Exploring genetic variations in threatened medicinal orchids using start codon targeted (SCoT) polymorphism and marker-association with seed morphometric traits

Soheila Gholami[1] · Yavar Vafaee[1,2] · Farzad Nazari[1] · Abdolbaset Ghorbani[3]

Abstract We aimed to study the genetic diversity and population structure of eight Iranian terrestrial orchid species, including *Anacamptis coriophora* (L.) R. M. Bateman, Pridgeon and M. W. Chase, *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski, *Himantoglossum affine* (Boiss.) Schltr., *Orchis collina* Banks and Solander, *Orchis mascula* (L.) L., *Orchis simia* Lam., *Ophrys schulzei* Bornm. and Fleischm., and *Ophrys straussii* H. Fleischm. and Bornm. using start target codon markers (SCoT) and finding markers associated with seed morphometric traits. A total of 254 reproducible SCoT fragments were generated, of which 248 fragments were polymorphic (average polymorphism of 96.18%). The SCoT markers showed a narrow range of polymorphism information content (*PIC*) varied from 0.397 for S9 primer to 0.499 for S11 and S20 primers. Based on the population analysis results, the *Orchis simia* accessions collected from Paveh region (Os.P) represented the lowest observed number of alleles (*Na*) (1.13) and effective number of alleles (*Ne*) (1.09). At the same time, the highest *Na* (1.29) and *Ne* (1.18) values

were obtained in *O. schulzei* collected from Javanrood (Oyst.JA). Shannon's information index (*I*) was ranged from 0.03 for *D. umbrosa* accessions collected from Marivan (Du.M population) to 0.263 for Ha.Ja population (*H. affine* accessions collected from Javanrood). The UPGMA dendrogram obtained with the Jaccard similarity coefficient (*r* = 0.97295) divided 97 studied terrestrial orchid accessions into eight groups mainly based on species type and geographical origin. Based on the Bayesian statistical index, the highest probability of the data was achieved when accessions were divided into eight groups (K = 8). Multiple association analysis (MRA) revealed significant associations between some of SCoT bands with seed morphometric traits. Our findings can be useful for germplasm characterization, conservation, and improvement of Iranian terrestrial orchid species.

Abbreviations

CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
MRA	Multiple Association Analysis
PCoA	Principal Coordinate Analysis
PIC	Polymorphism Information Content
SEM	Scanning Electron Microscope
UPGMA	Un-weighted Paired Group Methods with Arithmetic Mean

✉ Yavar Vafaee
y.vafaee@uok.ac.ir

1 Department of Horticultural Science and Engineering, Faculty of Agriculture, University of Kurdistan, 66177-15175 Sanandaj, Iran

2 Medicinal Plants Breeding and Development Research Institute, University of Kurdistan, 66177-15175 Sanandaj, Iran

3 Department of Organismal Biology, Evolutionary Biology Centre, Uppsala University, Norbyvagen 18D, SE-75236 Uppsala, Sweden

Introduction

With more than 28,000 species comprising over 763 genera, *Orchidaceae* is the second largest family within the plant kingdom. Orchids have spread worldwide from tropical to high alpine areas (Phillips et al. 2020). Remarkable flower beauty and diversity, unusual seeds, exceptional and complicated pollination, and reproduction system, as well as particular symbiotic interactions with mycorrhiza fungi, have led to focusing comprehensive scientific researches on various biological aspects of orchid species (Cozzolino and Widmer 2005; Sherif et al. 2020). Among different life forms of orchids, terrestrial orchids are usually grown in soil and generate a round or palmate underground fleshy tuber (de Boer et al. 2017). These fleshy tubers are currently used to make traditional ice-cream and beverages with unique rheological properties in the minor Asia and the Middle East (Farhoosh and Riazi 2007).

Compared with neighboring regions, orchids are rather rare and usually not numerous in Iran. This is due to its situation, where the western, north-western and eastern marginal zones are the only hotspots of much richer orchid centers. The Iranian orchids have spread over various habitats not only over the Hyrcanian and dry oak forests but also throughout the alpine wet meadows, primarily the Alborz and the Zagros Mountains. They mainly belong to European-Mediterranean species within *Dactylorhiza*, *Orchis*, *Cephalanthera*, *Epipactis* and *Ophrys* genera (Renz 1978). No connection is present between the two hotspots of orchids distribution in the western and eastern periphery of the Iran plateau. This is because the deserts are located at the center of the country which has separated the European-Mediterranean species from the Himalayan species that can be seen in the far east of the country (Kreutz and Spencer 2011). This wide gap has limited the possible gene flow of orchids in either direction. Based on the Flora Iranica, 46 tuberous orchid species are grown in the Iran, mainly in western regions, including Kurdistan, Kermanshah, and West Azerbaijan provinces (Kreutz and Spencer 2011; Renz 1978). Among Iranian terrestrial orchids, 36 species have been exploited for their fleshy tubers, and only in 2013 around 7–11 million orchid tubers have been dug and collected causing depletion of natural populations (Ghorbani et al. 2014a, b). All Iranian orchids are listed in the Convention on International Trade of Endangered Species of Fauna and Flora (CITES) appendices (Appendix I or II) (CITES 2014). Besides, over-harvesting, the Iranian terrestrial orchids have also experienced an extensive range of fragmentation and decreased the population sizes due to climate change (Nosrati et al. 2011). Despite the extensive commercial usage, Iranian tuberous orchids have not already been domesticated and are exclusively collected from nature, leading to even the local distinction of some species (Moradi et al. 2015; Ghorbani et al. 2014b).

Comprehensive and detailed information on genetic variability and population structure is fundamental to highlight the long-term population viability and adaptation under dynamic environmental conditions (Ebadi et al. 2019; Vafaee et al. 2008). The knowledge on genetic variability of terrestrial orchids can provide baseline genetic information for the development of sustainable conservation programs and for breeding and improvement strategies (Vafaee et al. 2017).

Little information is available on the genetic diversity of Iranian terrestrial orchid species, and there are only few studies available on these threatened species. Vafaee et al. (2021) evaluated genetic diversity of a collection of 107 individuals of Iranian terrestrial orchids using floral- and tuber-related traits and seed micromorphology characteristics. Ghorbani et al. (2017) determined the phylogeny of the Iranian orchids based on DNA-metabarcoding analyses at the genus and species levels. Kaki et al. (2020) investigated intra- and inter-population genetic variation and genetic distance among five medicinal orchid species using the IRAP marker. ISSR marker was also successfully applied to elucidate the genetic variation among individuals and inter- and intra-population diversity in a collection of Iranian terrestrial orchids (Gholami et al. 2021). Hence, DNA-based molecular markers like SCoT have not yet been used to study genetic variation in medicinal Iranian orchid species. On other side, seed and testa micro-morphological traits have been used as taxonomical and phylogenetic markers on a vast range of orchid species (Barthlott et al. 2014). In this regard, scanning electron microscopy (SEM) has come to aid the analyses of orchid seeds and their microstructures (Aybeke 2007).

Among DNA-based molecular markers, start codon targeted (SCoT) polymorphism has been introduced as a gene-targeted molecular marker system principally different from random DNA markers and it generally relies on the short conserved nucleotide region surrounding the ATG initiation codon of plant genes (Collard and Mackill 2009). SCoT markers are advantageous over other PCR-based molecular markers because of low cost, easy operation, high loci amplification and polymorphism, high reproducibility and reliability and suitability for marker-assisted selection programs (Bhattacharyya et al. 2013; Collard and Mackill 2009). The SCoT markers have been effectively employed to study the genetic variation and population structure of different species within the *Orchidaceae* family, including *Dendrobium nobile* (Bhattacharyya et al. 2013), *Cypripedium japonicum* (Tian et al. 2018), *Eulophia* sp. (Bhattacharyya and van Staden 2018), *Huperzia serrata* (Minh et al. 2019), and *Paris polyphylla* (Zhao et al. 2020).

