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Investigation in the intergenic transcribed sequences to determination of genetic variation for Iranian species of salvia

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Abstract

The nuclear ribosomal DNA internal transcribed spacer (ITS) has been established as a suitable genomic region to clarify genomic and phylogenetic relationships of plant species. This study aimed to evaluate the performance of ITS2 DNA barcoding for distinguishing *Salvia* species and determine genetic relationships among *Salvia* species from different regions of Iran. The seeds of 12 *Salvia* species were provided and planted. Total genomic DNA was extracted and ITS2 regions were amplified by PCR with specific primers and were sequenced by Bioneer Company. Genetic distance between *Salvia* species was estimated according to the Kimura 2-Parameter (K2P) model. ITS2 secondary structures predicted based on ITS2 databases. Multiple sequences were aligned using ClustalW algorithm and phylogenetic analyses were conducted using maximum likelihood (ML). The lengths of ITS2 sequences ranged from 270 to 282 bp and the mean of GC content was 59.3%. The greatest variation in helix length (10 to 22 bp) in the secondary structure of ITS2 at different species of *Salvia* was related to the third helix. All of the Salvia species formed five major clades. The results showed that the species of *Salvia* genus in Iran can be clearly distinguished from each other using ITS2 DNA barcoding.

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Introduction

Lamiaceae is a family of medicinal plants and the genus Salvia L. belongs to this family. This genus containing approximately 1000 species and has been widely used in the pharmaceutical, food, spice, cosmetic, and ornamental industries (Farka *et al.,* 2005). About 58 species of Salvia are distributed throughout Iran; in which 17 of these species are endemic to Iran (Walker *et al.,* 2004; Sepehry-Javan *et al.,* 2012). So, genetic material is unique for each species; DNA markers are reliable for molecular phylogenetic studies.

DNA barcoding, when morphologic traits are absent, aids in global species identification that has been successfully used in several studies (Savolainen *et al.,* 2005; Marshall, 2005; Schindel and Miller, 2005). Identifying of species using a short segment of DNA is used frequently in animal studies (Thomas, 2009). But, DNA barcoding studies on classifying of plant species are limited. The nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) has been established as a suitable genomic region to clarify genomic and phylogenetic relationships of plants (Wang *et al.,* 2013). The internal transcribed spacer 2 (ITS2), one of the most important molecular markers in systematic and evolution studies (Coleman, 2003; Thornhill *et al.,* 2007), shows significant sequence diversity at the levels of species. The diversity in its structural information permits higher taxonomic level analysis (Coleman, 2003; Aguilar and Sanchez, 2007; Schultz and Wolf, 2009; Coleman, 2009), which provides additional information for betterment accuracy and strength in the reconstruction of phylogenetic trees (Keller *et al.,* 2010). Furthermore, ITS2 is potentially useful as a standard DNA barcode to identify medicinal plants (Chen *et al.,* 2010; Luo *et al.,* 2010). Also, ITS2 is one of the molecular markers served as candidate DNA barcodes because of its valuable characteristics, including the sufficient variability to distinguish closely related species and the availability of conserved regions for designing universal primers. ITS2, which separates the nuclear ribosomal genes 5.8SrRNA and 28SrRNA (Fig. 1) (correspondingly 25S or 26SrRNA in several fungi), provides completely different associated characteristics for phylogenetic analyses (Alvarez and Wendel, 2003; Coleman, 2003; Feliner and Rossello, 2007). Another feature of the ITS2 renders possible an alternate way to infer phylogenies from a low to a high level of species relationships that does not face the mentioned problems in model based approaches. The other fortunate characteristics make the ITS2 an interesting marker for biodiversity research beside its usability in phylogenetic studies. With that and the high amount of nucleotide substitutions even on a generic level, it meets the requirements to be used as a DNA barcoding marker, i.e. to distinguish species by a short fragment of the genomic sequence. Utilization of ITS phylogeny in Lamiaceae has also been well established. In previous studies, ITS markers successfully have been used to investigate the genetic diversity and phylogenetic relationships for 7 accessions of *Salvia miltiorrhiza* (Zhang *et al.,* 2013). In addition to primary sequences, secondary structures of ITS2 were used to distinguish the medical plants such as Salvia species (Wang *et al.,* 2013). Wang *et al.* (2013) conducted nrDNA sequences among species of Salvia. Their results illustrated that the ITS regions is apotent barcode for identifying Salvia species, especially *Salvia miltiorrhiza*. Therefore, these markers are very useful and informative for phylogenetic studies and genetic barcoding.

