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Full Length Research Paper

Inter-simple sequence repeats (ISSR) and morphological diversity in *Onosma* L. (*Boraginaceae*) species in Iran

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Morphological characteristics as well as inter-simple sequence repeats (ISSR) molecular markers were used to study the species relationship in *Onosma* species (Boraginaceae) in Iran. Principal component analysis (PCA) showed that morphological characters like caule leaf size and shape, bract shape, corolla shape, nutlet length, venation and corolla teeth size and anther length are the most variable morphological characters among *Onosma* species studied. Out of 17 ISSR primers used, 6 primers produced 41 polymorphic and reproducible bands. Few specific ISSR bands were obtained for the species studied which show genetic material change during species diversification. Such bands may be used in the species identification too. Morphological and molecular trees obtained partly agreed with each other. In both trees, *Onosma dasytrichum* and *Onosma microcarpum* are placed close to each other, *Onosma procerum* and *Onosma pachypodum* show affinity to each other and *Onosma araraticum*, *Onosma bodeanum*, *Onosma bistonensis*, *Onosma stenosiphon* and *Onosma bulbotrichum* are placed close to each other while, *Onosma rostellatum* stands far from the other species. The combined morphological and ISSR tree obtained separated the members of three sections of *Onosma*, *Podnosma* and *Protonosma* from each other. Moreover, almost a good separation of different subsections in the section *Onosma* was observed in the combined tree.

Key words: Inter-simple sequence repeats, morphometry, *Onosma.*

INTRODUCTION

Onosma is a genus with about 150 species occurring in dry, cliffy and sunny habitats, distributed mainly in Eurasia and Mediterranean regions, having its center of distribution and maximum concentration of species in Iran (Ball, 1972; Willis, 1973).

The genus *Onosma* contains biennial or perennial, hispid herbs, with flowers in terminal cymes, calyx accrescent, stamens inserted at the middle of the corolla and generally 4 nutlets flat at the base (Binzet et al., 2010).

It contains about 60 species in Flora Iranica region (Riedl, 1968), with 39 species growing in Iran (Khatamsaz, 2002; Attar and Jouharchi, 2006; Attar, 2007).

Molecular studies in the genus *Onosma* are very limited, mainly confined to amplified fragment length polymorphism (AFLP) study of genetic diversity in populations of *Onosma echioides* L. (Mengoni et al., 2006). Therefore, the present study considers numerical analysis of morphological and inter simple sequence repeats (ISSR) characteristics in 29 *Onosma* species growing in Iran and tries to show the species relationship based on these features.

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Table 1. Species studied and their respective sections and subsections divisions.

Species	Section	Subsection
O. albo-roseum Fisch.& C. A. Mey	Onosma	Asterotricha (Boiss) Gürke.
O. araraticum Riedl.	Onosma	Haplotricha (Boiss) Gürke.
O. armenum DC.	Onosma	Asterotricha
O. bistonensis Attar.	Onosma	Asterotricha
O. bodeanum Boiss.	Onosma	Haplotricha
O. bulbotrichum DC.	Onosma	Haplotricha
O. chlorotrichum Boiss.	Onosma	Heterotricha Boiss.
O. cornotum Riedl.	Onosma	Haplotricha
O. dasytrichum Boiss.	Onosma	Asterotricha
O. demavendicum Riedl.	Onosma	Heterotricha Boiss.
O. dichoroanthum Boiss.	Onosma	Haplotricha
O. elwedicum Wettst.	Onosma	Heterotricha
O. intertextum Hub.	Onosma	Asterotricha
O. kilouyense Boiss.	Onosma	Heterotricha
O. kotschy Boiss.	Onosma	Haplotricha
O. longilobum Bge.	Onosma	Haplotricha
O. macrophyllum Bornm.	Onosma	Heterotricha
O. microcarpum Steven ex DC.	Onosma	Haplotricha
O. olivieri Boiss.	Onosma	Heterotricha
O. pachypodum Boiss.	Onosma	Haplotricha
O. platyphyllum Riedl.	Onosma	Haplotricha
O. procerum Boiss.	Onosma	Haplotricha
O. rasychaenum Boiss.	Onosma	Asterotricha
O. sabalanicum	Onosma	Haplotricha
O. sericeum Willd.	Onosma	Haplotricha
O. stenosiphon Boiss.	Onosma	Haplotricha
O. straussii (Riedl) Khatamsaz.	Onosma	Haplotricha
O. rostellatum Lehm.	Protonosma	-
O. orientale L.	Podnosma	-