We assume that there is different genetic variation pattern among individuals of geographically distant populations and SCoT marker could be a good choice to elucidate this diversity pattern. On the other hand, as the seed morphometry of orchids are species-specific, we hypothesize that some seed micro-morphology traits can possibly be associated with some SCoT loci. This is the first study on the usability of SCoT markers on genetic diversity of endangered Iranian orchid species as well as the first report on the possible association between seed microstructure traits with molecular markers in species within *Orchidaceae* family which could also be exploited for breeding and conservation purposes. Therefore, the present research study was aimed to (i) evaluate important seed microstructure characteristics and their variation, (ii) study genetic diversity and population structure using 15 SCoT primers, and (iii) estimate associations between SCoT loci and evaluated seed micro-structure characteristics in a collection of 97 accessions belonging to eight terrestrial orchid species.

Material and methods

Plant material

A collection of 97 accessions belonging to sixteen geographically isolated populations of eight Iranian terrestrial orchids species including *Anacamptis coriophora* (L.) R.M.Bateman, Pridgeon and M.W.Chase, *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski, *Himantoglossum affine* (Boiss.) Schltr., *Orchis collina* Banks and Solander, *Orchis mascula* (L.) L., *Orchis simia* Lam., *Ophrys schulzei* Bornm. and Fleischm., and *Ophrys straussii* H. Fleischm. and Bornm. were evaluated (Fig. 1). Table 1 represents the population origin, code, accession number, and geographical coordinates of studied orchid populations. The distribution map of the collected populations has been shown in Fig. 1. The systematic identification of plant material was performed in the Herbarium of University of Kurdistan, where the voucher specimens of collected species have been deposited. A minimum of five accessions per population was considered for genetic diversity analysis. For SCoT analysis, young leaf samples were collected, labeled, and frozen in liquid nitrogen and finally kept at − 80 °C until DNA extraction.

DNA extraction and quantification

The total DNA was extracted from ∼ 100 mg leaf tissue according to the cetyltrimethylammonium (CTAB) based on the Doyle and Doyle (1987) procedure with minor modifications. The quantity and quality of the extracted DNAs were determined through both spectrophotometry (using a ND 1000 spectrophotometer, NanoDrop Technologies, Wilmington, DC, USA) and agarose gel electrophoresis methods. The DNA samples with optimal quality and purity were used for PCR amplification.

PCR reactions and SCoT analysis

To optimize PCR amplification, two DNA samples for each population were chosen for preliminary evaluation of primers by determining samples with satisfactory band profile and exclusion of primers with low quality and weak bands. From 19 assessed SCoT primers, 15 primers produced clear and consistent bands, and these primers were used for the final SCoT amplification. PCR reactions carried out in a final volume of 10 µl, comprising 20 ng pure DNA, 200 µM dNTPs, 0.4 U *Taq* DNA polymerase (SinaClone, Tehran, Iran), 1 µl 10X PCR buffer, 2 mM MgCl$_2$ and 10 pmol/µl SCoT primer. PCR reactions performed in a Biorad thermocycler (Bio-Rad, Hercules, CA, USA) based on the following program: 5 min initial denaturation at 94 °C followed by 30 cycles of 45 s denaturation at 94 °C, 60 s primer annealing at 50.7–58 °C and 90 s extension at 72 °C, with a final extension of 7 min at 72 °C. In addition to the main PCR amplifications, negative (water) and positive control samples were also tested to identify possible self-dimers or DNA contaminations and to check the consistency of amplified bands, respectively. The obtained DNA bands were separated on 1.5% agarose gel (SinaGene, Iran) at 90 V in 1 × TAE buffer for 120–150 min. The amplified DNA fragments were visualized by staining the agarose gels in 0.0015% w/v ethidium bromide solution and photographing under UV light by a Bio-Rad Gel Doc 2000 model (Bio-Rad, Hercules, CA, USA). Weak and ambiguous fragments were eliminated from scoring, and only clear and sharp fragments were considered for the next steps where the bands scored as 1 and 0 for the presence and absence, respectively. The PCR product size was determined using a 1 kb SinaGene DNA ladder (250–3500 bp).

Seed morphometric analysis

To analyze seed morphometric traits, fully ripen capsules were obtained for each individual and transported to the research lab at the Department of Horticultural Sciences and Engineering, University of Kurdistan. The obtained seed were further dehydrated for a minimum of 1 month and were maintained in closed paper bags. The light microscopic images taken by Olympus BX51 stereomicroscope (fitted with an Olympus DP72 camera) were used to determine seed testa color. To perform scanning electron microscope (SEM) experiment, the seed of each terrestrial

Fig. 1 Geographic locations and collection sites of the sample populations and inflorescence of studied terrestrial orchid species. **a** *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski, **b** *Himantoglossum affine* (Boiss.) Schltr., **c** *Orchis simia* Lam., **d** *Orchis mascula* (L.) L., **e** *Anacamptis coriophora* (L.) R.M.Bateman, Pridgeon & M.W.Chase, **f** *Ophrys schulzei* Bornm. & Fleischm., **g** *Ophrys straussii* H. Fleischm. & Bornm., **h** *Orchis collina* Banks and Solander
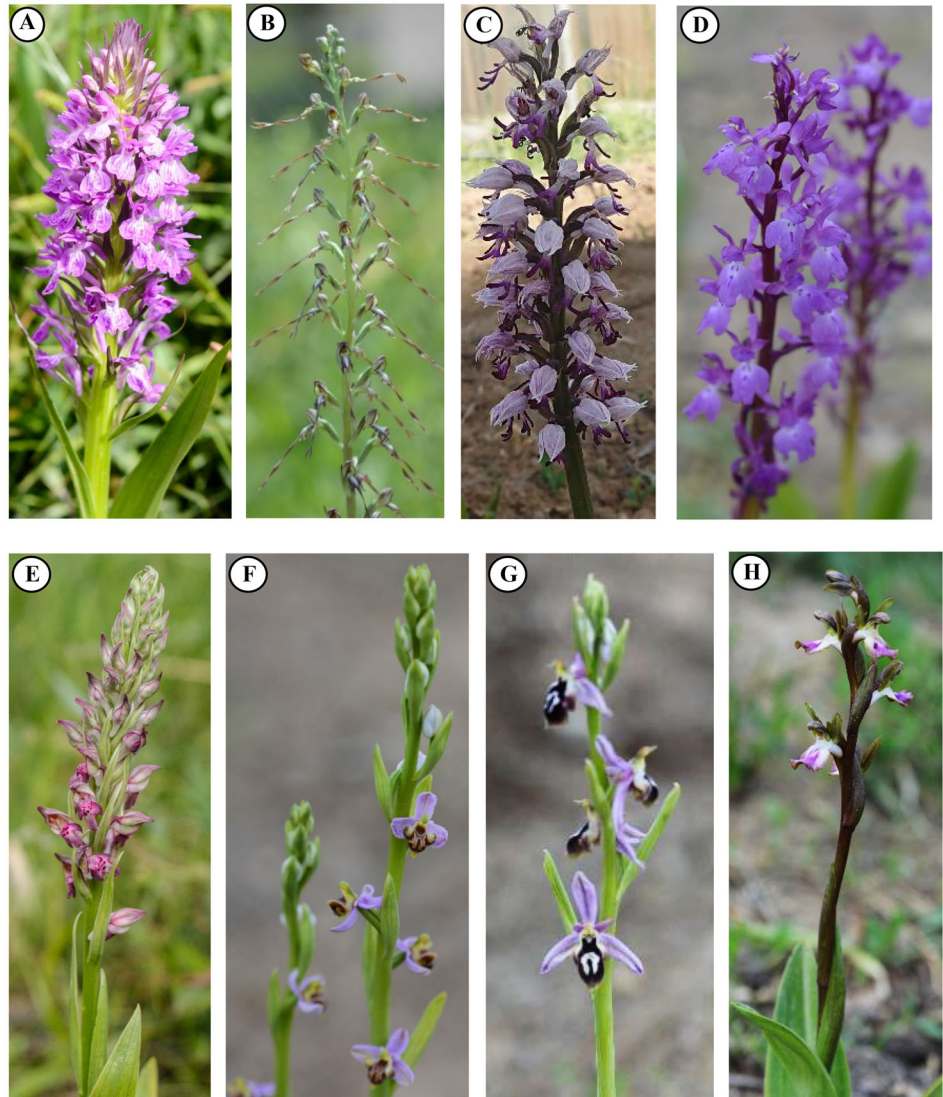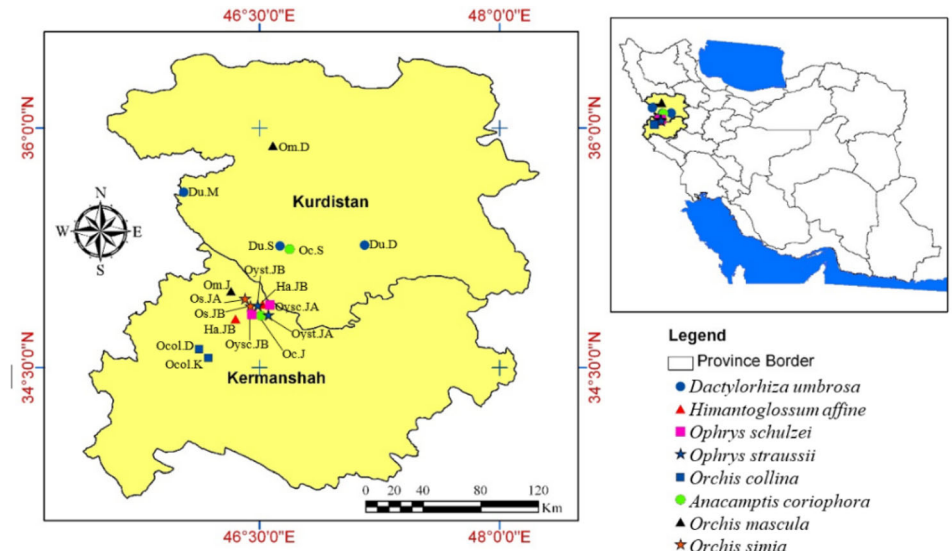
Table 1 Information on species, origin, sample size, population code and location of the sixteen Iranian terrestrial orchid populations

