Bangladesh J. Plant Taxon. **22**(2): 67-75, 2015 (December)

GENETIC DIVERSITY AND INTERSPECIFIC RELATIONSHIPS OF SOME *ALLIUM* L. SPECIES USING INTER SIMPLE SEQUENCE REPEAT MARKERS

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Keywords: *Allium* L.; Genetic relationship; ISSR; Molecular marker.

Abstract

In this study genetic diversity and interspecific relationships of 11 *Allium* L. species from Khorassan province of Iran including 32 accessions were investigated by inter simple sequence repeat (ISSR) markers. Nine ISSR primers produced a total of 80 polymorphic markers and revealed high polymorphism among the studied species. The average gene diversity, effective number of alleles and Shannon's information index were 0.2, 1.28 and 0.3, respectively. *Allium kuhsorkhense* exhibited the greatest level of variation (H_e: 0.18), whereas *A. stipitatum* demonstrated the lowest level of variability (H_e: 0.05). UPGMA (Unweighted Pair Group Method with Arithmetic mean) analysis showed that *Allium* accessions have a similarity range of 0.60 to 0.95. *Allium scapriscapum* composed the most distant group in the dendrogram. The clustered groups of *Allium* species clearly reflect the recent taxonomic concept of the genus at the subgenus and section levels. The present study showed that the ISSR technique is an effective molecular approach for analyzing genetic diversity and relationship in *Allium* species.

Introduction

The genus *Allium* L. is a member of Amaryllidaceae (APG III, 2009), subfamily Allioideae, tribe Allieae (Chase *et al*., 2009; Reveal and Chase, 2011). It is one of the largest genera of monocots and comprises more than 900 species naturally occurring in the Northern Hemisphere (Fritsch and Abbasi, 2013). This genus has a main centre of diversity in the eastern Mediterranean area as well as southwest and central Asia (Fritsch and Friesen, 2002). *Allium* is a typical genus for Irano-Turanian floristic region and displays a high level of specific endemism there (Matin, 1992). There are nearly 50 *Allium* species, which are cultivated widely in the world and many more wild species are utilized locally for human consumption as spices, vegetables, medicinal and ornamental plants (Friesen *et al*., 2006).

Allium consists of perennial herbs mostly characterized by tunicate bulbs, narrow basal leaves, umbellate or head-like inflorescences, flowers with 6 free or almost free tepals, and an onion-like odour and taste due to the presence of cystine sulphoxides (Li *et al*., 2010). Many studies assessing morphological and anatomical characters of *Allium* species have been performed and numerous data have so far been published (Friesen, 1995; Mathew, 1996; Fritsch and Friesen, 2002; Kovtonyuk *et al*., 2009). However, due to the close morphological similarities of the species, over reliance on dried specimens, and high degree of polymorphism of specific morphological traits (Khassanov and Fritsch, 1994; Mes *et al*., 1997), many gaps still remain in

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our knowledge of infrageneric taxonomy and differentiation and evolution in the genus (Rabinowitch and Brewster, 1990; Rabinowitch and Currah, 2002).

DNA-based molecular markers have been used previously in the studies of genetic diversity and phylogenetic analysis of *Allium* (Mes *et al.*, 1999; Friesen *et al.*, 2006; Gurushidze *et al.*, 2008; Mukherjee *et al.*, 2013). Of the different molecular markers, inter sample sequence repeat (ISSR) marker has been widely used to access species genetic diversity and relationships because of its cost effectiveness, simple operation as well as the need of very little starting DNA template (Lin *et al.*, 2009; Uysal *et al.*, 2010). In addition, previous studies of evaluating the phylogenetic relationship of Korean *Allium* species using ISSR marker indicated that these markers were highly informative in *Allium* (Hao *et al.*, 2002).