ISSR markers show high level of repeatability and have been used as useful molecular markers in studying genetic diversity and species relationships (Pharmawati et al., 2004; Dogan et al., 2007). Morphometric analysis has also been used to clarify taxonomic status of *Onosma* species (Peruzzi and Passalacqua, 2008).

MATERIALS AND METHODS

Plant materials

27 *Onosma* species from two sections of *Onosma* and *Protonosma* were studied (Tables 1 and 2). The species studied have been placed in 3 subsections of *Asterotricha* (Boiss) Gürke. *Haplotricha* (Boiss) Gürke., and *Heterotricha* Boiss (Riedl, 1968; Khatamsaz, 2002; Attar and Jouharchi, 2006; Attar, 2007). Voucher specimens have been deposited in Herbarium of Shahid Beheshti University (HSBU) and Iran National Botanical Garden Herbarium (Iran).

Morphometry

In total, 36 morphological characters (quantitative and qualitative) were used for morphometry and coded accordingly (Table 3). Minimum of 10 randomly selected plants from each species from different populations were used for obtaining morphological data.

For multivariate analyses, the mean of quantitative characters were used, while qualitative characters were coded as binary/ multistate characters. Standardized variables (mean = 0, variance = 1) were used for multivariate statistical analyses (Podani, 2000). Principal components analysis (PCA) was performed to identify the most variable morphological characters among the species studied and PCA plot of the components obtained were used to get species groupings (Podani, 2000).

Unweighted paired group with arithmetic average (UPGMA) and Neighbor joining (NJ) clustering methods were performed for grouping of the species based on morphological characters by using PAUP vers. 4b (2000), while PCA analysis was performed by SPSS ver. 9 (1998) software.

ISSR assay

Total genomic DNA was extracted from fresh leaves using the CTAB method by Murry and Tompson (1980) with the modification described by De la Rosa et al. (2002). Six ISSR primers used were $(GA)_{9T}$, UBC810, UBC811, UBC834, UBC849 and CA7GT commercialized by the University of British Columbia (UBC).

Table 2. Species locality and their vouchers.