Species name	Origin	No.	Pop. Code	Latitude (N)	Longitude (E)	Altitude(M)
Dactylorhiza umbrosa	Sanandaj, Kurdistan	6	Du.S	46°37′48.5"	35°15′35.0"	1937
Dactylorhiza umbrosa	Dehgolan, Kurdistan	5	Du.D	47°09′32.9"	35°15′55.1"	2042
Dactylorhiza umbrosa	Marivan, Kurdistan	6	Du.M	46°01′47.8"	35°35′47.5"	1351
Himantoglossum affine	Javanrood, Kermanshah	6	Ha.JA	46°26′38.9"	34°53′17.6"	1644
Himantoglossum affine	Javanrood, Kermanshah	7	Ha.JB	46°24′18.4"	34°51′39.8"	1339
Orchis mascula	Paveh, Kermanshah	5	Om.Pa	46°19′31.8"	34°58′42.1"	1860
Orchis simia	Paveh, Kermanshah	5	Os.Pa	46°25′44.9"	34°53′18.0"	1644
Orchis simia	Javanrood, Kermanshah	5	Os.J	46°24′45.7"	34°55′58.9"	1767
Anacmptis coriophora	Salian, Kurdistan	7	Oc.S	46°38′26.6"	35°15′19.6"	1737
Anacmptis coriophora	Javanrood, Kermanshah	8	Oc.J	46°26′41.1"	34°53′7.3"	1280
Orchis collina	Kerend, Kermanshah	8	Ocol.K	46°7′31.2"	34°37′3.79"	2045
Orchis collina	Dalahoo, Kermanshah	5	Ocol.D	46°10′56.3"	34°33′45.8"	2149
Ophrys straussii	Javanrood, Kermanshah	7	Oyst.JA	46°26′33.9"	34°53′18.8"	1690
Ophrys straussii	Javanrood, Kermanshah	5	Oyst.JB	46°27′1.74"	34°53′10.5"	1710
Ophrys schulzei	Javanrood, Kermanshah	6	Oysc.JA	46°25′53.0"	34°53′10.2"	1470
Ophrys schulzei	Javanrood, Kermanshah	6	Oysc.JB	46°26′57.0"	34°53′18.2"	1390
Total		97				

orchid species were fixed on an aluminum SEM stub and were then coated with 10-nm gold particles. The SEM experiment was carried out using a FESEM Mira 3 scanning electron microscope (Tescan, Breno, Czech Republic) at 30 kV voltage. Rating and scaling were used to analyze seed qualitative micromorphology characteristics including testa color (TeCo), testa cell pattern (TeCePa), overall seed shape (OvSeSh), ridges on periclinal cell walls (RiPeWa), height of anticlinal walls (HeAnWa), and anticlinal wall curvature (AnWaCu). The obtained SEM images were used to evaluate quantitative attributes including seed width (SeWi), seed length (SeLe), seed length/width ratio (SeLe/Wi), seed cell length (SeCeLe), seed/cell length ratio (Se/CeLe), cell number per seed (CeNu/Se), and cell number in the longitudinal axis (CeNuLoAx). In this connection, the terminologies and procedures introduced by Barthlott et al. (2014), Akçin et al. (2009); Arditti and Ghani (2000), Szendrak (1997), Molvray and Kores (1995), and Chase and Pippen (1988) were used. The SEM experiment was performed in triplicate (3 capsules) where five seed were randomly selected for analyses.

Data analyses

The obtained mean values for seed micro-structure quantitative traits were used for statistical analysis. The descriptive statistical measures such as mean, minimum and maximum values, standard deviation (SD) and coefficient of variation (CV%) were estimated for the evaluated seed traits using PAST software (Hammer et al. 2001). The CV percentage as a variation index was also calculated using PAST software.

The genetic similarity among terrestrial orchid accessions based on Jaccard's coefficient was estimaed using the SIMQUAL implemented in the numerical taxonomy multivariate analysis system NTSYS-pc version 2.10 (Rohlf 2000) and the dendrogram were drawn using SAHN clustering program with the unweighted pair group method with arithmetic means (UPGMA) as recommended in most diversity studies. To assess the efficiency of SCoT primers, the total number of polymorphic bands, polymorphism information content (*PIC*), effective multiplex ratio (*EMR*), marker index (*MI*), and resolving power (*RP*) parameters were estimated according to Powell et al. (1996). GenAlEx software version 6.5 (Peakall and Smouse 2006) was used to calculate genetic diversity indices including the number of different alleles (Na), the number of effective alleles (Ne), Shannon index (I), Nei's gene diversity (H) and the percentage of polymorphic loci (Pp%) at both population and species levels. Moreover, the principal coordinate analysis (PCoA) was carried out to show the relationships among the terrestrial orchid accessions based on the variance–covariance matrix obtained from SCoT data, and a biplot was then drawn using the first two main coordinates (PCo1 and PCo2).

To analyze population structure, a clustering method based on the Bayesian model implemented in the STRUCTURE software package 2.3.4 was used (Falush

et al. 2007; Pritchard et al. 2000). This model identifies subpopulations, assigns their individuals, and estimates the allele frequency. The structure analysis was conducted by 10 independent runs per each K with a cluster number ranging from K = 1 to K = 10. For each individual run, a 50,000 burn-in length was set with 5000 Markov Chain Monte Carlo (MCMC) replication using the admixture model and independent allele frequency. To calculate the best K value, both log-probability of the data (LnP(D)) and delta K values determined with the online program Structure Harvester (Earl and vonHoldt 2011).

Multiple regression analysis (MRA) was used to identify SCoT loci (as independent variables) associated with the studied seed micro-structure characteristics (as dependent variables). For this purpose, stepping criteria using SPSS software were employed. The model $Y = a + b_1m_1 + b_2m_2 + \ldots + b_jm_j + \ldots + b_nm_n + d + e$ was used to perform MRA. This model attributes the variation in the dependent variable Y to a linear function of the set of SCoT markers (defined as independent variables, mj). The coefficient of partial regression (bj) indicates the empirical connection between Y and mj, d shows residuals among individuals (remaining after regression) representing the random error of Y, and e represents the environmental variation (Virk et al. 1996). The SCoT loci with significant regression values were assumed to be associated with the related seed micro-structure trait. The independent variables were selected based on F-values with the probability of 0.045 and 0.099 for entry and removal, respectively. Besides, the evaluated SCoT loci were individually tested with a linear curve fitting by linear models to verify the significance of standardized beta coefficients (β-statistics) for each locus calculated by MRA.

