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GENETIC CHARACTERIZATION OF SELECTED GENERA OF FAMILY RHAMNACEAE BASED ON *rps* 11 GENE

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Abstract

Rhamnaceae family is geographically distributed all over the world; its representation both in tropical and subtropical regions presents difficulties in tracing out its history of origin. Rhamnaceae contains 58 genera enclosing a total of 984 species. In the present study, eleven species (*Z. mauritiana*, *Z. spina- crhristi*, *Z. jujuba*, *Z. mauritiana var. spontanea*, *Z. oxyphylla*, *Z. nummularia*, *S. thea*, *S. filiformis*, *R.pentapomica*, *R. purpurea* and *H. lanceolatus*) representing different genera of family Rhamnaceae were investigated for the assessment of genetic variability. Genomic DNA from all the selected species was isolated, used as template for the amplification of *rps*11 gene by a set of *rps* 11 primers and amplified fragments was sequenced. Sequence data was analyzed by molecular evolutionary genetics analysis (MEGA 6). It was observed that *S. thea* and *S. filiformis* in cluster I sharing same genus exhibited 99 % bootstrap value while *Z. jujuba* and *H. lanceolatus* of two different genera also showed 99 % homology manifesting close kinship among them. The value of evolutionary divergence was found to be in the range of 0.073-0.407 among the investigated species. Furthermore, genetic characterization by use of molecular markers would help in better understanding of diversity among plant species at different taxonomic ranks.

Key words: Rhamnaceae, *rps* 11, *Z. mauritiana*, *Z. spina- crhristi*, *S. thea*, *S. filiformis*,

Introduction

Rhamnaceae is multiethnic family enclosing 57 different genera (Hauenschild *et al*., 2016; Norouzi *et al*., 2017) and 900 species (Cronquist, 1981), popularly known as buckthorn family (Nasir and Ali, 1972; Qaisar and Nazimmudin, 1981). Latest taxonomic reconsideration of whole family states, Hooker's (1862) five tribes containing 58 genera and 984 species (Suessenguth, 1953). Michel Adanson was responsible for the establishment of this taxon in the *familles des plantes* as 'ziziphi' (Adason, 1763), afterward Antoine- Laurent de Jussieu in *Genera Plantarum* treated it as Rhamni (de Jessieu, 1789). Mostly members of family Rhamnaceae are trees, shrubs, climber and rarely herbs (only one) (Cronquist, 1981; Richardson *et al*., 2000a; Norouzi *et al*., 2017). Buckthorn is allocated in many parts of the world (Nasir and Ali, 1972; Qaisar and Nazimmudin, 1981), its representation in both tropical and temperate regions presents difficulties in tracing out its history (Raven & Axelord, 1974). Although family Rhamnaceae is widely distributed worldwide but major diversity zone is toward the Southern hemisphere (Rhichardson *et al*., 2000a). The mega fossil is the first unequivocal evidence of the distribution of Rhamnaceae family in Southern hemisphere (Jud *et al*., 2017). Australia exhibit a well representation of family Rhamnaceae with total 200-250 species (Kellermann *et al*., 2005), 90 % of which are endemic (Ladiges *et al*., 2005). In Pakistan family Rhamnaceae is represented by 6 genera and 21 species (Nasir and Ali, 1972; Qaisar & Nazimmudin, 1981).

Rhamnaceae show obhaplostemony arrangement of its stamen and petals a relatively rare feature of angiosperms evaluating its association with Vitacae and Cornaceae having the same arrangement (Richardson *et al*., 2000b). While vegetative features depicts close taxonomic relation with Elaeagnaceae (Takhtajan, 1997). Morphological characters of fruits are emphasized mainly in tribal classification of family Rhamnaceae, resulting two large and heterogeneous tribes Rhamneae and Zizipheae by Sussenguth's system. Both *Ziziphus* and *Berchemia* on the basis of drupaceous fruits were included in tribe Zizipheae, differentiated from each other in number of other characters like venation patterns and position of ovary indicating relationship to other tribes. Moreover other tribes were monophyletic including Colletieae, Ventilagineae and Gouanieae (Richardson *et al*., 2000a).

DNA technology is the proposed alternative to traditional morphological taxonomy, where researchers tried to gain advantage of variability in the genome to identified species (Hebert *et al*., 2003; Pons *et al*., 2006). Scientists are investigating viruses, bacteria and protists through micro genomics system of identification as the identification of these groups is almost impossible by utilizing only morphological basis (Zettler *et al*., 2002; Hebert *et al*., 2003; Lewis & Lewis, 2005; Abriouel *et al*., 2008; Iliff *et al*., 2008; Shinwari *et al.*, 1994a, 1994b).