Species	Locality	Collector	Voucher number
O. albo-roseum	Kermanshah, Ghasre- Shrin to Gilanegharb	Sharif	IRAN-2684
O. araraticum	Zanjan, Belgheis Mt.	Mehrabian	HSBU-2010200
O. armenum	Azarbaidjan, Khoy to Ghotor	Sharif	IRAN-2693
O. bistonensis	Kermanshah, Biston Mt.	Mehrabian	HSBU-2010207
O. bodeanum	Eastern Azarbaidjan, Mishodagh Mt.	Mehrabian	HSBU-2010211
O. bulbotrichum	Kurdestan, Marivan to Saghez	Mehrabian	HSBU-2010203
O. cornotum	Eastern Azarbaidjan, Mishodagh Mt.	Mehrabian	HSBU-2010204
O. dasytrichum	Kermanshah, Javanrod to Paveh	Mehrabian	HSBU-2010216
O. demavendicum	Markazi, Arak	Mehrabian	HSBU-2010228
O. dichoroanthum	Khorasan Razavi, Mashhad	Sheikhakbari & Ghorbani	HSBU-2010222
O. elwedicum	Tehran, Lashkarak	Mehrabian	HSBU-20110205
O. kilouyense	Kermanshah, Gahvareh	Mehrabian	HSBU-2010209
O. kotschy	Lorestan, Aligodarz to Ghalikuh	Iranshahr	IRAN-2697
O. longilobum	Khorasan, Darehgaz, Allah-o- Akbar Mt.	Iranshahr & Zargani	IRAN-2831
O. macrophyllum	Kermanshah, Gahvareh, Baba Shah Ahmad	Mehrabian	HSBU-2010226
O. microcarpum	Mt. Kurdestan, Dizli to Nodesheh	Mehrabian	HSBU-2010219
O. olivieri	Kurdestan, Nosoud to Marivan	Mehrabian	HSBU-2010202
O. orientale	Kohkilouye, Dogonbadan	Zairi	IRAN-2907
O. pachypodum	Tehran, Lashkarak	Mehrabian	HSBU-2020224
O. platyphyllum	Lorestan, Oshtorankuh, Dsht-e-Takht	Iranshahr	IRAN-2010217
O. procerum	Esfahan, Semirom, Sivar Village	Mehrabian	HSBU-2010221
O. rasychaenum	Kurdestan, Sanandaj, Salavatabad,	Pahlevani & Amini	IRAN-2919
O. rostellatum	Kurdestan, Bayangan, 4555ft	Mehrabian	HSBU-2010225
O. sabalanicum	Ardebil, Meshkinshahr, Sabalan mountain	Mehrabian	HSBU-2010201
O. sericeum	Tehran, Lashkarak	Mehrabian	HSBU-2010227
O. stenosiphon	Kerman, Rafsanjan, Sarcheshmeh	Riedl & Etemad	IRAN-2797
O. straussii	Markazi, Arak, Gavar village	Mehrabian	HSBU-2010212

Polymerase chain reaction (PCR) reactions were performed 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; 0.2 µM of a single primer; 20 ng genomic DNA and 3 unit of Taq DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 1 min at 50°C, 1 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Fermentas, Germany).

ISSR bands obtained were treated as binary characters and coded accordingly (presence =1, absence = 0). The average taxonomic distance and Manhatan distance were used as dissimilarity coefficient in cluster analysis of data (Podani, 2000). UPGMA and NJ clustering methods were performed for grouping of the species by using PAUP vers. 4b (2000), while PCA analysis was performed by SPSS ver. 9 (1998) software.

RESULTS

Morphometry

The PCA analysis performed to identify the most variable morphological characters among *Onosma* species studied, revealed that the first five PCA factors comprise about 60% of total variance (data not given). In the first factor, characters like caule leaf size, bract shape, corolla shape and nutlet length showed th highest positive correlation (r>0.60), while in the second factor with about 12% of total variance, morphological characters like venation and corolla teeth size, showed the highest negative correlation (r= -0.60). In the third and forth factors with about 7 and 6% of total variance respectively, characters like caule leaf shape and anther long had the highest positive correlation (r>0.69).

UPGMA and NJ clustering of morphological characters produced similar results and therefore UPGMA tree is discussed here. The first major cluster is formed by *Onosma araraticum, Onosma armenum, Onosma rasychaenum, Onosma demavendicum* and *Onosma bodeanum*. The second cluster is formed by *Onosma bistonensis, Onosma orientale, Onosma sabalanicum* and *Onosma stenosiphon* (Figure 1).

Onosma bulbotrichum, Onosma kotschy, Onosma platyphyllum, Onosma oliveri, Onosma elwedicum and *Onosma kilouyense* formed the third cluster, while

Table 3. Morphological characters and their coding.