Therefore, this study aimed to evaluate the performance of ITS2 DNA barcoding for distinguishing Salvias species with important economical values and determine genetic relationships among 12 *Salvia* species from different regions of Iran.

Materials and methods

Plant material

The seeds of 12 Salvia species were provided by Research Institute of Forests and Rangelands (RIFR) of Iran. All of the materials were planted in the botanical garden of Agricultural Research Center in Qazvin. Table 1 lists the species names and their locality.

DNA isolation, PCR amplification, and sequencing

Total genomic DNA was extracted from young fresh leaves according to the CTAB method (Piccolo *et al.,* 2012). The purity and quantity of genomic DNA were determined by a nanodrop (BIO-RAD) and 0.8% agarose gel electrophoresis. The DNA samples were stored at −20 °C prior to amplification. nrDNA ITS2 regions were amplified by polymerase chain reaction (PCR) with primers ITS2F: 5'-ATGCGATACTTGGTGTGAAT-3' and ITS2R: 5'-GACGCTTCTCCAGACTACAAT-3 (Chen *et al.,* 2010). The PCR amplification was performed using Taq Amplicon PCR Master Mix in a 50 μL reaction mixture according to the manufacturer's instructions. The thermal cycling program for PCR consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation (30 s at 95 °C), annealing (30 s at 56 °C), extension (45 s at 72 °C), and a final extension at 72 °C for 10 min. The PCR products for each species were sequenced by Bioneer Company with both forward and reverse primers.

Data analysis

Sequence statistics were calculated with Edit Seq software (DNA Star, Inc., Madison, WI, USA) (Thompson *et al.,* 1994). Genetic distance between Salvia species was estimated using MEGA 4.0 based on the ITS region according to the Kimura 2-Parameter (K2P) model (Tamura *et al.,* 2007). Internal transcribed spacer (ITS2) secondary structures predicted based on ITS2 databases (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) (Schultz *et al.,* 2006).

Phylogenetic analysis

Multiple sequences were aligned using the Clustal W algorithm followed by manual adjustment implemented by CLC Main Workbench 5.5. Phylogenetic analyses were conducted using maximum likelihood (ML). Maximum likelihood analysis was performed in CLC Main Workbench 5.5.

Topological robustness ML analysis was assessed by bootstrap analysis with 1000 replicates using simple taxon addition. *Verbena officinalis* was selected as the out-group for drawing *Salvia* phylogenetic tree.

Results and discussion

PCR amplifications and sequencing

The PCR amplification and sequencing success rate of the ITS2 sequences from medicinal plants of *Salvia* was 100%.

Table 1. Salvia species used in this study for evaluation of genetic diversity.

Species	Collection site	Province	Gene Bank Accession No.
S. aethiopis	Rostamabad	Guilan, Iran	MF289967
S. virgata	Qazvin	Qazvin, Iran	MF289971
S. macrosiphon	Dashtestan	Bushehr, Iran	MF289972
S. reuterana	Tehran	Tehran, Iran	MF289973
S. sclarea	Meshkinshahr	Ardabil, Iran	MF289974
S. verticillata	Qazvin	Qazvin, Iran	MF289975
S. officinalis	Hamedan	Hamedan, Iran	MF289970
S. spinosa	Qazvin	Qazvin, Iran	MF289969
S. syriaca	Ilam	Ilam, Iran	MF289968
S. nemorosa	Najafabad	Isfahan, Iran	MF289976
S. eremophila	Khatam	yazd, Iran	-
S. eremophila	kerman	kerman, Iran	-

The ITS sequences in this study included three regions: 5.8S rRNA gene, the complete sequences of ITS2, and 28S rRNA gene. Sequences of the 5.8S rRNA and 28S rRNA genes were completely identical for all cloned sequences of the 12 species/accessions and showed no variations for all species included in this study.