Chase *et al*., (1993) investigated 499 species of angiosperm through *rbc*L plastid gene executing a weak relationship of Rhamnaceae with Rosaceae, Urticales and Fagales. Furthermore, close relation between Rhamnaceae and Elaeagnaceae was disclosed by *rbc*L, *atp*β and 18S nuclear ribosomal DNA (Soltis *et al*., 1995; Savolainen *et al*., 2000), on the other hand noncoding region of plastid has placed Rhamnaceae in relation with Dirachmaceae and Barbeyaceae (Thulin *et al*., 1998). Process of nitrogen fixation is common among Elaeagnaceae, Rosaceae, Ulmaceae and Rhamnaceae figuring out a close association across them (Soltis *et al*., 1995; Swensen,

1996). Richerdson *et al*., (2000a) concluded family Rhamnaceaeas an old monophyletic in association with Dirachmaceae and Barbeyaceae. The underlying objective of present investigation was to assess the taxonomic status (monophyly and polyphyly) of family Rhamnaceae through its selected genera and evolutionary relationship among them based on a chloroplast gene *rps* 11.

Material and Method

The main objective of the designed study was to check genetic variability among selected species of family Rhamnaceae. For this purpose different species of this cosmopolite family was collected from different areas of Pakistan and Azad Kashmir. Identification of specimens was accomplished with the help of online data available and herbarium, and then stored at 4ºC for further processing. Species included in the present study are enlisted in table 1.

Table 1. List of species from selected genera of family Rhamnaceae.

Sr. No.	Species	Location
	Ziziphus	
1.	*Ziziphus mauritian* Lam	KPK,Hazara, Sawat
2.	*Ziziphus spina-christi* L.	Kashmir, Baluchistan
3.	*Ziziphus oxyphylla* Edgew	Swat, Buner, Hazara
4.	*Ziziphus jujuba* Mill.	Kashmir, Islamabad
5.	*Ziziphus mauritiana* var. *spontanea* Edgeworth	Kashmir, Pakistan
6.	*Ziziphus nummularia* Brum. f.	Attock, D.G. Khan
	Sageretia	
1.	*Sageretia thea* (Osbeck) Jhonst.	Islamabad
2.	*Sageretia filiformis* Roth	Pakistan
	Rhamnus	
1.	*Rhamnus pentapomica* R. Parker	Kashmir, Pakistan
2.	*Rhamnus purpurea* Edgew	Kashmir, Pakistan
	Helinus	
1.	*Helinus lenceolatus* Brandis	Pakistan, Mirpur

Key words: KPK; Khyber Pakhtunkhwa, D.G Khan: Dera Ghazi Khan

Isolation of genomic DNA: Genomic DNA was isolated by CTAB (cetyltrimethyl ammonium bromide) method with little amendments and 1% agrose gel was used to check the quality of quality of isolated DNA.

Primer designing and PCR amplification: Chloroplast genome of *Tobbacum* (Accession # Z00044.2) was used to design a pair of primer to amplify *rps*11 gene with the help of primer '3' online available program. The sequence of designed primers is,

rps 11 F: 5' TGGCAAAAGCTATACCGAAAA 3'
rps 11 R: 5' TTCGGAGGTCTACAGCCATT 3'

Chloroplast genes encoding ribosomal proteins have already been used in past for assessing genetic diversity (Mahmood *et al.*, 2011; Wali *et al.*, 2013; Ibrahim *et al.*, 2014; Rehman *et al*., 2015) and this marker system was found to be useful.

PCR reaction mixture and PCR conditions: A reaction mixture of 25 µL was prepared for the amplification of *rps* 11 gene through polymerase chain reaction (PCR). In reaction mixture, 2.5 µL of *Taq* buffer (10 ×), 1.5 µL of $MgCl_2$ (25 mM), 1 µL of forward primer and reverse primer each (25 pM), 1.5 µL of dNTPs (2 mM), 16.2 µL nano pure water, 0.3 µL of *Taq* polymerase (1.5 U) and 1 µL template DNA (50ng/ µL) were added. PCR conditions used were as follows: 5 minutes pre-denaturation at 94ºC, then 35 cycles of denaturation for 1 minute at 94ºC, annealing for 30 seconds at 55ºC proceeded by 1 minute extension at 72ºC. Last programmed cycle is alike other cycles with the exception of extension time period which in this case was 20 minutes.