Morphological character	0	1	2	3	4	5	6	7	8	9
Anther exertion	Absent	Present								
Corolla trichome	Absent	Low	High							
Height		≥15 cm	25-15	35-25	45-35	55-45	≥55 cm			
Anther-connection	Absent	Present								
Anther connection length		1/3	1/2							
Trichome kind		Simple	Branched basal	Stellate						
Nectar trichome	Absent	Present								
Corolla color		Yellow	Purple	White	Yellow-pink	White-blue	Yellow-blue	Red	Red-purple-yellow	Red-blue
Corolla length		10-15	15.1-20	20.1-25	25.1-30	30.1-35				
Corolla lobe	Absent	Present								
Corolla teeth size		0.5-1	1.1-1.5	1.6-2	2.1-2.5	2.6-3				
Calyx length		5-10	10.1-15	15.1-20	20.1-25	25.1-30				
Bract size		1-5	5-10	10-15	15-20					
Bract shape		Lanceoate	Wide lanceolate	Large						
Pedicle		1-2	2.1-3	3.1-4	4.1-5	5.1-6	6.1-7	7.1-8		
Basal leaf length		5-10	10.1-20	20.1-30	30.1-40	40.1-50	50.1-80	80≤		
Caule leaf length		cm								
Nutlet curve	Absent	Present								
Venation	Absent	Reticulate								
Small trichomes	Absent	Present								
Trichome color		White	Yellow							
Corolla shape		Tubular	Campanulate							
Basal leaf shape		Spathulate	Linear-spathulate	Obovate	Oblong-Spathulate	Lanceolate-Spathulate	Lanceolate-oblong	Lanceolate	Linear-lanceolate	Ovate-Lanceolate
Caule leaf shape		Spathulate	Linear spathulate	Obovate	Oblong-Spathulate	Lanceolate-Spathulate	Lanceolate-Oblong	Lanceolate	Linear-Lanceolate	Ovate-Lanceolate
Trichome orientation		Vertical	Repent							
Inflorescent dense		Scant	Medium	Dense						
Style exertion	Absent	Present								
Style length		5-10	11-15	16-20	21-25	26-30				
Anther length		5-7	7-9	10≤						
Nutlet length		2-3	3-4	4-5	5-6	6-7	7-8			
Nutlet wide		2-3	3-4	4-5	5-6					
Nutlet orientation		Vertical	curve							
Nutlet beak		1-1.5	1.6-2	2.1-3						
Nutlet shape		Trapeziform	Ovate	Deltoid	Wide ovate	Linear				
Nutlet color		Black-yellow	Brown-white	Brown	Green-white	White				
Nutlet surface		Smooth	Wrinkle	Verrucate						