Recent advances in taxonomy and classification of *Allium* have shown that, there are about 135 species of *Allium* including 7 subgenera and 32 sections in Iran (Fritsch and Maroofi, 2010; Memariani *et al.*, 2012; Fritsch and Abbasi, 2013). North-eastern part of Iran (Khorassan provinces) with about 35 species is one of the most important centres of diversity of genus *Allium* in the country (Memariani *et al*., 2007). Previously there have been some studies on the taxonomy of *Allium* (Fritsch *et al.*, 2006; Fritsch and Abbasi, 2013), however, except for a few studies focusing on the diversity of one species (Abdoli *et al.*, 2009; Ebrahimi *et al.*, 2009), there has not been any reports corresponding to molecular genetic diversity and genetic relationship of *Allium* in Iran. The present study was designed to explore the genetic diversity and interspecific relationships of some *Allium* species in north-east Iran and to evaluate the potential of ISSR marker in detecting the genetic variability of native Alliums.

Materials and Methods

Plant materials:

A total of 32 accessions representing 11 species of *Allium* were collected from North Khorassan and Razavi Khorassan provinces, located in northeast of Iran during 2012−2013 (Table 1). The samples were identified based on morphological characteristics and diagnostic descriptions of the species in the relevant literature (Wendelbo, 1971; Fritsch and Abbasi, 2013). Modern concepts of infrageneric classification of the genus are based on Friesen *et al.* (2006), Fritsch *et al.* (2010), and Fritsch and Abbasi (2013).

DNA isolation:

Total genomic DNA was extracted based on CTAB method (Doyle and Doyle, 1990) using Accuprep genomic DNA extraction kit (Bioneer, Korea) following manufacturer's instructions. The relative purity and concentration of extracted DNA was estimated with spectroscopy and Lambda DNA (Thermo scientific, USA) using a known concentration as a reference.

ISSR amplification:

A set of 20 ISSR primers (University of British Colombia, Canada) was screened to generate the molecular profiles. Nine out of 20 primers were selected because of their consistent amplification and clear banding pattern. The primers sequences are listed in Table 2. PCR condition was optimized using different concentration of template DNA and Mg as well as different annealing temperature. PCR was done with 10 ng template genomic DNA, 5 µl of Taq DNA Polymerase, 2× Master Mix RED (Ampliqon, Denmark), 1.5 mM $MgCl_2$, 0.3 µM primer, in a total volume of 10 µL. DNA amplification was performed on Ependorf Master cycler gradient (Ependorf Scientific, Germany) using the following condition: an initial denaturation step of 94˚C for 5 min followed by 37 cycles of 94˚C for 25 s, optimized annealing temperature for 25 s, 72˚C for 1 min and a final extension at 72˚C for 5 min. The amplification products were separated by electrophoresis on 1.5% (W/V) agarose gel in 0.5× tris-borate-ethylenediamin tetra acetic acid

Table 1. List of species of *Allium* L. employed in the present study.