Table 2. Analysis of ITS2 nucleotide sequences in salvia species studied in this research.

Species names	Product size (bp)	Size of ITS 2	Count of nucleotides					
			A	C	G	T	C+G	A+T
S. aethiopis	457	271	38	106	84	43	190	81
S. virgata	463	270	39	100	86	45	186	84
S. macrosiphon	467	270	40	101	84	45	185	85
S. reuterana	460	270	40	101	85	44	186	84
S. sclarea	460	270	37	104	86	43	190	80
S. verticillata	457	270	40	101	84	45	185	85
S. officinalis	479	282	41	107	87	47	194	88
S. spinosa	462	270	40	101	85	44	186	84
S. syriaca	459	270	39	102	85	44	187	83
S. eremophila(kerman)	472	282	36	109	93	44	202	80
S. eremophila (yazd)	460	270	39	103	84	44	187	83
S. nemorosa	460	272	41	103	84	44	187	85

The amplified sequence lengths ranged from 457 to 479 bp (Fig. 2). After removing the conserved 5.8S rRNA and 28S rRNA sequences, the lengths of the ITS2 sequences used in the analysis ranged from 270 to 282 bp, with an average length of 272 bp (Table 2). The mean GC content was 59.3% and ranged from 68.5% (*S. macrosiphon*and *S. verticillata*) to 71.6% (*S. eremophila*-kerman) (Table 2). Therefore, the GC content of Salvia species ITS2 sequences is high and relatively variable.

Table 3. Matrix of similarity (upper main diameter) and distance (lower main diameter) among salvia species

Salvia species	*S.syriaca*	*S.spinosa*	*S.reuterana*	*S.verticillata*	*S.sclarea*	*S.virgata*	*S.aethiopis*	*S.nemorosa*	*S.officinalis*	*S.macrosiphon*	*S.eremophila*(Y)	*S.eremophila*(K)
S. syriaca		99.63	99.63	99.26	98.15	97.42	95.57	95.22	83.03	99.26	99.26	81.88
S. spinosa	0.00		99.26	98.89	97.78	97.05	95.20	94.85	83.68	98.89	98.89	81.53
S. reuterana	0.00	0.01		98.89	97.78	97.05	95.2	94.85	83.68	98.89	98.89	81.88
S. verticillata	0.01	0.01	0.01		97.41	96.68	95.2	94.49	83.33	100	98.52	81.88
S. sclarea	0.02	0.02	0.02	0.03		95.94	95.94	93.75	83.33	97.41	98.89	81.18
S. virgata	0.03	0.03	0.03	0.03	0.04		93.38	97.06	86.06	96.68	97.05	83.28
S. aethiopis	0.05	0.05	0.05	0.05	0.04	0.07		91.21	80.9	95.2	96.31	79.79
S. nemorosa	0.05	0.05	0.05	0.06	0.07	0.03	0.09		84.72	94.49	94.85	82.58
S. officinalis	0.18	0.18	0.18	0.19	0.19	0.15	0.22	0.17		83.33	83.68	83.04
S. macrosiphon	0.01	0.01	0.01	0.00	0.03	0.03	0.05	0.06	0.19		98.52	81.88
S. eremophila(Y)	0.01	0.01	0.01	0.01	0.01	0.03	0.04	0.05	0.18	0.01		81.53
S. eremophila(K)	0.21	0.21	0.21	0.21	0.22	0.19	0.24	0.20	0.19	0.21	0.21	