Successfully amplified PCR products of *rps*11 gene were later on confirmed on 1.5% agrose gel. Through thermo scientific GeneJET purification kit the resultant PCR products were purified and sent to Marcogen (Korea) for sequencing.

Data analysis: The sequenced data obtained for different species of family Rhamnaceae by using *rps* 11 primers was analyzed using various computational and bioinformatics tools. Sequences were submitted to Genbank to get accession numbers.

Molecular evolutionary genetics analysis (MEGA 6): A software "molecular evolutionary genetics analysis"(MEGA 6) is predominately designed for comparative investigation of DNA and proteins sequences to check the evolutionary pattern at molecular level (Kumar *et al*., 1994; Tamura *et al*., 2011). Online available program MEGA 6 was used to check the homology and diversity among the species in order to draw the evolutionary tree.

Results and Discussion

A sequence from cpDNA provides primary data for the assessment of phylogenetic relations in plants (Shinwari, 1995, Small *et al*., 2005). For the evaluation of genetic variability in higher taxonomic ranks, protein coding sequences were favorably used while non coding gene sequences were extensively used to assess genetic diversity at lower taxonomic ranks (Chase *et al*., 1993; Taberlet *et al*., 2007) with the exception of few protein coding genes like *rbc*L (Santoso *et al*., 2005; Shinwari & Shinwari, 2010).

In the present study, genomic DNA of good quality was isolated from leaves of selected species of family Rhamnaceae via CTAB method. Acquired good quality template DNA was used to amplify *rps* 11 gene using a pair of primer under optimized PCR conditions. Purified PCR products (using Thermo scientific Jet PCR purification kit) sent to Marcogen Company for sequencing, the data was later on aligned and subjected to nucleotide BLAST (basic local alignment and search tool) through NCBI. Comparison of nucleotide sequences of *rps* 11 gene by nucleotide BLAST with already sequenced data illustrate high similarity value which ranges from 89-99 %. The sequences were submitted to NCBI in order to get accession number given in the table 2.

Table 2. Accession number of the selected species of family Rhamnaceae based on *rps*11 gene.

Sr. No.	Species	Accession No.
1.	*Ziziphus spina-christi*	KT581955
2.	*Ziziphus nummularia*	KT698857
3.	*Ziziphus jujuba*	KT698859
4.	*Ziziphus mauritiana var. spontanea*	KT698860
5.	*Ziziphus mauritiana*	KT698861
6.	*Ziziphus oxyphylla*	KT719382
7.	*Sageretia thea*	KT698862
8.	*Sageretia filiformis*	KT581956
9.	*Rhamnus purpurea*	KT698858
10.	*Rhamnus pentapomica*	KT698863
11.	*Helinus lanceolatus*	KT719383

Table 3. Estimation of nucleotides composition.

Sr. No.	Species	T/U	C	A	G	Total
1.	*Ziziphus muritiana* var. *spontanea*	24.2	19.4	30.5	25.9	417.0
2.	*Ziziphus spina-christae*	23.7	20.9	29.0	26.4	417.0
3.	*Ziziphus nummularia*	24.0	20.6	29.0	26.4	417.0
4.	*Sageretia thea*	25.4	20.9	28.8	24.9	417.0
5.	*Sageretia filiformis*	24.2	20.4	30.2	25.2	417.0
6.	*Rhamnus purpurae*	23.7	19.4	30.7	26.1	417.0
7.	*Rhamnus pentapomica*	24.2	26.6	29.3	19.9	417.0
8.	*Ziziphus oxyphylla*	24.2	19.2	31.4	25.2	417.0
9.	*Ziziphus mauritiana*	23.0	20.9	28.8	27.3	417.0
10.	*Helinus lenceolatus*	26.1	18.9	30.9	24.0	417.0
11.	*Ziziphus jujuba*	28.3	21.6	27.3	22.8	417.0
	Average	24.7	20.9	29.6	24.9	417.0

Table 4. Estimation of nucleotide diversity among selected species of family Rhamnaceae from *rps* 11 gene via Tajima's test.

No. of sequences "m"	No. of segregation site "S"	Ps= S/n	T= Ps/al	Nucleotide diversity "P"	Tajma test statics "D"
11	221	0.529976	0.180943	0.175234	-0.152432

Table 5. Assessment of evolutionary divergence among *rps* 11 gene sequence from different species of family Rhamnaceae through pairwise distance MEGA6.