Accession code	Species	Section	Subgenus	Location
1	*Allium ampeloprasum* L.	*Allium*	*Allium*	North Khorassan: Rein
2	*A. ampeloprasum* L.	*Allium*	*Allium*	Razavi Khorassan: Ferizi
3	*A. ampeloprasum* L.	*Allium*	*Allium*	Razavi Khorassan: Andishish village
4	*A. ampeloprasum* L.	*Allium*	*Allium*	Razavi Khorassan: Ferizi
5	*A. atroviolaceum* Boiss.	*Allium*	*Allium*	Razavi Khorassan: Ferizi
6	*A. atroviolaceum* Boiss.	*Allium*	*Allium*	Razavi Khorassan: Ferizi
7	*A. atroviolaceum* Boiss.	*Allium*	*Allium*	Razavi Khorassan: Ferdowsi Univ. campus
8	*A. umbilicatum* Boiss.	*Avulsea*	*Allium*	Razavi Khorassan: Tandooreh
9	*A. umbilicatum* Boiss.	*Avulsea*	*Allium*	Razavi Khorassan: Tandooreh
10	*A. umbilicatum* Boiss.	*Avulsea*	*Allium*	Razavi Khorassan: Akhlamad
11	*A. cristophii* Trautv.	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Tandooreh
12	*A. cristophii* Trautv.	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Akhlamad
13	*A. cristophii* Trautv.	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Bazangan
14	*A. ellisii* Hook. f.	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Hezar Masjed Mountain
15	*A. altissimum* Regel	*Procerallium*	*Melanocrommyum*	Razavi Khorassan: Tandooreh
16	*A. altissimum* Regel	*Procerallium*	*Melanocrommyum*	Razavi Khorassan: Shamkhal
17	*A. altissimum* Regel	*Procerallium*	*Melanocrommyum*	North Khorassan: Zu-e Eram
18	*A. stipitatum* Regel	*Procerallium*	*Melanocrommyum*	Razavi Khorassan: Ferizi
19	*A. stipitatum* Regel	*Procerallium*	*Melanocrommyum*	Razavi Khorassan: Ferizi
20	*A. stipitatum* Regel	*Procerallium*	*Melanocrommyum*	Razavi Khorassan: Ferizi
21	*A. sarawschanicum* Regel	*Megaloprason*	*Melanocrommyum*	Razavi Khorassan: Shamkhal
22	*A. sarawschanicum* Regel	*Megaloprason*	*Melanocrommyum*	Razavi Khorassan: Shamkhal
23	*A. kuhsorkhense* R.M. Fritsch & Joharchi	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Ferizi
24	*A. kuhsorkhense* R.M. Fritsch & Joharchi	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Ferizi
25	*A. kuhsorkhense* R.M. Fritsch & Joharchi	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Ferizi
26	*A. kuhsorkhense* R.M. Fritsch & Joharchi	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Torbat-e-Jam
27	*A. kuhsorkhense* R.M. Fritsch & Joharchi	*Asteroprason*	*Melanocrommyum*	Razavi Khorassan: Torogh
28	*A. scabriscapum* Boiss.	*Scapriscapa*	*Reticulatobulbosa*	Razavi Khorassan: Tandooreh
29	*A. scabriscapum* Boiss.	*Scapriscapa*	*Reticulatobulbosa*	Razavi Khorassan: Allah-o Akbar Mountain
30	*A. oschaninii* O. Fedtsch.	*Cepa*	*Cepa*	Razavi Khorassan: Ferizi
31	*A. oschaninii* O. Fedtsch.	*Cepa*	*Cepa*	Razavi Khorassan: Ferizi
32	*A. oschaninii* O. Fedtsch.	*Cepa*	*Cepa*	Razavi Khorassan: Akhlamad

(TBE) buffer at 90 V, stained with DNA green viewer (Pars Tous, Iran) and visualized under ultraviolet (UV) light in gel documentation system (UVI doc, UK). A 100 bp DNA ladder (Thermo scientific, USA) was used as molecular size standard. PCR amplification was repeated twice or sometimes more for each primer to ensure the reproducibility of the results.

Table 2. Characteristics of ISSR markers and genetic diversity statistics.

Primers	Sequence	N	A_e	H_e	I
UBC 807	AGA GAG AGA GAG AGA GT	9	1.34 (0.182)	0.24 (0.102)	0.40 (0.132)
UBC 808	AGA GAG AGA GAG AGA GC	7	1.23 (0.103)	0.18 (0.068)	0.32 (0.095)
UBC 809	AGA GAG AGA GAG AGA GG	8	1.20 (0.136)	0.16 (0.085)	0.29 (0.118)
UBC 811	GAG AGA GAG AGA GAG AC	7	1.21 (0.091)	0.17 (0.061)	0.31 (0.085)
UBC 827	ACA CAC ACA CAC ACA CG	9	1.16 (0.079)	0.13 (0.057)	0.25 (0.084)
UBC 834	AGA GAG AGA GAG AGA GYT	11	1.26 (0.154)	0.19 (0.097)	0.33 (0.134)
UBC 835	AGA GAG AGA GAG AGA GYC	7	1.60 (0.365)	0.35 (0.160)	0.52 (0.193)
UBC 840	GAG AGA GAG AGA GAG AYT	6	1.68 (0.268)	0.39 (0.122)	0.57 (0.146)
UBC 855	ACA CAC ACA CAC ACA CYT	16	1.15 (0.061)	0.13 (0.047)	0.25 (0.074)
Average		8.88	1.28	0.2	0.34

N = number of bands; A_e = number of effective alleles; H_e = expected heterozigosity; I = Shannon's information index; Standard errors are in parentheses.