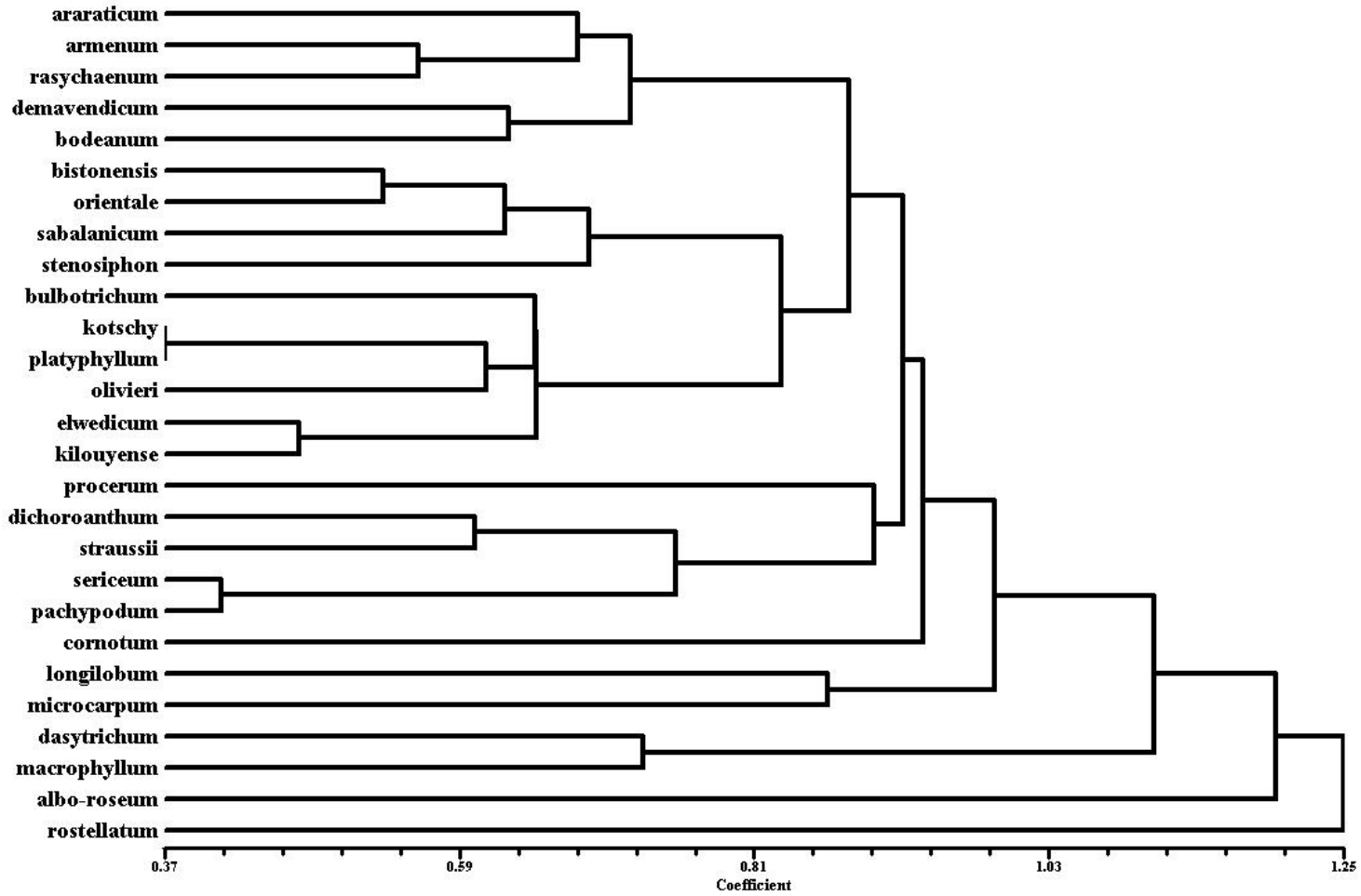

Figure 1. UPGMA tree of morphological characters.

Onosma procerum, Onosma dichoroanthum, Onosma straussii, Onosma sericeum and *Onosma pachypodum* comprised the fourth cluster. Two species of *Onosma longilobum* and *Onosma microcarpum* showed morphological affinity and form a separate cluster. The same is true for two species of *Onosma dasytrichum* and *Onosma macrophyllum* showing close affinity and form a distinct cluster. The last two species showing similarity to each other were *Onosma albo-roseum* and *Onosma rostellatum* which stand far from the other species (Figure 1).

ISSR analysis

Out of the 17 ISSR primers used (alone and in combination), 6 primers produced 41 polymorphic and reproducible bands. Bands No. 4 and (450 and 750 bp respectively) of the primer 807, band No. 6 (450 bp) of the primer 834 and band No. 8 (750 bp) of the primer 849 were specific for *O. rostellatum*, band No. 8 (480 bp) of the primer 834, occurred only in *O. procerum*. Few bands were present only in two species, for example, band No. 4 of the primer 834 (400 bp) occurred in *O. bistonensis* and *O. bulbotrichum*, while the bands No. 5 and 7 (420 and 460 bp respectively) occurred in, *O. rostellatum* and *O. sericeum*. UPGMA and NJ trees of combined morphological and ISSR data produced similar results and therefore NJ tree is only discussed thus (Figure 2). *O. araraticum* and *O. straussii* from the subsect. *Haplotricha* showed affinity and stand far from the other species forming the first major cluster. The second major cluster contains three subclusters. *O. armenum*, *rasychaenum* (subsect. *Asterotricha*) and *O. orientale* (sect. *Protonosma*), *O. demavendicum* and *O. cornotum* (subsect. *Haplotricha*) and *O. macrophyllum* (subsect. *Heterotricha*) are placed close to each other comprising the first subcluster, while *O. orientale* (subsect. *Asterotricha*), *O. dichoroanthum*, *O. kilouyense* and *O. elwedicum* (subsect. *Heterotricha*) comprise the second subcluster. The third subcluster is formed by *O. dasytrichum* (subsect. *Asterotricha*) and *O. sericeum*