Results

Seed morphometric analysis

Seed micro-structure characteristics were studied by SEM in all individuals within collected populations, and there was a high variation among these traits (Table 2). The CV values for qualitative characteristics, including anticlinal wall curvature (138.23%), ridges on periclinal cell walls (82.06%), testa color (54.19%), and overall seed shape (53.47%) were higher compared to quantitative traits. For the height of anticlinal wall, *H. affine* accessions and all accessions within the genus Orchis excluding *O. mascula* was prominent. On the other hand, the height of anticlinal wall was low in *D. umbrosa* accessions (Fig. 2a), while it was medium–high in *O. mascula* and *Ophrys* species. Overall, in most of evaluated terrestrial orchid accessions,

the cells in apical and basal ends were short and smaller, while the cells in the middle part of seed were rectangular and elongated. The seed testa color of studied terrestrial orchid accessions was mainly brown and dark brown. Regarding seed dimensions, the highest and the lowest seed width was observed in Os.Pa1 (127.6 μm) and Du.M3 (276.6 μm), respectively (Electronic Supplementary 1). On the contrary, Ocol.K6 and Os.J1 had the shortest (304.4 μm) and longest (788.4 μm) seed, respectively. Fusiform was the most prevalent seed shape among the studied terrestrial orchid accessions (Fig. 2). This type of seed shape was observed in accessions within *O. simia* (Fig. 2d), *O. straussii* (Fig. 2f), and *O. schulzei* (Fig. 2g) species. The studied terrestrial orchid accessions represented a significant difference in seed cell number. In this connection, Du.S1 (*D. umbrosa*, Salian population) and Ac.S4 (*A. coriophora*, Salian population) represented the highest (139.3) and the lowest (36.6) seed cell number, respectively.

Molecular analysis

SCoT polymorphism

Among 19 preliminary studied SCoT primers, 15 primers produced clear and sharp banding patterns, which were considered for final reactions and analyses. As represented in Table 3, a total of 254 fragments were generated, of which 248 fragments were polymorphic (average polymorphism of 96.18%). The total band per primer was varied from 9 (S9 primer) to 22 (S20, S21, and S23 primers) with a size range 100–2800 bp. Most of the studied SCoT primers represented 100% of polymorphism, and only S5, S9 and S11 had lower polymorphism percentages. The allele frequency of each primer was used to calculate polymorphism information content (PIC). The highest PIC value (0.499) was obtained with S11 and S20 primers, confirming their higher efficiency to determine genetic diversity and distance (Table 3). On the contrary, the lowest PIC value (0.397) was calculated for S9 primer. The marker index (MI) was ranged from 2.19 by S5 primer to 7.50 by S20 primer with a mean of 5.26 for all evaluated SCoT primers. The average RP value of the SCoT primers used in the present study was observed as 15.66. The highest (29.89) and the lowest (9.19) RP values were obtained for S5 and S3 primers, respectively.

Genetic diversity and differentiation

The genetic diversity indices of the studied populations are represented in Table 4. The *O. simia* accessions collected from the Paveh region (Os.P) represented the lowest observed number of alleles (*Na*) (1.13) and effective

Table 2 Descriptive statistics for the thirteen seed microstructure traits over 97 terrestrial orchid accessions

No.	Trait	Abbreviation	Unit	Min	Max	Mean	SD	CV%
1	Seed width	SeWi	μm	127.6	276.6	190.53	35.56	18.66
2	Seed length	SeLe	μm	304.4	768.4	588.22	124.41	21.15
3	Seed length/width ratio	SeLe/Wi	ratio	1.47	4.58	3.171	0.83	26.40
4	Testa color	TeCo	code	1	9	4.89	2.65	54.14
5	Seed cell length	SeCeLe	μm	58.8	166.4	106.37	26.18	23.68
6	Seed/cell length ratio	Se/CeLe	ratio	3.09	9.04	5.76	1.58	23.68
7	Cell number per seed	CeNu/Se	number	36.8	139.3	69.08	29.17	42.22
8	Cells number in longitudinal axis	CeNuLoAx	number	4.6	11.8	6.76	1.38	20.53
9	Overall seed shape	OvSeSh	code	1	7	3.74	2.00	53.62
10	Ridges on periclinal cell walls	RiPeWa	code	0	3	1.45	1.19	81.89
11	Height of anticlinal walls	HeAnWa	code	1	5	3.70	1.52	41.30
12	Anticlinal wall curvature	AnWaCu	code	0	1	0.34	0.47	37.20
13	Testa cell pattern	TeCePa	code	0	1	0.30	0.46	150.27

number of alleles (*Ne*) (1.09), while the highest *Na* (1.29) and *Ne* (1.18) values were obtained in *O. schulzei* collected from Javanrood region (Oyst.JA) (Table 4). With an average of 0.114, heterozygosity (*H*) was found to be highest (0.195) in population D of *A. collina* (O.colK) collected from Divandareh region, while the lowest *H* (0.51) was obtained in Os.P population of *O. simia* collected from Paveh region. Furthermore, Shannon's information index (*I*) was ranged from 0.03 for *D. umbrosa* accessions collected from Marivan region (Du.M population) to 0.263 for Ha.Ja population (*H. affine* accessions collected from Javanrood region). Based on AMOVA results, a significant (*P* < 0.001) genetic differences were obtained within and among populations (Table 5). Of the total genetic variation, inter-population variations were 68%, and the rest 32% was intra-population variation.

Cluster analysis

The most appropriate cut-off line to classify orchid individuals and assigning clusters and sub-clusters was set based on the inter- and intra-group variance. Based on obtained dendrogram, the 97 studied terrestrial orchid accessions were clustered into eight main groups (Fig. 3). The first group included 17 accessions of *D. umbrosa* belonged to population Du.M (collected from Marivan region), Du.S (collected from Salian region), and Du.D (collected from Dehgolan region). All samples of *A. coriophora* belonging to Oc.J (collected from Javanrood) and Oc.S (collected from Salian) fell within the IInd group. In the IIIrd group, 15 accessions within Ocol.K and Ocol.D populations belonging to *O. collina* species were grouped

together. The individuals within Om.P from Pave region belonging to *O. mascula* were classified under the IVth group. The *Orchis simia* accessions of Os.Pa and Os.J populations (from Paveh and Javanrood regions, respectively) were clustered under the Vth group. All accessions within VIth group were *H. affine* accessions belonging to A (Ha.JA1–Ha.JA5) and B (Ha.JB1–Ha.JB7) populations collected from Javanrood region. Interestingly, the accessions within Oysc.JA, Oysc.JB, Oyst.JA, and Oyst.JB belonging to *O. sculzei* and *O. straussii* species were closed under VIIth group.