Data analysis:

The reproducible and well resolved fragments obtained from ISSR analysis were scored as binary code, *viz.* presence (1) and absence (0) of homologous bands. The binary data matrix was analyzed using NTSYS-PC version 2.1 software package (Rohlf, 2000). The pairwise genetic distances among all accessions was calculated based on Nei (1978) similarity coefficient. Genetic diversity (H_e) was calculated per primer and for each species (except for *Allium ellisii* Hook. f. for which only one accession was available) using POPGENE software, version 1.32 (Yeh and Boyle, 1997). A dendrogram was constructed by using the unweighted pair group method with arithmetic mean **(**UPGMA) employing the SAHN (Sequential Agglomerative Hierarchical and Nested) module of NTSYS-PC to show a phenetic representation of genetic relationships as revealed by similarity coefficient. Percentage of polymorphic bands (PPB) was calculated by dividing the number of polymorphic bands by total number of bands surveyed.

Results

Nine ISSR primers generated 80 bands corresponding to an average of 8.8 bands per primer (Table 2). The fragment size varied from 100 to 2200 bp and the number of bands ranged from 6 (UBC 840) to 16 (UBC 855). All of the 80 bands detected by ISSR primers were polymorphic among the individuals, *i.e.* the percentage of polymorphic bands was 100% for each primer. Considering all accessions, the average gene diversity, effective number of alleles and Shannon's information index was 0.2, 1.28 and 0.3, respectively. The genetic diversity generated by each primer varied from 0.38 (primer UBC 840) to 0.12 (UBC 855). The average effective number of alleles and Shannon's information index was 1.28 and 0.34, respectively. Among the 11 species, *A. kuhsorkhense* R.M. Fritsch & Joharchi and *A. ampeloprasum* L. exhibited the highest variability (PPB: 42.5% and 37.5%, H_e: 0.18 and 0.14, respectively) whereas the species *A. stipitatum* Regel and *A. sarawschanicum* Regel presented the least variability (PPB: 12.5% and 16.25%, and H_e: 0.05 and 0.07, respectively) as shown in Table 3.

A dendrogram generated based on Nei's genetic distances and UPGMA method revealed genetic relationships among *Allium* species and accessions (Fig. 1). The high cophenetic correlation (r = 0.95) obtained indicating a good fit between the dendrogram clusters and the distance matrix. The dendrogram displayed four main groups corresponding to four subgenera: *Allium*, *Melanocrommyum* (Webb & Berthel.) Rouy, *Cepa* (Mill.) Radić, and *Reticulatobulbosa* (Kamelin) N. Friesen. The most distant group comprised two accessions of *A. scabriscapum* with low genetic similarity coefficient (Nei = 0.22) belonging to the subgenus *Reticulatobulbosa*. The only species present in section *Cepa* in this study was *A. oschaninii* B. Fedtsch. which formed a distinct group. The largest group corresponds to the subgenus *Melanocrommyum*. It, however, is divided into three subclusters each composing the species of the same section: section *Procerallium* comprises the accessions of *A. altissimum* and *A. stipitatum*; *A. sarawshanicum* Regel (section *Megaloprason* Wendelbo) makes a separate cluster; and *A. cristophii* Trautv. and *A. ellisii* comprise the section *Asteroprason* R.M. Fritsch (subsection *Christophiana* Tscholok.). Five accessions of *A. kuhsorkhense* with the highest genetic diversity (0.18) placed in section *Asteroprason* (subsection *Asteroprason*). Species belonging to the subgenus *Allium* formed a separate cluster. This cluster, however, is divided into two subclusters correspond to sections *Allium* and *Avulsea* F.O. Khassanov.

Table 3. Diversity parameters of *Allium* species.

Species	Sample size	H_e	PPB (%)
A. altissimum Regel	3	0.1237	35
A. ampeloprasum L.	4	0.1445	37.5
A. atroviolaceum Bioss.	3	0.0802	21.25
A. cristophii Trautv.	3	0.0778	21.25
A. kuhsorkhense R.M. Fritsch & Joharchi	5	0.1779	42.5
A. oschaninii O. Fedtsch	3	0.0904	26.25
A. sarawschanicum Regel	2	0.0673	16.25
A. scabriscapum Bioss.	2	0.0725	17.5
A. stipitatum Regel	3	0.0516	12.5
A. umbilicatum Bioss.	3	0.1102	25

H_e = expected heterozygosity; PPB = percentage of polymorphic bands.

