03 (Table 1).

ISSR analysis revealed polymorphic and monomorphic bands, in total, 37 bands by using three primers. The number of amplified fragments varied from 10 to 14, with average12 bands per primer. Out of 37 bands, 25 bands were polymorphic, and the polymorphism percentage average was 0.65 across all the genotypes. A maximum number of polymorphic bands. The average polymorphic information content (PIC) was 0.54, ranging from 0.53 (GA9A) to 0.56. The polymorphism per primer among the samples ranged from 57.4% (GA9A) to 80% (AGC5GG), with an average of 67.56%. (Table 1).The highest values for Ne (0.97), He (0.079), I (0.112) and H (1.155) were obtained in *Neckera crispa* population while the lowest values of them were obtained in *Neckera complanata*. (Table 2). Rp values ranged from 4-6.88 using ISSR marker. The EMR values for this marker indicated a range of 5.66 to 10.66. Also, MI values were about 0.03 (Table 1).

AMOVA test

The analysis of Molecular variance (AMOVA) using SCoT marker indicated a significant molecular difference (PhipT=0.701, P=0.03) and a great level of genetic differentiation among the studied populations. It also revealed that 70% of total genetic diversity was related to inter-population differences, while 30% was related to intra-population differences. Similarly, in DAMD, the inter-population genetic differentiation was at the highest level (71%) and (29%) within populations (PhipT=0.711, P=0.03). ISSR data indicated that the genetic variations observed are due to genetic differences among the populations 77% (PhipT=0.770, P=0.01). Genetic variance among the populations (Est) were 15.55, 8.74, and 5.59 in DAMD, ISSR, and SCOT markers, respectively.

Species-specific marker

The applied DNA markers revealed a total of 55 species-specific bands. Bands occurred within and among species. Among used markers, SCoT primers, which revealed 32 bands, were found to be most effective for the molecular identification between species. S7 primer

Table 2. Genetic diversity parameters obtained by each marker in three different species.

GP	Sp	V. No.	PPB (%)			Na			Ne			I			He			NSB		
			S	D	IS	S	D	IS	S	D	IS	S	D	IS	S	D	IS	S	D	IS
1	*Neckera complanata*	HSBU201901	37.75	20.83	5.41	1.088	0.708	0.568	1.28	1.177	1.037	0.231	0.133	0.031	0.159	0.094	0.021	6	0	0
2	*Holalothecium sericeum*	HSBU201907	17.50	27.08	18.09	0.75	0.917	0.919	1.14	1.182	1.138	0.109	0.155	0.112	0.076	0.105	0.077	11	6	0
3	*Neckera crispa*	HSBU201909	17.50	18.75	16.2	0.663	0.771	0.973	1.12	1.135	1.155	0.101	0.111	0.110	0.069	0.076	0.079	12	8	3

V. No.: Voucher number, Ne: Mean number of effective alleles, He: Gene diversity, PPB: Genetic polymorphism percentage, Na: The mean number of different alleles over all loci, S: SCoT, D: DAMD, IS: ISSR, NSB: Number of species-specific band.

revealed 14 species-specific bands, while 12 bands were amplified by S18 and six bands were amplified by S1.

DAMD markers revealed a total of 20 species-specific bands. Nine species-specific bands were amplified by URP9F. Eight amplicons were amplified by URP38F, whereas three bands were amplified by URFOGR.

ISSR markers were less efficient in the identification of mosses species. Only three species-specific loci were revealed by AGC5GG (Table 2).

Apart from the number of revealed species-specific markers, another critical consideration is the ratio between them and the total number of amplified bands. The highest ratio between species-specific bands and amplified bands was obtained in SCoT markers (0.4) and DAMD (0.41). This ratio was substantially lower (0.08) in ISSR markers revealing the minimum number of species-specific bands. Among the mosses species studied, the highest number of bands were noted for *Neckera crispa*. A total of 23 species-specific bands were distinguished, of which 12 were revealed by SCoT markers and eight by DADM markers and three were revealed by ISSR marker. The molecular identification of *Homalothecium sericeum* was carried out based on 18 bands. Similarly, as in the case of *Neckera crispa*, SCoT revealed 12 species-specific loci, while DAMD marker amplified 6 species-specific bands. SCoT primers were found to be the most effective ones in molecular identification of *Neckrea complanata*, as they revealed 10 out of 16 species-specific markers.