Secondary structures of ITS2 in Salvia species

To identify the species, we focused not only on the divergence of primary sequences of ITS2, but also on the use of variations in the secondary structures of ITS2. Secondary structure predictions of the ITS2 for six species of Salvia were shown in Fig. 3, from which

a general secondary structure model consisting of a multi-branch loop with several paired regions has reconstructed (Fig. 3). After drawing the secondary structure of ITS2 in the studied Salvia species using the online tools and counting the number of nucleotide base pairs of each helix and the number of unpaired loops (Fig. 3), it was found that the secondary structure of ITS2 for the *Salvia* species was similar to the conventional structure of ITS2 in the flowering plants that have four helixes (Schultz *et al.,* 2005).

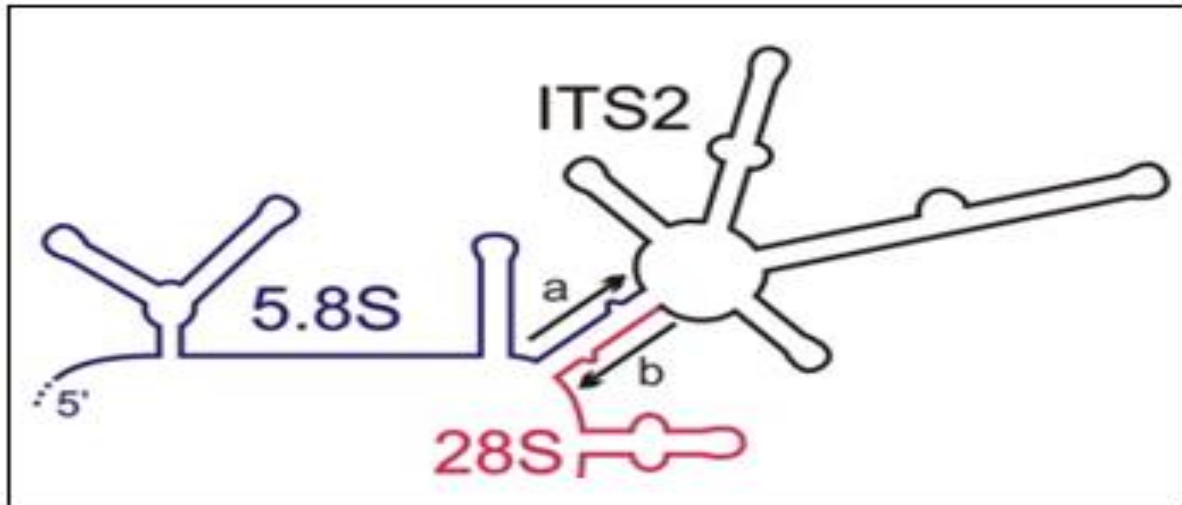

Fig. 1. The intergenic transcribed sequences region is flanked by 5.8S and 28S ribosomal DNA.

The study of the secondary structure of ITS2 showed different forms for the studied species (Fig. 3). The greatest variation in helix length in the secondary structure of ITS2 at different species of Salvia was related to the third helix, the length of which varied from 10 to 22 bp. The fourth helix had the longest length, varied from 40 to 51 bp, compared to other helixes. The lengths of helixes I, II varied from 10 to 16 bp and 13 to 21 bp, respectively.