Discussion

In the present study, nine ISSR primers yielded a total of 80 reproducibale bands with an average of 8.8 bands per primer, which was higher than in some other studies that have applied ISSR markers to *Allium* species. For example, in an analysis of 24 accessions of 13 *Allium* species, Son *et al.* (2012) detected 3 to 11 alleles per locus (average 7.5 alleles per primer) using 20 ISSR markers. In our study, a very high level of ISSR polymorphisms was detected in the *Allium* species indicating ISSR-PCR as a reliable technique for fingerprinting in the genus. ISSR markers have been widely employed in assessment of genetic relationships within and between plant species (Thul *et al.*, 2012; Liu *et al.*, 2013). Although there are not many reports on application of ISSR markers for analyzing the genetic relationships among *Allium*, the efficacy of ISSR markers on revealing the classification of *Allium* species has been strongly supported by previous studies (Hao *et al.*, 2002; Son *et al.*, 2012). Furthermore, the homology of ISSR bands between *Allium* species has been formerly confirmed by sequence analysis (Son *et al.*, 2012).

In this study, the genetic diversity of the endemic species *A. kuhsorkhense*, as well as the genetic relationships among *Allium* species were studied for the first time. The study revealed that *A. kuhsorkhense* is the most diverse species among the species employed in Khorassan. The accessions of *A. kuhsorkhense* were collected from nearly distant area. This endemic species has the widest distribution range among the other species of the endemic *Allium* section *Asteroprason* (Memariani *et al.*, 2012). Hamrick (1989) stated that the wide distribution of a species can increase the rate of genetic diversity among the accessions. The clustered groups of *Allium* species clearly reflect the recent taxonomic concept of the genus at the subgenus and section levels. Subgenus *Melanocrommyum* comprises the largest group in the dendrogram. Three sections within the subgenus were detected and clearly identified, which is in consistent with previous studies (Gurushidze *et al.*, 2008; Fritsch *et al.*, 2010). In section *Asteroprason*, two main subclusters support its morphological classification into two subsections *Christophiana* and *Asteroprason* (Fritsch and Maroofi, 2010; Memariani *et al.*, 2012*).*