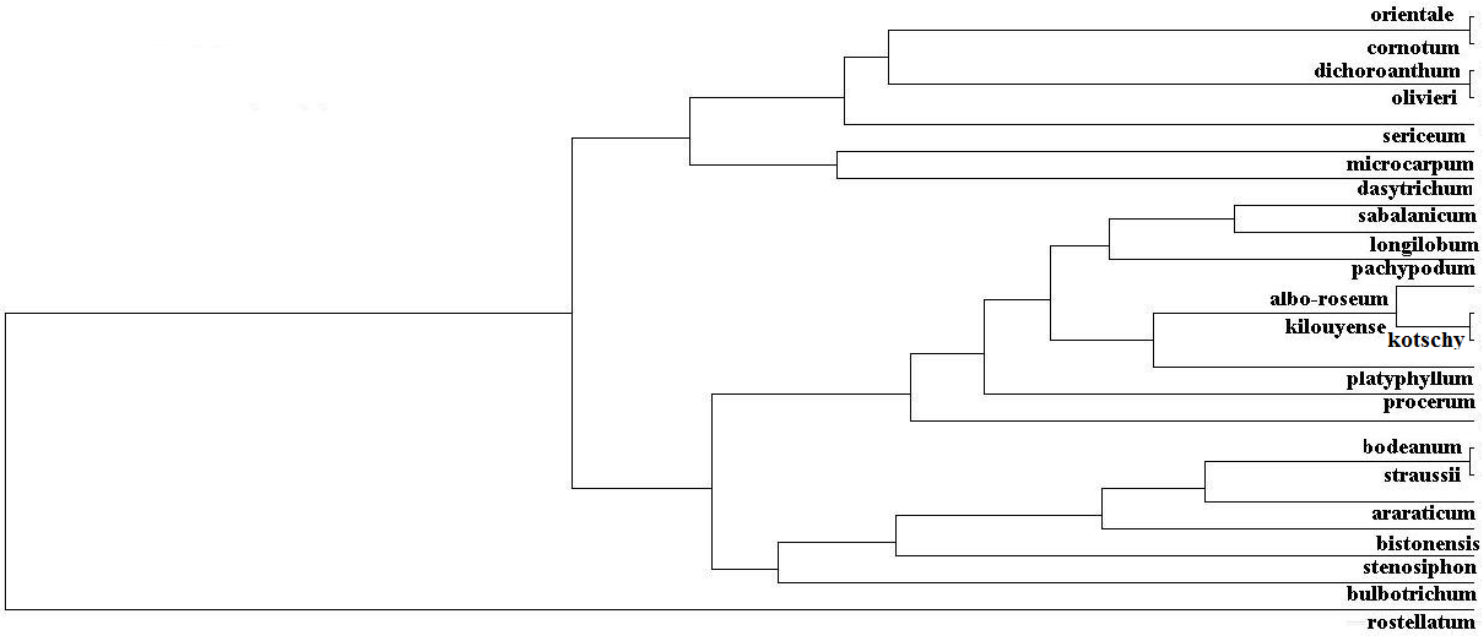

Figure 2. UPGMA tree of ISSR data.

(subsect. *Haplotricha*).