The PCoA biplot was constructed based on the the first two principal coordinates (PCo1 and PCo2), which somewhat confirmed the results of UPGMA clustering. As illustrated in Fig. 4, most of the studied terrestrial orchid accessions were separated mainly based on species taxonomy except for *O. schulzei*, *O. straussi*, *O. collina*, and *A. coriophora* while their accessions were placed under common groups. Phylogenetically, *O. collina* and *A. coriophora* are the species with a close genetic relationship, which can also generate interspecific hybrids (Dafni and Ivri 1979). Both the UPGMA clustering and PCoA plot represented the close relationship between *A. coriophora* and *O. collina* accessions. On the other hand, few individuals from different populations of each species were clustered together. This genetic similarity between individuals of different regions can probably be due to anemochoric dispersal of their seeds over long distances and during long times (Pinheiro et al. 2012).
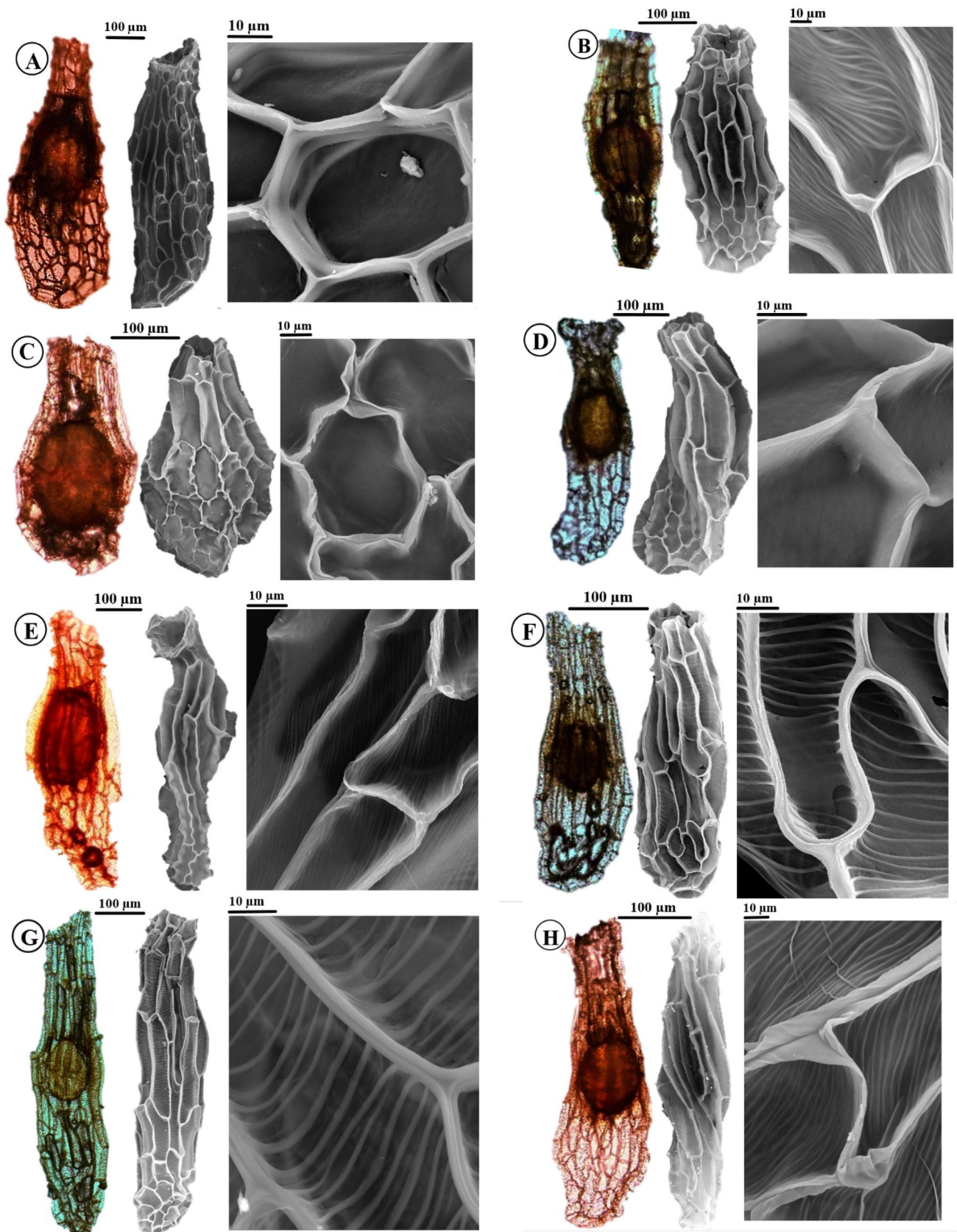
Fig. 2 Seed morphology, view in light microscopy and SEM. **a** *Dactylorhiza umbrosa*, **b** *Himantoglossum affine*, **c** *Orchis mascula*, **d** *Orchis simia*, **e** *Anacamptis coriophora*, **f** *Ophrys schulzei*, **g** *Ophrys straussii*, **h** *Orchis collina*

Table 3 Summary of SCoT primers used in the present study and their extent of polymorphism

Primer	Sequence (5′ → 3′)	TM	TB	PB	MB	PPB%	PIC	EMR
S1	CAACAATGGCTACCACCA	52	17	8	9	47.59	0.481	15.56
S3	CAACAATGGCTACCACCG	52	14	14	0	100	0.498	14.00
S4	CAACAATGGCTACCACCT	50.7	21	21	0	100	0.485	21.00
S5	CAACAATGGCTACCACGA	52	12	9	3	75	0.429	6.75
S7	CAACAATGGCTACCACGG	51.5	17	17	0	100	0.434	17.00
S9	CAACAATGGCTACCAGCA	52	9	7	2	77.7	0.397	5.44
S10	CAACAATGGCTACCAGCC	53	16	16	0	100	0.404	16.00
S11	AAGCAATGGCTACCACCA	53	12	11	1	91.6	0.499	10.08
S13	ACGACATGGCGACCATCG	58	18	18	0	100	0.478	18.00
S14	ACGACATGGCGACCACGC	57	11	11	0	100	0.470	11.00
S17	ACCATGGCTACCACCGAG	55	20	20	0	100	0.487	20.00
S20	ACCATGGCTACCACCGCG	53	22	22	0	100	0.499	22.00
S21	ACGACATGGCGACCCACA	55	22	22	0	100	0.483	22.00
S22	AACCATGGCTACCACCAC	54	21	21	0	100	0.483	21.00
S23	CACCATGGCTACCACCAG	56	22	22	0	100	0.498	22.00
Average	–		16.93	16.53	0.4	96.28	0.468	16.21
Total			254	248	6	–	–	–

TB Total number of amplified bands; *PB* Polymorphic bands; *MB* Monomorphic bands; *PPB%* The percentage of polymorphism; *PIC* Polymorphism information content; *EMR* Effective multiplex ratio; *MI* Marker index; *RP* Resolving power

Table 4 Mean number of different alleles (*Na*), number of effective alleles (*Ne*), Shannon index (*I*), genetic diversity (*H*) and percentage of polymorphic loci (*Pp%*) in terrestrial Iranian orchids populations

Population	*Na*	Ne	*H*	*I*	*Pp* (%)
Du.S	1.24	1.16	0.093	0.137	24.41
Du.D	1.16	1.12	0.069	0.100	16.54
Du.M	1.18	1.12	0.070	0.03	18.50
Ha.JA	1.45	1.32	0.180	0.263	45.67
Ha.JB	1.40	1.27	0.152	0.224	40.16
Om.P	1.32	1.24	0.131	0.190	32.28
Os.Pa	1.13	1.09	0.051	0.076	13.78
Os.J	1.18	1.13	0.073	0.106	18.50
Ac.S	1.31	1.23	0.130	0.188	31.89
Ac.J	1.24	1.17	0.098	0.142	24.41
Oysc.JA	1.25	1.19	0.109	0.157	25.98
Oysc.JB	1.23	1.18	0.099	0.142	23.62
Oyst.JA	1.32	1.24	0.132	0.192	32.68
Oyst.JB	1.27	1.21	0.117	0.169	27.95
Acol.K	1.31	1.25	0.136	0.195	31.89
Acol.D	1.40	1.28	0.195	0.233	40.55
Average	1.27	1.2	0.114	0.159	28.05

Table 5 Calculated AMOVA ($P \leq 0.01$) using SCoT markers in Iranian terrestrial orchid populations

Source of variation	DF	SS	MS	Est. Var	%
Among population	15	3392.42	226.161	34.723	68
Within population	81	1300.63	16.057	16.057	32
Total	96	4693.05		50.780	100