Species	1	2	3	4	5	6	7	8	9	10	11
Z. spina-christi											
S. filiformis	0.148										
H. lanceolatus	0.226	0.181									
Z. nummularia	0.136	0.151	0.234								
R. purpurea	0.103	0.108	0.183	0.106							
R. pentapomica	0.291	0.314	0.400	0.322	0.251						
S. thea	0.194	0.073	0.209	0.172	0.139	0.348					
Z. mauritiana	0.106	0.134	0.216	0.087	0.098	0.286	0.158				
Z. oxyphylla	0.243	0.222	0.310	0.222	0.200	0.407	0.224	0.199			
Z. mauritiana var. *spontanea*	0.140	0.089	0.193	0.108	0.097	0.314	0.126	0.084	0.189		
Z. jujuba	0.254	0.213	0.204	0.270	0.191	0.380	0.258	0.230	0.327	0.233	

Tajima's test of neutrality and nucleotide composition was assessed via MEGA6 software to figure out the genetic diversity between *rps* 11 gene from selected species of family Rhamnacea which reveals overall average value of nucleotide composition of latter was 417, dominated by A (adenine) =29.6 (Table 3). While overall average value of nucleotide diversity among selected species of family Rhamnaceae was 0.175234 indicating greater diversity among the investigated species (Table 4). Pair wise distance through MEGA6 software was also applied which reveals *S. thea* and *S. filiformis* exhibits lowest divergence with pairwise distance of 0.073 revealing their close association while highest genetic divergence was observed between distantly related *Z. oxyphylla* and *R. pentapomica* with 0.407 pair wise distance among investigated species. An overall assessment of evolutionary divergence among *rps* 11 gene sequence representing different species of family Rhamnaceae was calculated and its average was 0.205 (Table 5).

MEGA6 was used to construct the phylogram in order to illustrate the genetic divergence by Neighbor-Joining method among the selected species of family Rhamnaceae. Overall phylogram exhibits greater diversity among the species under study. Phylogram consists of two main clusters, cluster I and cluster II and an out group. An overall bootstrap value ranges between 19-99%. Cluster I comprises of total six species which includes *S. filiformis*, *S. thea*, *Z. nummularia*, *Z. mauritiana*, *Z. oxyphylla* and *Z. mauritiana* var. *spontanea*. In this cluster *Z. mauritiana* and *Z. nummularia* were grouped together with 74% bootstrap value assessing greater diversification between them. A recent study on genetic variability of *Z. nummularia* assessed by ISSR and UPGMA also depicted the moderate to high diversity among the cultivars (Akhtar *et al*., 2017). Whereas *Z. mauritiana* var. *spontanea* demonstrated 19% likeness towards *Z. mauritiana* and *Z. nummularia*, while 22% kinship by *Z. oxyphylla*. Moreover, *S. thea* and *S. filiformis* of same genus exhibited greater homology with 99% bootstrap value demonstrating close affinity and less diversification between them; additionally 25% affinity was demonstrated towards rest of the species of cluster I. Norouzi *et al*., (2017) conducted multivariate analysis of genus *Ziziphus* by including *Z. nummularia*, *Z. oxyphylla* and *Z. spina-christi* found high phenotypic diversity within and among the studied species.

Cluster II encloses two species, which includes *H. lanceolatus* and *Z. jujuba* both of which exhibited 99% similarity, illustrating a very close kinship between them. Moreover, cluster II showed 50% bootstrap value to the species of cluster I. Outgroup encloses *R. purpurea*, *Z. spina-christi* and *R. pentapomica*. *R. purpurea* exhibited 33% bootstrap value while no significant bootstrap values are demonstrated by *Z. spina-christi* and *R. pentapomica* which illustrates their distinct relation with rest of species in the phylogram. It is unveiled by phylogram that *R. pentapomica* is primitive most, among the investigated species and there might be probability that rest of species have originated from it (Fig. 1).