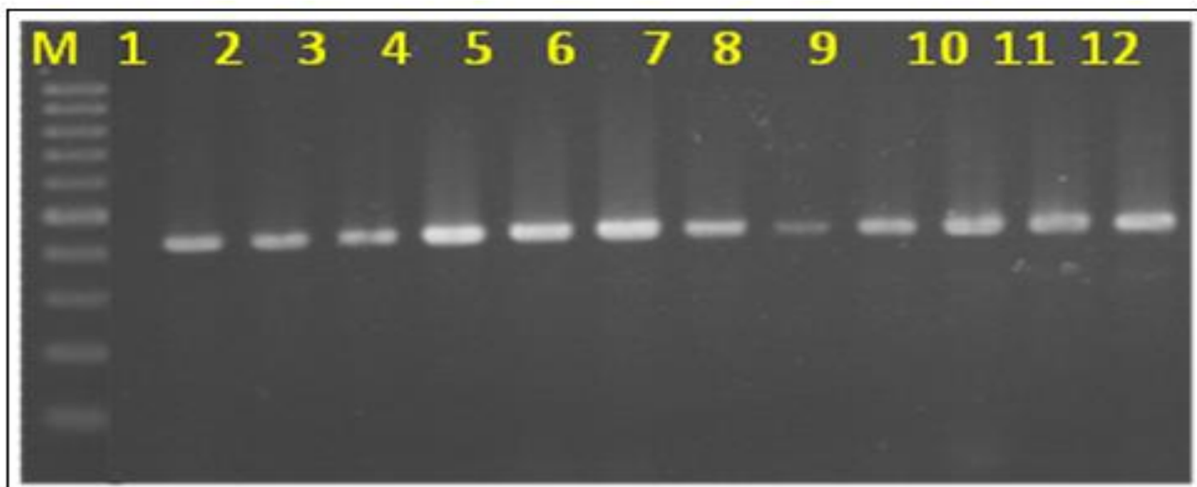

Fig. 2. Electrophoretogram of PCR products from the ITS2 region of salvia species. 1) *S. aethiopis* (MF289967), 2) *S. macrosiphon* (MF289972), 3) *S. reuterana* (MF289973), 4). *S. sclarea* (MF289974), 5) *S. verticillata* (MF289975), 6) *S. officinalis* (MF289970), 7) *S. spinosa* (MF289969), 8) *S. nemorosa* (MF289976), 9) *S. eremophila* (Yazd), 10) *S. eremophila* (kerman), 11) *S. virgata* (MF289971) and 12) *S. syriaca* (MF289968).

The studied species had a unique secondary structure that differed in this structure in two respects with others, length of helixes and number of loops on their helixs (Fig. 3). All species had two loops on their first helix, but *S. sclarea* in the first helix had only one loop. The species *S. virgata* and *S. officinalis* had three loops on their second helix, but *S. macrosiphon*, *S. reuterana*, *S. sclarea* and *S. verticillata* had two loops on their second helix.