Df Degree of freedom

Population structure

In our study, the highest probability of the data was obtained when individuals were divided into eight populations (K = 8). Therefore, K = 8 was optimum to elucidate population structure and to estimate the membership matrix of individual clusters. Figure 5 shows the bar plot obtained for K values 7, 8, and 9. The obtained Bayesian matrix clustering was matched with the UPGMA dendrogram grouping and particularly with the PCoA plot, which also divided accessions into eight major clusters. In the bar plot obtained for K = 8, the studied accessions of *D. umbrosa*, *H. affine*, *O. mascula*, *O. simia*, *A. coriophora*, *O. schulzei*, *O. straussii* and *O. collina* were placed in corresponding clusters, respectively.
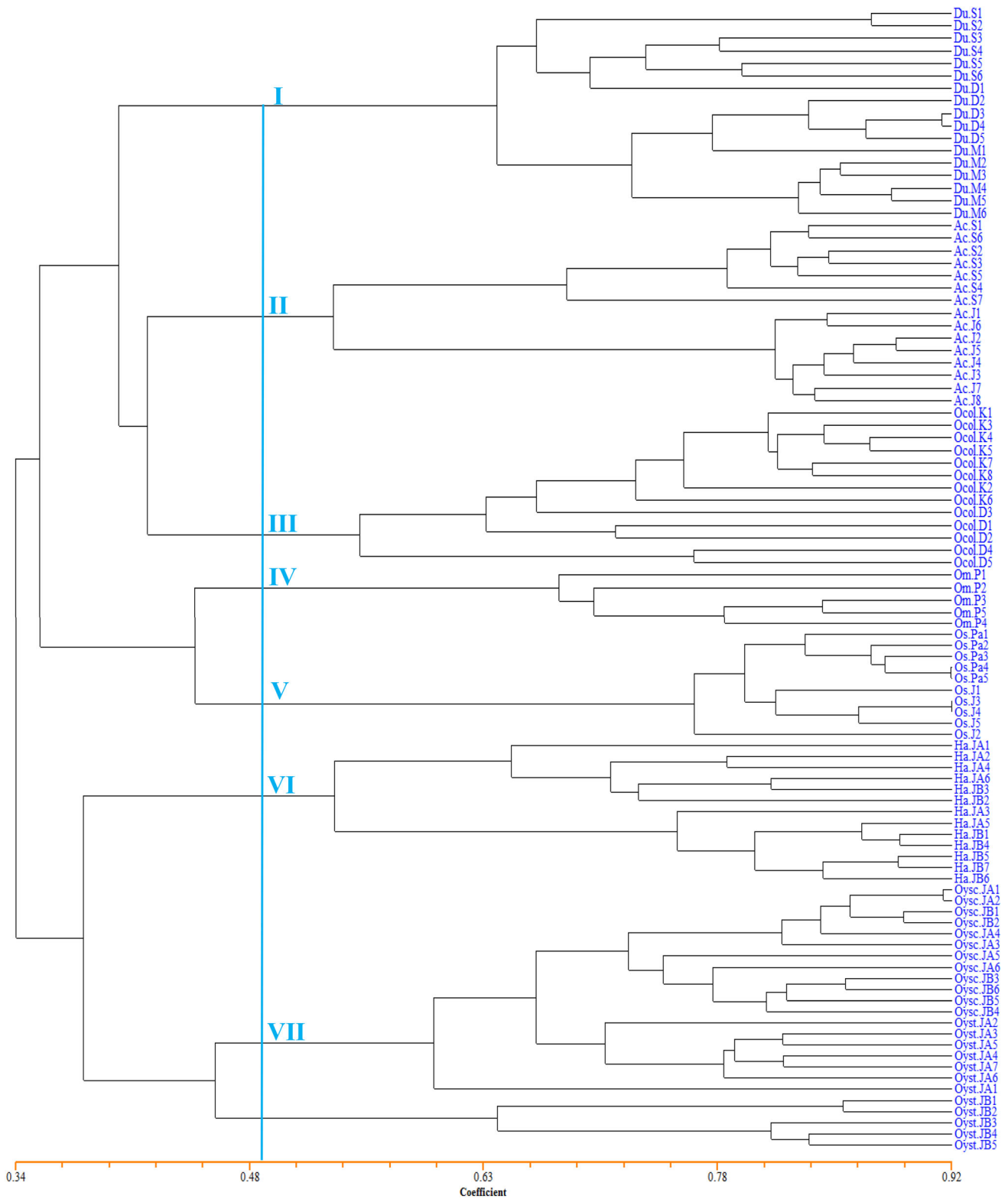
Fig. 3 UPGMA dendrogram of genetic similarity based on the SCoT data generated from Jaccard coefficients

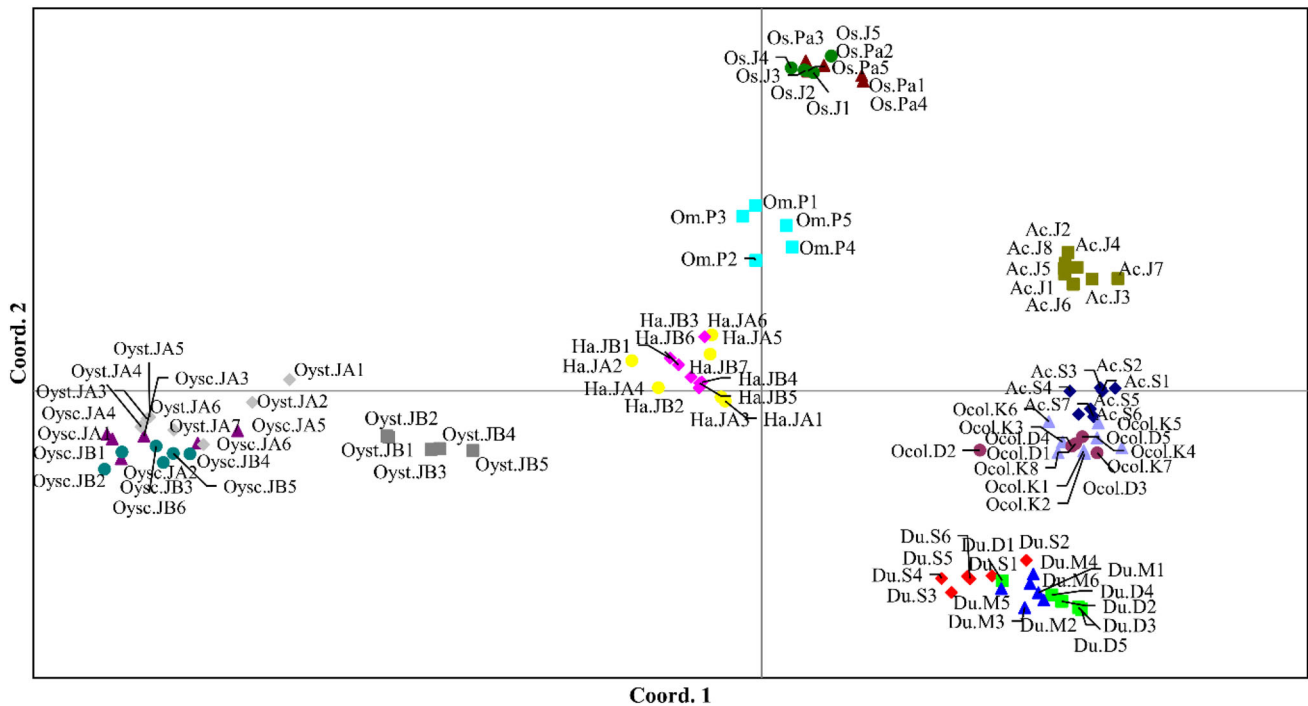## Principal Coordinates (PCoA)

Fig. 4 The PCoA biplot of terrestrial orchid populations based on the two first principal coordinates (PCo1 = 39.40%, PCo2 = 30.76%) using SCoT data

Association between seed micro-structure traits and SCoT markers

The associations between SCoT amplified bands and seed micro-structure traits were determined using multiple regression analysis (MRA) (Table 6). Seed morphometric characteristics and SCoT markers were considered as dependent and independent variables where they were associated and showed highly significant correlation values. From 254 amplified SCoT bands, 63 bands were associated with seed microstructure traits. In this regard, S1-15, S13-13, S13-11, and S13-7 loci represented the highest associations with seed width (SeWe). Seed Length (SeLe) was significantly correlated with S11-2, S22-16, S20-22, and S21-10 markers. Significant associations were also observed between seed length/width ratio (SeLe/Wi) with S7-4, S20-22, S11-5, and S21-2 SCoT loci. Four SCoT bands, including S17-11, S7-5, S23-22, and S22-16 represented association with seed cell length (SeCeLe). Seed/Cell Length Ratio (Se/CeLe) was associated with S5-5, S22-12, S1-8, and S21-11, where S5-5 provided the highest positive β-value (0.646) among all analyzed SCoT bands. Cell number per seed (CeNu/Se) was significantly associated with S1-15, S23-19, S23-7, and S14-9 markers. Finally, there were significant association values between

cells number in longitudinal axis (CeNuLoAx) with S3-2, S3-10, S7-11, and S17-10.