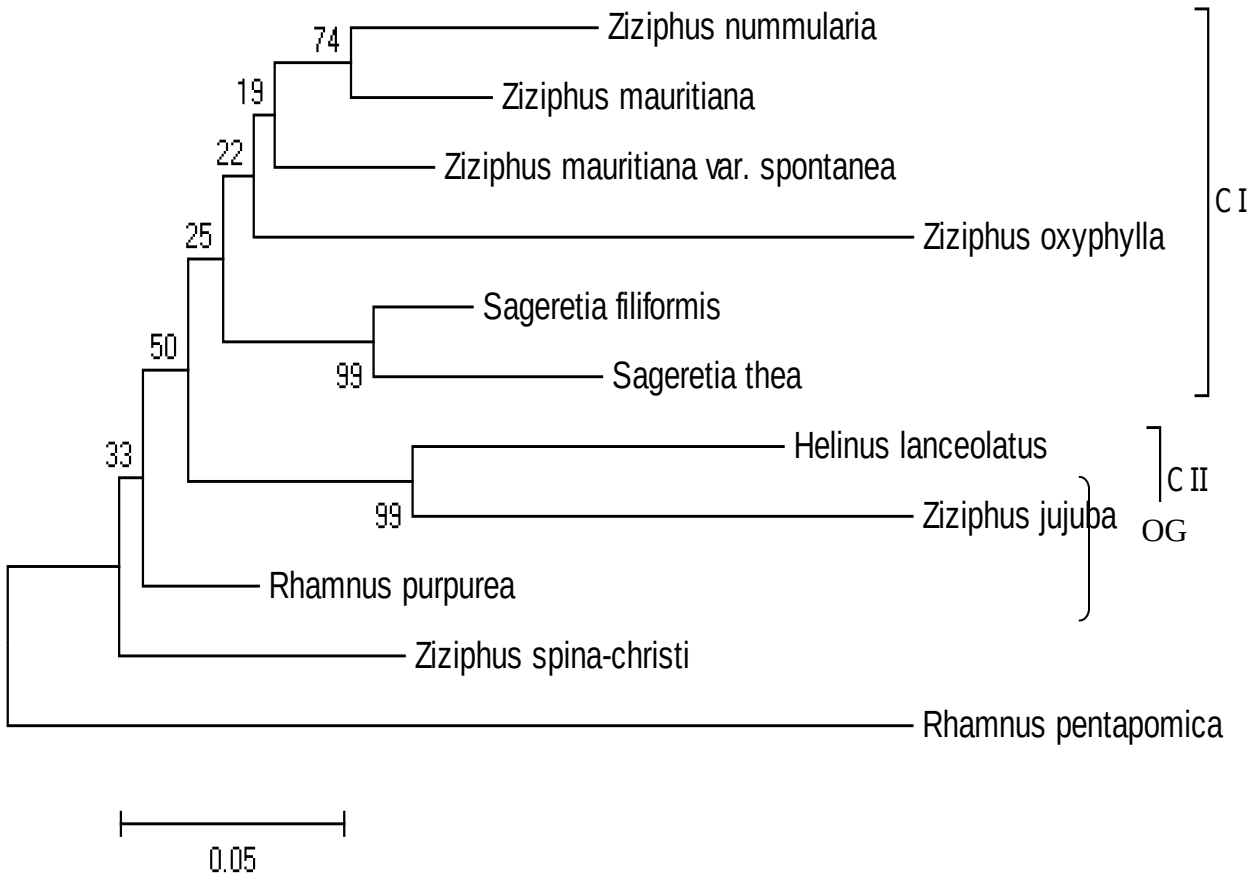

Fig. 1. Phylogentic tree of selected species of family Rhamnaceae based on *rps* 11 gene. C I: cluster I, C II: cluster II, OG: outgroup.

All the genera under investigation were monophyletic which appraises the monophyletic nature of family Rhamnaceae assessed by Richerdson *et al*., (2000a) on the basis of *rbc*L and combined tree analysis of *rbc*L/*trn*L-F data. Moreover, evolutionary divergence values among selected species varied between 0.073-0.407. *S. thea* and *S. filiformis* exhibited lowest divergence values reflecting their close association whereas *R. pentapomica* and *Z. oxyphylla* were distinctly related with highest divergence value 0.407. In past, Inter-Simple Sequence Repeats (ISSR) marker was used to check genetic diversity among 47 accessions of *Z. mauritiana* and one wild *Z. nummularia* with 18 ISSR primers. Data analysis exhibited genetic similarity among accessions ranging between 43.07- 90.30% illustrating the divergence among investigated accessions (Singh *et al*., 2007). Our result supports the findings of Singh *et al*., (2007) as high genetic diversity was observed in the present study where *Z. nummularia* and *Z. mauritiana* formed a cluster with a 74% bootstrap value.

Conclusion

The present investigation illustrates the presence of genetic variability among selected species of family Rhamnaceae and signifies *rps* 11 as a marker in phylogenetic assessment studies.

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