Cluster analysis by SCOT, DAMD, and ISSR markers

In the NJ tree of SCoT analysis, nine moss samples fell into two major groups. The first main cluster was partitioned into two subgroups that were formed by samples of *Neckera crispa* and *Neckrea complanata,* respectively. The second major cluster included three samples of *Homalothecium sericeum* (Figure 4).

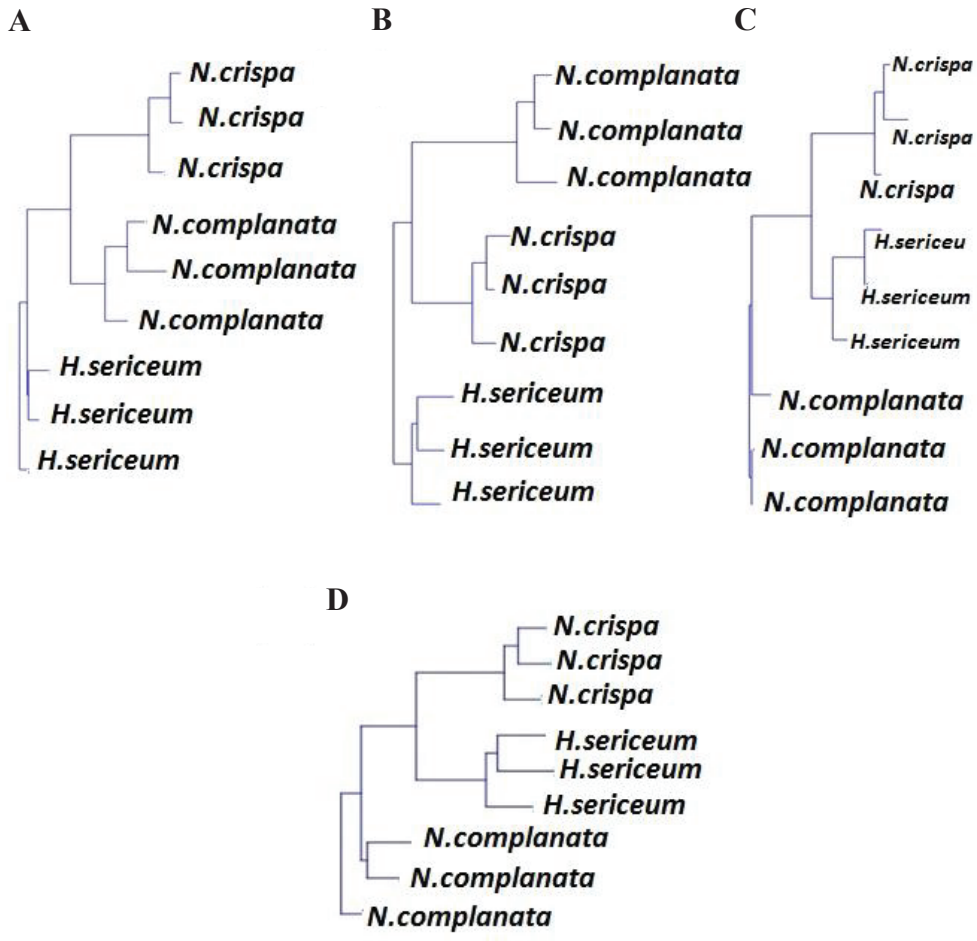

Figure 4. NJ tree based on Dice coefficient among three species revealed by **A:** SCOT, **B:** DAMD, **C:** ISSR markers and **D:** combined data of all three used markers.

NJ tree of DAMD analysis grouped nine populations into two major groups and exhibited comparatively similar grouping with the NJ tree of SCoT analysis. The first major cluster included two subclusters of three samples of *Neckrea complanata* and *Neckera crispa.* The second cluster consistd of samples of *Homalothecium sericeum.* The similarity between samples clustered by DAMD and SCoT analysis, was relatively higher than those obtained by ISSR markers.

NJ tree of the ISSR analysis also produced two major groups, but unlike the other two markers, the first major cluster consisted of two subgroups with *Neckrea complanata* and *Homalothecium sericeum* samples while *Neckera crispa* samples were in the second major cluster.

By using the combined data of the three sets of molecular markers, the general dendrogram was divided into three distinct clusters, and it was similar to those obtained separately with each marker (Figure 4). The dendrograms created by DAMD and SCoT data was most compatible with the general dendrogram.