Discussion

Seed microstructure traits of testa and embryos are important in genetic diversity and phylogeny studies of different taxa in the *Orchidaceae* family. Fusiform seed was the most prevalent seed shape in the present research which is known for their narrow apical and basal ends and broad median part due to embryo placentation. Fusiform has been suggested as the most common shape of seed in *Orchidoideae* subfamily (Chase and Pippen 1988). In contrast, the accessions of *D. umbrosa* and *H. affine* (2A and 2B) had flask- and oval-shaped seed, respectively. These results are in agreement with the findings of Akçin et al. (2009) and Arditti and Ghani (2000). The overall seed shape can be used as a complementary tool for molecular markers to study genetic diversity and classification of terrestrial orchid species (Phillips et al. 2020; Şeker and Şenel 2017; Gallo et al. 2016; Gamarra et al. 2015b). Seed cell number and cell surface pattern have also been proposed as important micro-morphology characteristics to study genetic diversity and phylogenetic classification of orchid species as they are consistent and typical for any
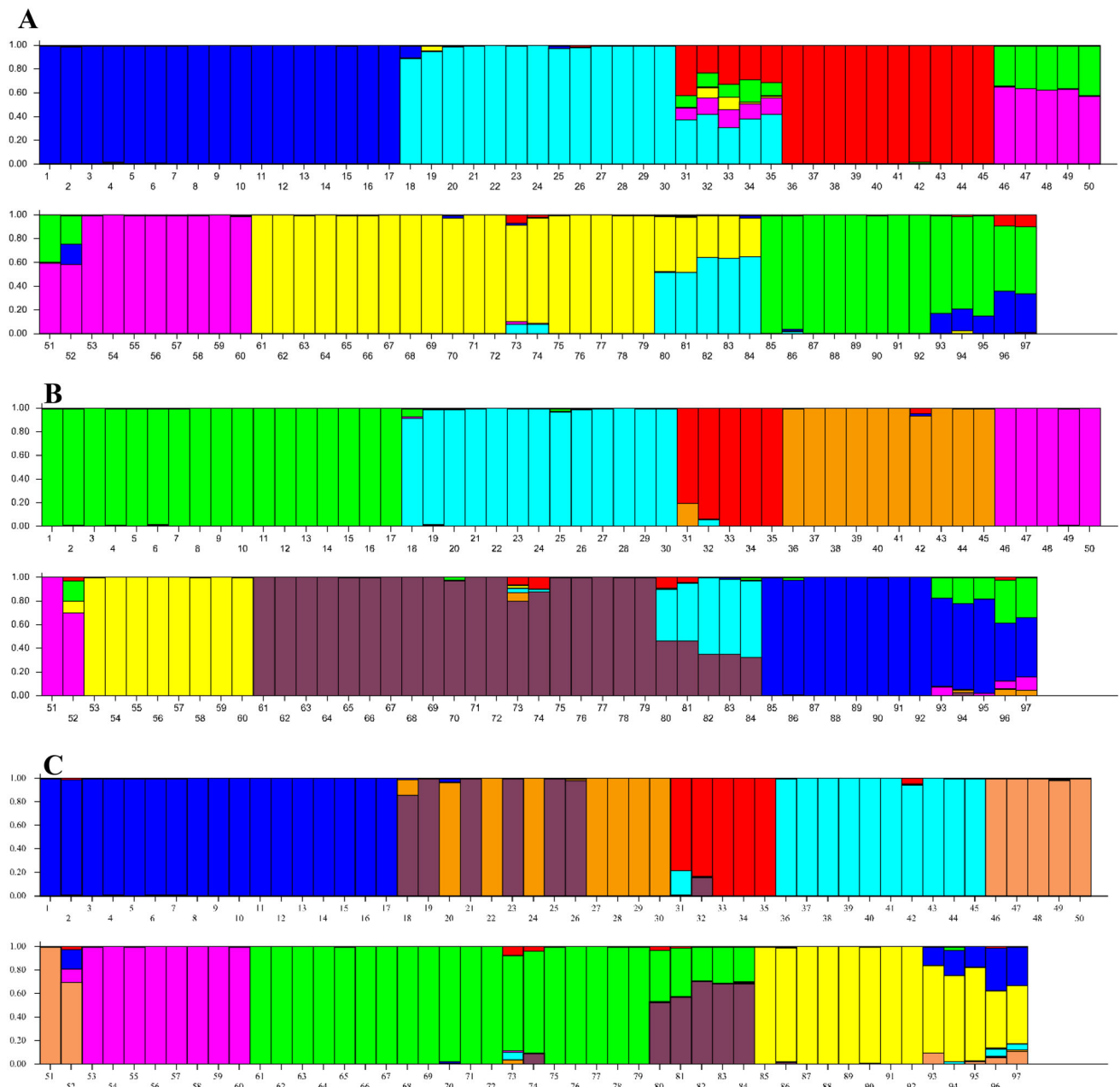
Fig. 5 Population structure of 97 Iranian terrestrial orchids based on SCoT data analyzed by STRUCTURE software and Bayesian model. **a** K = 7; **b** K = 8; **c** K = 9. Number of each horizontal lane is the individual code and each color shows a subpopulation

given orchid species (Gamarra et al. 2012, 2015a; Arditti and Ghani 2000). In line with previous reports, a species-specific pattern of seed microstructure traits was also revealed for Iranian terrestrial orchid species. From a conservational point of view, these findings can be particularly useful when the identity of ripen seed employed for in vitro conservation programs is unknown if the collection has been performed after flowering as the flower is the main tool to identify species (Verma et al. 2014). Therefore, the identification of orchid species using microstructure traits helps scientists and growers to

establish the best conditions specific for given species or genera from seed germination to tuber formation and flowering. More importantly, seed morphometry analysis of orchids could be important in managing seed samples maintained in gene banks to determine taxa identity for further usage in in vitro and in situ conservation programs, particularly in case of threatened and endangered species.

Different range of PIC, MI, and Rp value were obtained for evaluated SCoT primers. PIC can be efficiently employed in genetic diversity studies of plant populations and it shows the discriminating power of markers in

Table 6 SCoT markers associated with quantitative seed microstructure characteristics in terrestrial orchids as demonstrated by MRA

	SCoT Markers	r	R^2	β	t value	P value
Seed width (SeWi)	S1-15	0.742[a]	0.551	0.649	11.251	0
	S13-13	0.816[b]	0.665	− 0.267	− 5.412	0
	S13-11	0.839[c]	0.704	0.115	2.525	0.014
	S13-7	0.862[d]	0.743	0.252	5.71	0
Seed length (SeLe)	S11-2	0.749[a]	0.561	− 0.799	− 11.532	0
	S22-16	0.847[b]	0.718	0.209	5.131	0
	S21-10	0.902[c]	0.813	− 0.172	− 3.766	0
	S20-22	0.917[d]	0.841	0.278	7.536	0
Seed length/width ratio (SeLe/Wi)	S7-4	0.673[a]	0.453	− 0.341	− 8.336	0
	S20-22	0.745[b]	0.554	0.596	13.267	0
	S11-5	0.777[c]	0.604	0.364	4.599	0
	S21-2	0.826[d]	0.682	− 0.214	− 3.751	0
Seed cell length (SeCeLe)	S17-11	0.571[a]	0.326	− 0.135	− 2.824	0.006
	S7-5	0.690[b]	0.477	0.415	7.677	0
	S23-22	0.757[c]	0.573	0.155	3.216	0.002
	S22-16	0.793[d]	0.628	− 0.122	− 2.363	0.021
Seed/cell length ratio (Se/CeLe)	S5-5	0.804[a]	0.646	0.692	10.565	0
	S22-12	0.868[b]	0.754	− 0.492	− 10.494	0
	S1-8	0.906[c]	0.821	− 0.118	− 2.648	0.01
	S21-11	0.917[d]	0.84	− 0.161	− 4.611	0
Cell number per seed (CeNu/Se)	S1-15	0.875[a]	0.766	0.391	7.848	0
	S23-19	0.923[b]	0.852	− 0.232	− 6.18	0
	S23-7	0.946[c]	0.894	0.079	3.764	0
	S14-9	0.950[d]	0.903	0.209	7.152	0
Cells number in longitudinal axis (CeNuLoAx)	S3-2	0.703[a]	0.494	− 0.726	− 17.587	0
	S3-10	0.838[b]	0.703	− 0.255	− 7.605	0
	S7-11	0.874[c]	0.764	0.267	9.212	0
	S17-10	0.887[d]	0.787	− 0.311	− 9.559	0