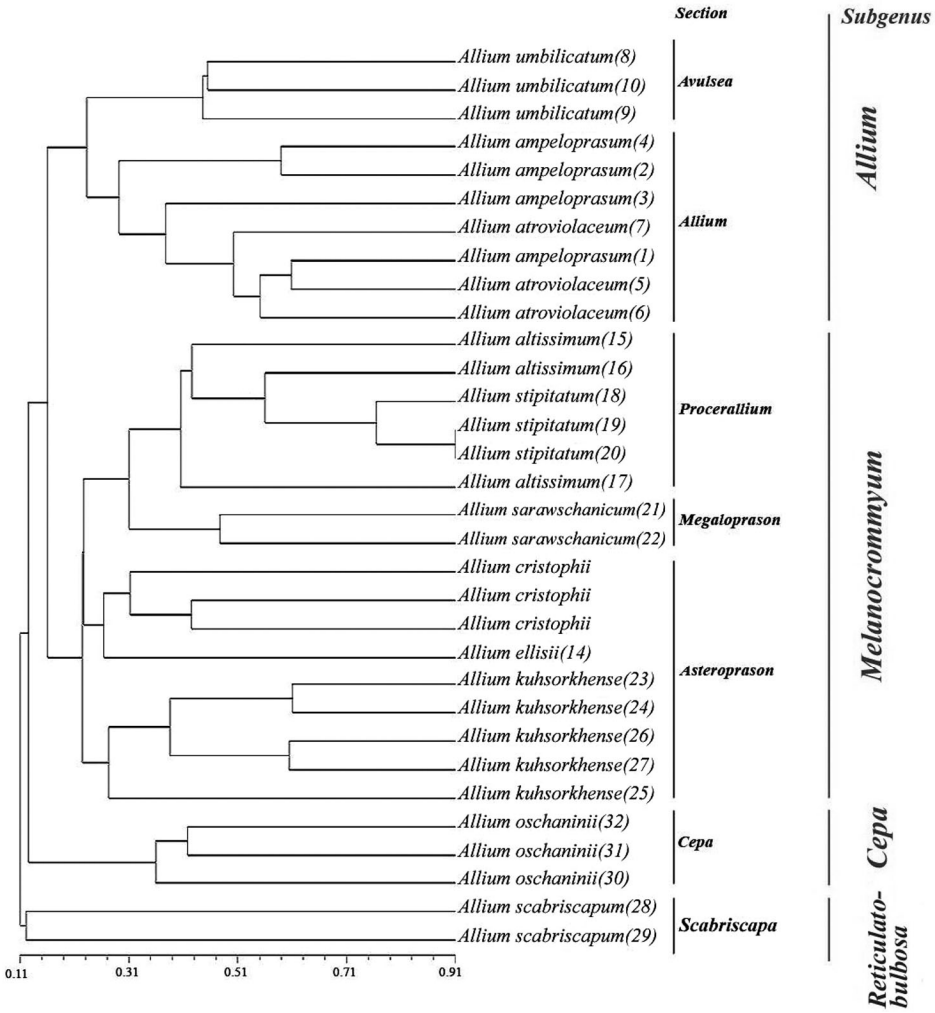

Fig. 1. UPGMA dendrogram showing species relationships of *Allium* based on Nei's genetic distance. The numbers in parentheses correspond accession codes of Table 1.

Within all sections of subgenus *Melanocrommyum*, the species were clearly distinguished except for *Procerallium* R.M. Fritsch. In this section, the accessions of *A. stipitatum* positioned among the accessions of *A. altissimum.* Indeed these two species are morphologically very similar and difficult to be distinguished properly. However, it is accepted that *A. altissimum* is more slender and somewhat smaller than typical *A. stipitatum* possessing narrower and glabrous (or at the most only sparsely toothed) leaves, a smaller umbel, and more intensely coloured, sublinear, in late anthesis spirally enrolled tepals (Fritsch and Abbasi, 2013). Our ISSR analysis is in agreement with the molecular analyses based on ITS sequences of nuclear rDNA, sequences of the plastid *trn*L-*trn*F region in which the cultivated strain of *A. altissimum* positioned among many accessions of Central Asian *A. stipitatum* underlining a high genetic similarity (Fritsch and Maroofi, 2010; Gurushidze *et al.*, 2010). Based on our analysis, the accessions of *A. ampeloprasum* and *A. atroviolaceum* (section *Allium*) are similarly positioned among each other, however, are well-separated from the accessions of *A. umblicatum* Boiss. (section *Avulsea*). Hirschegger *et al*. (2010) found similar results for *A. ampeloprasum* group based on nuclear and chloroplast DNA sequences analyses.

ISSR markers were highly informative at the section level as well as at the species level in the genus *Allium*. The resulting dendrogram was found consistent with the modern taxonomic classification, confirming that ISSR marker data can be used for taxonomic studies in the genus *Allium*. Genetic variation among wild species may assist plant taxonomists, and also breeders in identifying and introducing valuable traits into new hybrids. The collection and inclusion of more accessions of endemic and newly described *Allium* species will be useful for confirming their infrageneric classification, especially in morphologically diverse subgenus *Melanocrommyum* and also in economically important species and their wild relatives in subgenus *Allium* (garlic and leek).

About one-third of Iranian *Allium* species are native to Khorassan-Kopetdagh floristic province, located in the northeast of Iran and partly in southern Turkmenistan which is a transitional zone connecting different floristic provinces of Irano-Turanian region. Several *Allium* species occur in the eastern or western limits of their distribution ranges in Khorassan-Kopetdagh as well as many narrow and local endemics (Wendelbo, 1971; Memariani *et al*., 2007, 2012). Molecular analyses on newly described and rare *Allium* species, especially using ISSR markers, may helpfully reveal their taxonomic position among the infrageneric classification of the genus. Moreover, the assessment of genetic diversity among populations of *Allium* species can help to prioritize conservation efforts in order to prevent the extinction of the rare and threatened taxa with lower genetic diversity and also effective conservation of the genetically variable taxa.

Acknowledgement

This work was supported by the Ferdowsi University of Mashhad (grant no. 17812).

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(Manuscript received on 1 November 2014; revised on 11 June 2015)