DISCUSSION

During the past two decades, the application of genomic information has played an essential role in several aspects of biology including taxonomy, phylogeny, biogeography and population studies (Crespo Pardo *et al*., 2014.). Even though sequence-related markers produce beneficial results in phylogeny and wide geographical scale of population studies, but they are not suitable for all research fields such as polymorphism assessment at small geographic scales and inter/ intraspecies. Banding-based molecular approaches are recognized as useful tools in these studies (Crespo Pardo *et al*., 2014). In this way, molecular techniques based on banding pattern have been used in different bryophyte studies. For example, RAPD, ISSR, AFLP and RFLP have been applied in bryophytes to study population genetic structure, polymorphism (Skotnicki *et al*., 2000, 2001; McDaniel *et al*., 2007; Natcheva and Cronberg, 2007) and species-level systematic (Boisselier-Dubayle and Bischler, 1994; Boisselier-Dubayle *et al*., 1995; Vanderpooter *et al*., 2003).

In the present study, ISSR, SCoT, and DAMD markers were used to compare DNA polymorphism and to estimate their efficacy in mosses species identification for the first time in Iran.

Sivaprakash *et al.* (2004) stated that the efficiency of a marker approach in genetic diversity studies is closely related to the degree of its polymorphism. The present investigation proved that SCoT and DAMD markers with an average of 97.5% and 91.66% polymorphism were more suitable markers for genetic variation assays than ISSR (65% polymorphism).

Spagnuolo *et al*. (2002) studied the population genetics of *Pleurochaete squarrosa* with ISSR marker and indicated 20-68% polymorphism and SHANNON index (I) of 0.09-0.31.

Also, EMR, PIC, and MI were investigated for detecting the efficiency of markers in showing polymorphism. EMR or multiplex ratio is the number of loci polymorphic in the germplasm set of interest analyzed per experiment. SCoT pointed out the highest value (12.58), followed by ISSR and DAMD. The PIC and MI of each primer are useful in showing the efficiency of markers for the analysis of genetic diversity (Heikrujam *et al*., 2015).

In our investigation, PIC and MI in three primers showed closely ranged values. However, for average PIC, both DAMD and SCoT scores were higher than ISSR. Similar results were reported in flowering plants and indicated high values of PIC obtained from SCoT markers comparing with ISSR (Gorji *et al*., 2011; Paliwal *et al*., 2013; Rajesh *et al*., 2015; Etminan *et al*., 2016; Yadav *et al*., 2016; Pour-Aboughadareh *et al*., 2017, 2018).

Moreover, another remarkable feature of an appropriate marker system is the discriminating power among different taxa (Crespo Pardo *et al*., 2014). Our results displayed that the average values of Rp obtained by SCoT primers (16.81) were higher than those obtained by DAMD and ISSR primers (8.95 and 5.18). This showed the high capability of the SCoT marker in the discriminating individuals. In this way, dendrograms produced by SCoT and DAMD data indicated similar results in the relative placement of the studied mosses.

In this study, the clustering pattern with ISSR markers showed an almost independent result. SCoT and DAMD were much more helpful than ISSR in species delimitation. The difference of clustering could be explained by several species-specific bands produced by ISSR (3 bands) compared to DAMD (20 bands) and SCoT (32 bands). There is no similar study in bryophytes with these three markers to compare, but Sawicki and Szczecinska (2011) compared two species of Sphagnum by RAPDs, ISJs and ISSRs markers and indicated that ISSR marker revealed the greatest number of species-specific bands (14 bands).

However, in flowering plants, SCoT molecular

system has been successfully used in genetic diversity and diagnostic delimitation like potato, grape, peanut, *Dendrobium nobile* , *Cicer* and mango (Gorji *et al*., 2011; Xiong *et al.,* 2011; Luo *et al*., 2011; Guo *et al*., 2012; Amirmoradi *et al*., 2012; Bhattacharyya *et al*., 2013).

Gene-targeted markers as DAMD and SCoT are derived from gene regions of the genome while ISSR primers amplify neutral regions of genomes. Therefore, this brings advantageous scores for DAMD and SCoT markers that causes them to be more capable in genetic diversity and related studies (Heidari *et al*., 2017).

In conclusion, SCoT markers demonstrated to be more efficient than DAMD and ISSR markers based on the multiplex ratio, Rp, genetic diversity parameters, and a number of species-specific bands. This is the first report of its type on the genetic diversity of mosses by application of SCoT and DAMD. The present results exhibited that the genetic assessment using these gene-targeted markers would be more applicable for theoretical and practical aspects of bryology like species delimitation, population genetic structure between related species and conservation (Heidari *et al*., 2017). More remarkably, the finding demonstrated that the SCoT and DAMD markers could be applied for the estimation of genetic relationships, which ultimately would be resulted in the separation of two closely related genus.

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