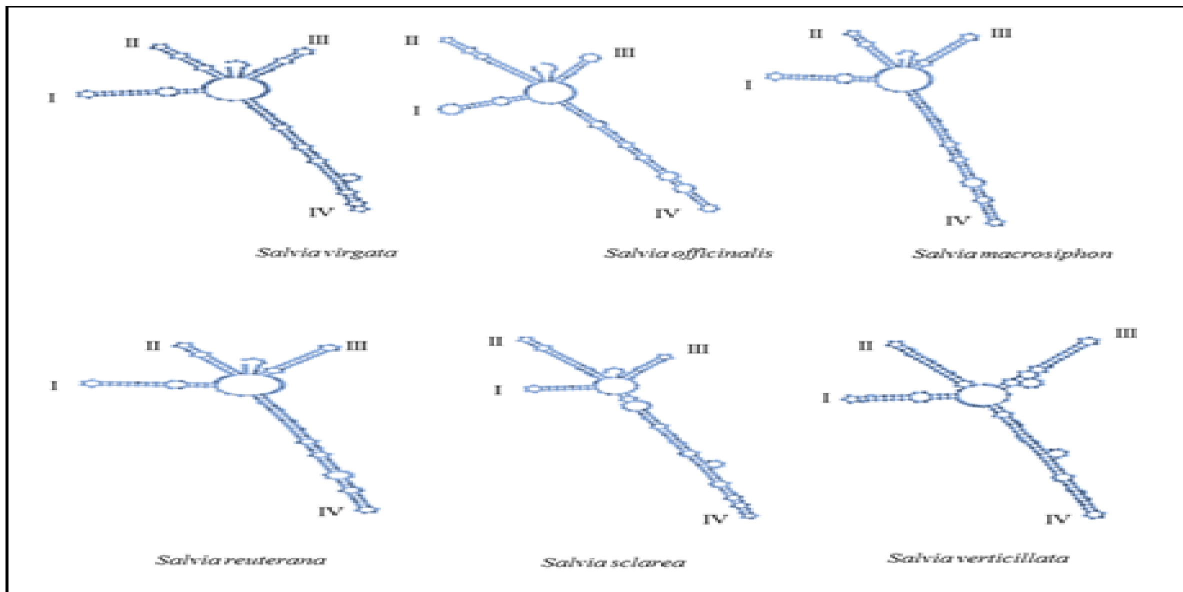

Fig. 3. The secondary structures of the predicted internal transcribed spacer (ITS2) regions in six species of salvia.

The number of loops in the third helix were 2, 1, 2, 2, 1 and 5 for *S. virgata, S. officinalis*, *S. macrosiphon, S. reuterana*, *S. sclarea* and *S. verticillata*, respectively. Also in fourth helix the numbers of loops were 7, 6, 5, 5, 9 and 4 for *S. virgata, S. officinalis*, *S. macrosiphon, S. reuterana*, *S. sclarea* and *S. verticillata*, respectively.

In this research, one of the most widely used regions in the DNA barcoding for plants, which in many cases has its efficiency for the correct diagnosis of species of the same genus, has been used. Considering the efficiency of ITS area, especially ITS2, in identifying and distinguishing plant species and increasing use of it, it was expected that this segment would be efficient in identifying the various species of Salvia. The secondary structure of ITS2 in Salvia was similar to that of other flowering plants with four helixes, and was different about the length of the fourth helix more than the other helixes instead of third helix as maximum length at flowering plants (Wilson, 2003; Schultz *et al.,* 2005; Coleman, 2007).

In previous studies on the secondary structure of ITS2 in different genus (Coleman, 2003; Keller *et al.,* 2008; Chen *et al.,* 2010), the secondary structure of ITS2 has always been one of the main tools for species identification and differentiation of different species within genus from each other. In accordance, the secondary structure of ITS2 could support the categorization of different species of Salvia species and also the secondary structure of the ITS2 region could be considered as a molecular morphological characteristic. In fact, in the genus whose nucleotide substitutions in the ITS region are more, the ITS2 secondary structure can succeed in differentiating of species (Meyer and Paulay, 2005). Therefore, according to dissimilarity of the ITS2 secondary structure in the studied Salvia species, it can be argued that one of the reasons for this region's ability to distinguish Salvia plants belonging to different species is its high variability among different species of Salvia genus.

Genetic distances and Phylogenetic analysis among Salvia species

In terms of genetic distance or similarity (Table 3), among the species of Salvia, the lowest genetic distances/the highest similarity was observed between *S. verticillata* and *S. macrosiphon, S. spinosa* and *S. syriaca*, *S. syriaca* and *S. reuterana*, *S. aethiopis* and *S. verticillata*, *S. reuterana* and *S. spinosa*. The highest genetic distance/the lowest similarity was observed between *S. eremophila* (accession kerman) with *S. aethiopis*, *S. sclarea*, *S. spinosa*, *S. eremophila* (accession Yazd) and between *S. aethiopis* and *S. officinalis*.