The third major cluster is formed by *O. bistonensis* and *O. bulbotrichum* (subsect. *Haplotricha*), *O. rostellatum* (sect. *Protonosma*), *O. stenosiphon* and *O. microcarpum* (subsect. *Haplotricha*). The fourth major cluster is formed by *O. procerum*, *O. sabalanicum*, *O. pachypodum*, *O. kotschy*, *O. longilobum*, *O.* platyphyllum, and *O. bodeanum* (subsect. *Haplotricha*) and *O. albo-roseum (subsect. Asterotricha).*

Combined data analysis

We made similar analysis on the combined data set of morphological and ISSR data (Figure 3). The first major cluster is comprised two subclusters. *O. araraticum*, *O. straussii* and *O. bulbotrichum* form the first subcluster, while *O. kilouyense*, *O. kotschy*, *O. platyphyllum*, *O. elwedicum*, *O. pachypodum*, *O. sabalanicum*, *O. albo-roseum*, *O. longilobum* and *O. procerum* form the second subcluster. These species all are from the section Onosma, subsect. *Haplotricha*.

The second major cluster contains 3 subclusters. The first subcluster is formed by *O. armenum*, O. *rasychaenum* (both from the sect. Onosma, subsect. Asterotricha), *O. demavendicum* (subsect. Heterotricha), and *O. bodeanum* (subsect. *Haplotricha*).

O. bistonensis (subsect. Asterotricha), *O. stenosiphon* and *O. microcarpum* (both from subsect. *Haplotricha*) comprise the second subcluster, while *O. dichoroanthum*, *O. sericeum* and *O. cornotum* (all from the subsect. *Haplotricha*) as well as *O. olivieri* (subsect. Heterotricha) form the third subcluster.

O. orientale (sect. Podnosma) stands alone in a separate cluster joining the members of second major cluster with some distance. Two species of *O. dasytrichum* (subsect. Asterotricha) and *O. macrophyllum* (subsect. *Haplotricha*) show affinity and form a cluster while, *O. rostellatum* (sect. Protonosma) stands far from the other species in a single cluster.

DISCUSSION

The variable qualitative morphological characters among *Onosma* species studied, like caule leaf shape, bract shape, corolla shape, venation may serve as useful taxonomic characters for the species delimitation which can also be supported by some quantitative characters including the caule leaf size, nutlet length, corolla teeth size and anther length.

Morphometric study was also used to study the species relationship in *Onosma echioides* complex (Peruzzi and Passalacqua, 2008), concluding to treat this complex as a single species subdivided in four subspecies. The names *O. angustifolia* Lehm., *O. canescens* J. and C. Presl, *O. dalmatica* Scheele, *O. echioides* var. *columnae* Lacaita, *O. echioides* var. *veronensis* Lacaita and *O. javorkae* Simonkai were typified and a new combinations of *O. echioides* subsp. *angustifolia* (Lehm.) Peruzzi and N. G. Passal., *O. echioides* subsp. *canescens* (J. and C. Presl) Peruzzi and N. G. Passal. and *O. echioides* subsp. *dalmatica* (Scheele) Peruzzi & N. G. Passal., were proposed.