The lower r values are more significant

r Pearson's correlation coefficient; R2 The square of R multiple adjusted for the number of predictors in the model; β Standardized beta coefficients

revealing polymorphism within the populations and differentiate genotypes based on the known allele frequency and distribution (Kaki et al. 2020; Botstein et al. 1980). PIC equals genetic variation, and higher PIC values imply a higher polymorphism and higher number of rare alleles in a single gene locus. In this regard, PIC determines the marker efficiency by providing a benchmark of primers employed in genetic diversity studies (Botstein et al. 1980). Variables including allele number generated by loci and genotype and primer number can affect the PIC range in genetic diversity and population structure analyses, which could be from 0 to 1 (Powell et al. 1996). MI is another important genetic index employed to determine the overall utility of marker systems and estimate primer efficiency to discriminate plant germplasm resources based on the polymorphic band frequency. The higher MI values thus suggest more coverage and information of the corresponding genome (Spooner et al. 2005). Similar to PIC, the higher MI implies the more efficiency and informativeness of the primer. This indicator is more informative when it exploits for inter-specific diversity studies (Medhi et al. 2014). RP refers to the primer ability to determine diversity level among sampled accessions based on the allele distribution and therefore, can be used as a powerful indicator to choose the suitable primers as it is impacted by the proportion of genotypes with bands and the number of bands produced (Azizi et al. 2009).

The fitness of loci to evaluate the genetic variation of plant accessions and populations depend on the average number of each marker (Rao and Hodgkin 2002). In this connection, primers that generate higher number of bands could be better for genetic variation studies as they are more effective in the coverage of genomic regions (Powell et al. 1996). The observed number of alleles is a pivotal index of genetic differentiation attributed to populations, types, and geographical locations. Na is 0 when accessions have the same allele frequency, and it equals 1 when the accessions are completely different for allele frequency. The effective number of alleles (*Ne*) is a reciprocal of expected homozygosity, so it is entirely correlated with the genetic diversity measured as the expected heterozygosity (or a sum of squares of allele frequencies at a locus) (Kimura and Crow 1963).

Both *I* and *H* are important genetic indices to study the polymorphism of populations gathered from geographically different locations. The higher *I* value represents higher diversity within populations, therefore, Ha.Ja had the highest diversity compared to other evaluated terrestrial orchid populations. This was further confirmed by its percentage of polymorphic loci (45.67%), which was also highest among all studied 16 populations. The level of genetic diversity is affected by different factors, including the current dynamical conditions of local populations, their adaptation to the climatic and micro-climatic conditions, and historical events. Size reduction and population fragmentation would decrease the level of genetic diversity within the population of terrestrial orchids due to genetic drift arising from restricted intra-population gene flow. Here, the observed low level of diversity among Iranian orchid populations, is probably due to exploitation and depletion of dried orchid tubers by local people of rural districts in West of Iran (de Boer et al. 2017; Kaki et al. 2020). The indices for intra-population variations estimated in our study were comparable with those obtained by the IRAP marker (Kaki et al. 2020) and ISSR markers (Gholami et al. 2021) in Iranian terrestrial orchids. This was also reported by Giri et al. (2017) (*Habenaria edgeworthii*), Liu et al. (2014) in *Paphiopedilum micranthum*, and Cai et al. (2011) in *Dendrobium loddigesii*.

Information on genetic variation is a key factor in defining conservation programs of rare orchid species (Bhattacharyya et al. 2013). The AMOVA results obtained in the present research were in line with the results obtained in *Dendrobium nobile* (Bhattacharyya et al. 2013, 2017), *Cypripedium kentuckiense* (Pandey et al. 2015) and *Cypripedium japonicum* (Tian et al. 2018) which shows a higher distribution of genetic variation amongst populations as compared to the distribution within populations. These findings further show the fragmentation of Iranian terrestrial orchid populations and their decreased

connectivity and relationship, which have been previously indicated by the IRAP marker (Kaki et al. 2020).

Clustering is among the most important multivariate analyses to study the genetic variation of plant species. In the present study, Jaccard similarity provided the highest correlation coefficient ($r = 0.97295$), and therefore, it was used to draw dendrogram based on the UPGMA clustering algorithm. Excluding *Ophrys* species, the studied species has been well-grouped in separate clusters and its populations within each species fall in individual sub-clusters. Only in case of Ophrys genera, SCoT marker data set couldn't differentiate species and population levels. In this regard, *O. schulzei* populations were not separated from *O. straussii* counterparts even as sub-clusters. This is probably because *Ophrys* has been known as the most diverse genus within terrestrial orchids (with over 250 identified species) where their interspecific relationship is a complicated classification of the *Orchidaceae* family due to the diverse intra-species hybridization (Breitkopf et al. 2015). The classification of remaining groups was mainly based on the species; however, the studied accessions were also separated relatively based on their populations and geographical collection site. A similar pattern of clustering was observed in Iranian terrestrial orchid species using IRAP molecular markers by Kaki et al. (2020). PCoA has been suggested as a suitable and complementary tool to interpret UPGMA or NJ clustering methods (Peakall and Smouse 2006). Therefore, PCoA was further performed to provide more details on the relationship among the evaluated accessions.

The Bayesian clustering method assigns accessions to hypothetical populations. The studied accesions of Iranian terrestrial orchis species were grouped mainly based on species where each species has been classified as a separate group. Population structure results were in agreement with the AMOVA results which revealed a higher level of the inter-population variation compared to intra-population diversity. Due to high sensitive to low degrees of genetic diversity, the SCoT technique is a powerful tool to study population genetics and structure in various plant species and also to identify species or population of the same species (Bhattacharyya et al. 2013).

In this paper, some SCoT markers were associated with more than one seed microstructure traits. This scheme of association could be due to correlation among traits and/or pleiotropy effect imposed by linked quantitative trait loci (QTL) on different characters (Culp et al. 1979). On the other hand, a single SCoT locus can include the tightly linked QTLs exhibiting different seed micromorphology traits. In this connection, S1-15 marker had association with both SeWi ($\beta = -0.799$) and CeNu/Se ($\beta = 1.268$). Furthermore, S22-16 marker showed a strong association with SeLe and SeCeLe traits. As the SCoT primers target

short conserved nucleotide regions surrounding the ATG initiation codon of plant genes, the loci associated with seed morphometric trait can contain the gene responsible for seed and testa development. This hypothesis can be tested by excision, purification, and sequencing of SCoT markers associated with seed morphometry traits, which can provide more genetic information on distinct seed coat features. This information could be useful in understanding seed germination process as seed testa is one of the main germination barriers in terrestrial orchids (Yeung 2017).

Conclusion

The preservation of genetic diversity is crucial in maintaining the evolutionary capacity of natural resources. The limited genetic variation generally results in reduced fitness of populations. Therefore, preliminary information on the genetic variability is pivotal to plan conservation and domestication programs for rare, endangered and threatened (RET) terrestrial orchid species. The SCoT primers employed in the current research considered to be appropriate to elucidate genetic variability and structure of 97 studied orchid accessions belonging to 16 populations and 8 Iranian tuberous orchid species. A high inter- population and low intra-population variability was observed in the present study, showing population fragmentation of terrestrial orchid species from the western part of Iran. This probably is the result of habitat deterioration mediated by anthropogenic activities and underground tuber over-harvesting, particularly in case of *D. umbrosa* and *O. simia*. All the studied Iranian orchids are classified as temperate hardy orchids with undeveloped embryos having internal seed dormancy. The features that result in low rate of germination in nature as these species need complicated symbiosis relationships with mycorrhiza fungi. Moreover, terrestrial orchid species need specific ecological niches and particular pollinators. Therefore, Iranian terrestrial orchid species deserve particular conservation attention on a national and international scale and our observations can be useful for germplasm characterization, conservation, and improvement of these valuable species.

Declarations

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