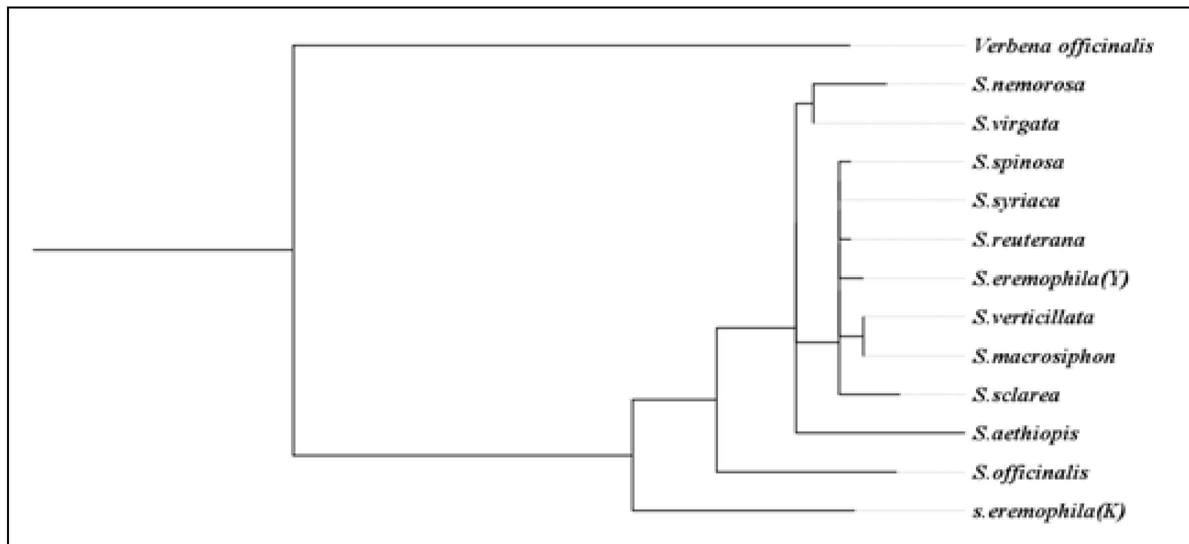

Fig. 4. Maximum Likelihood tree constructed from the ITS2 sequences of salvia species. The associated taxa clustered together in 1000 bootstraps.

In the Fig. 4, all of the Salvia species formed five major clades. Clade I consisted of two species, *S. nemorosa* and *S. virgata* and both of species were for Qazvin and Isfahan provinces from north and central Iran. Clade II consisted of *S. syriaca*, *S. spinosa*, *S. reuterana*, *S. eremophila* (accession Yazd), *S. macrosiphon*, *S. verticillata* and *S. sclarea*, and these are distributed in the southwestern, central and northwestern of Iran. In Clade II, it was obvious that four subclades (subclade A, subclade B, subclade C, and subclade D) were formed. Subclade A consisted of two species, *S. syriaca, S. spinosa* and *S. reuterana* belonged to Ilam, Qazvin and Tehran provinces. Subclade B included the species of *S. eremophila* (accession Yazd). *S. macrosiphon* and *S. verticillata* from Bushehr and Qazvin formed Subclade C. Subclade D included the species of *S. sclarea* from Ardabil province. Each of clades III, IV, V comprised one species of *S. aethiopis* (Guilan), *S. officinalis* (Hamedan), *S. eremophila* (accession kerman), respectively.

The Salvia genus consisted of species that were distributed widely all over the world with a large number of endemic species. Alziar (1988) recognized that Salvia has undergone marked species radiations in three regions of the world: Central Asia/Mediterranean, Central and South America, and Eastern Asia. In this study, all of the Salvia species formed five major clades based on nrDNA ITS2 sequences. The five Salvia individual clades/subclades were distinguished by five main geographical distributions (Guilan, Hamedan, kerman, Ardabil, Yazd). The individual clades/subclades indicated that the native species in Iran had not close relationships. There were closer relationships between Clade I and Clade II as compared with Clades III, IV, and V. The results showed that the species of Salvia genus in Iran can be clearly distinguished from each other as well as accessions within one species such as *S. eremophila* (Yazd) *and S. eremophila* (Kerman).

The phylogenetic study (Walker *et al.,* 2004) suggested that Salvia was not monophyletic but comprised at least two and possibly three distinct lineages. Epling (1939) found that Salvia genus has a high proportion of endemic small species groups. However, Steward *et al.* (1958) reported that the derivations of the Asian species were less clear because there is not as much information available about the species in this region.

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