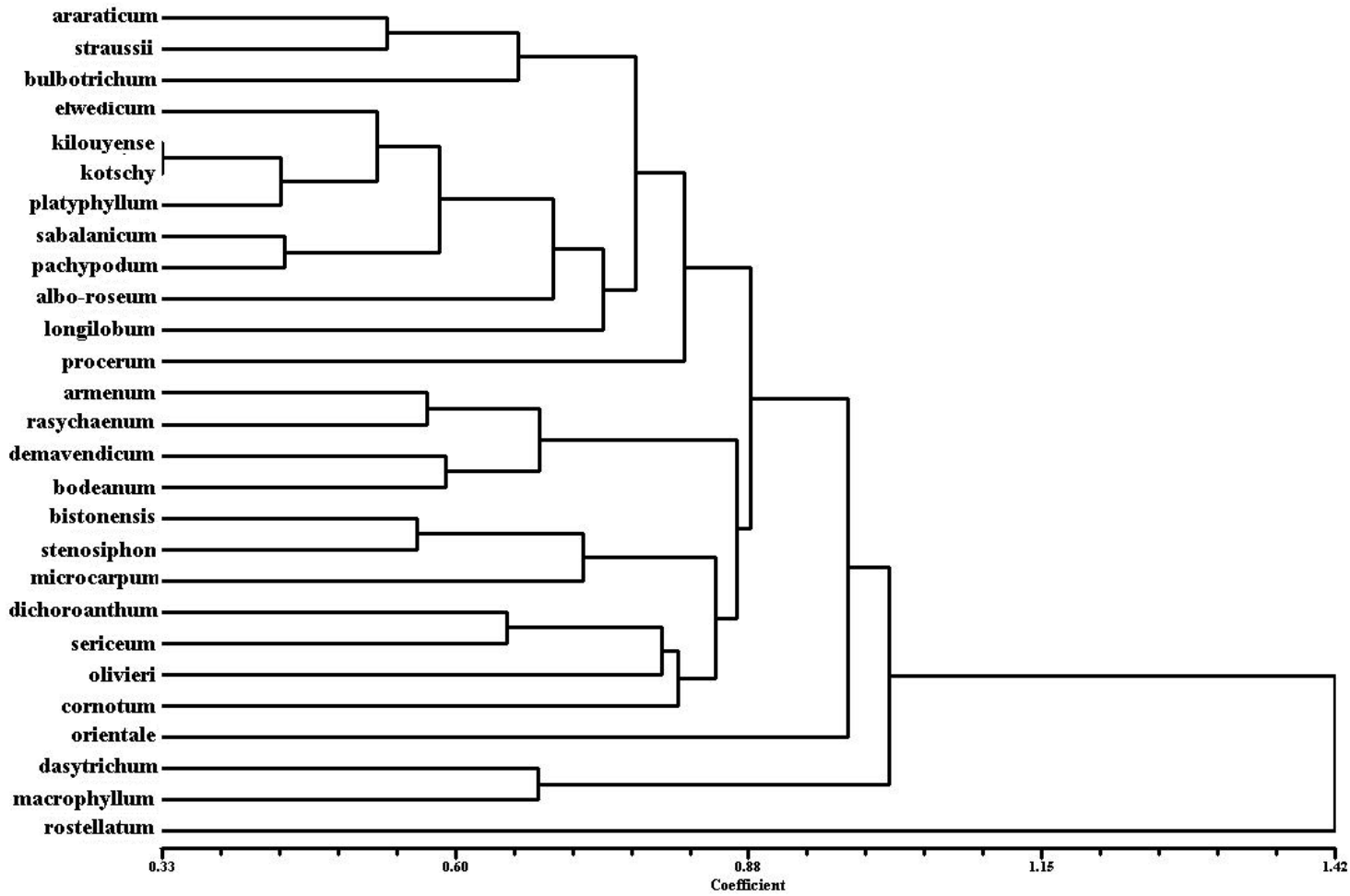

Figure 3. UPGMA tree of combined data.

The specific ISSR bands obtained in *O. rostellatum*, *O. procerum*, showed the occurrence of genetic material change during species diversification and that such molecular markers may be used in the species delimitation. Possibly, by using more number of ISSR markers we may find more specific bands for the other species too. Moreover, presence of common bands in two or more species, for example ISSR bands obtained in *O. bistonensis* and *O. bulbotrichum*, as well as in, *O. rostellatum* and *O. sericeum* indicate the presence of synapomorphic characters to be used in sister group identification.

The NJ and UPGMA trees obtained from morphological and molecular data partly agrees with each other. In both trees, *O. dasytrichum* and *O. microcarpum* are placed close to each other, *O. procerum* and *O. pachypodum* showed affinity to each other and *O. araraticum*, *O. bodeanum*, *O. bistonensis*, *O. stenosiphon* and *O. bulbotrichum* are placed close to each other while, *O. rostellatum* stands far from the other species.

The tree of combined morphological and ISSR data (Figure 3), clearly separates the members of three sections of *Onosma*, *Podnosma* and *Protonosma* from each other. Moreover, almost a good separation of different subsections in the sect. *Onosma* occurs in this combined tree. For example all members of the first major cluster are from the subsect. *Haplotricha*. Therefore, molecular data along with morphological characters may be of use in taxonomic treatment of the genus *Onosma*.

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