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MOLECULAR PHYLOGENY OF DIFFERENT SPECIES OF FAMILY VERBENACEAE USING CHLOROPLAST *rps*14 GENE

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Abstract

Family Verbenaceae has immense importance for ornamental, forage resources, aromatic and medicinal purposes. In the present study chloroplast *rps*14 gene is used for determination of phylogenetic relationship among Verbenaceae species and structural validation of *rps*14 protein through Ramachandran plots. The *rps*14 gene was amplified and sequenced analysis was done by MEGA7, I-TASSER and RAMPAGE. In phylogram the Verbenaceae species show little genetic distance of 0.0050 that revealed close genetic relationship between them. The genetic diversity ranges from 0.092 to 0.017 and value of overall mean distance was 0.067. The nucleotide diversity in Tajima's Neutrality Test was 0.063165. The phylogenetic tree based on *rps*14 gene depicted close relationship of *Verbena tenuisecta, V. officinalis*, *V. bonariensis* and *V. incisa* among them with BS value of 67% The *Duranta erecta* and *Citharexylum spinosum* show close relationship in group I with BS value of 57%. In Group II *V. tenuisecta* and *V. officinalis* depicted close phylogenetic association with BS value of 100%. The *rps*14 protein structure validation by RAMPAGE revealed that *V. tenuisecta, C. spinosum, V. bonariensis, V. incisa* and *P. volubilis* had good quality protein structural models because these species had ≤ 2% amino acid residues occurred in the outlier region. This study shows close genetic relationship and low genetic diversity between Verbenaceae species. The low nucleotide diversity also revealed close relationship between Verbenaceae species. The phylogenetic tree indicated close genetic relationship between Verbenaceae species with well supported BS values. The validation of *rps*14 protein structure by RAMPAGE predicted the best quality protein structural models. This study demonstrated the *rps*14 gene as useful marker for assessment of phylogenetic studies.

Key words: Verbenaceae , Genetic diversity, Polymerase chain reaction (PCR), Phylogeny.

Introduction

The molecular phylogenetic studies are conducted for assessment of relationship between organisms or genes through comparison of DNA or protein sequences (Patwardhan *et al*., 2014; Jan *et al*., 2016; Jan *et al*., 2017). The cp genome is used for phylogenetic studies in plants because chloroplast genome is conserved and smaller effective population size, shorter coalescent time in comparison with the nuclear genome (Birky *et al*., 1983; Jansen *et al*., 2007). The cp genome comprised of large single copy, smaller single copy separating the inverted repeat regions (Palmer, 1985). The substitution rate of cp genome is lower in comparison with nuclear genome and also reduced in the inverted repeat regions of cp genome (Wolfe *et al*., 1987). The noncoding regions of cp genome can be used for intergeneric, interspecific and intraspecific studies. The noncoding regions show more variation on per site basis in comparison with coding region due to less functional constraints (Taberlet *et al*., 1991). The *psbA-trnH* intergenic spacer is used for phylogenetic studies at various taxonomic levels (Pornpongrungrueng *et al*., 2007). The *psbA-trnH* intergenic spacer can also be a suitable barcode for land plants (Kress *et al*., 2005; Zahra *et al*., 2014; Zahra *et al*., 2016; Shinwari *et al*., 2018; Khan *et al*., 2019).

The *psaA* and *psaB* plastid genes in rice encoded two apoproteins of P700 chlorophyll *a* protein complex of photosystem I. The *rps*14 gene for ribosomal protein S14 was organized to a transcription unit (Chen *et al*., 1992). The *rps*14 gene has been found in the cp genome of *N. tabacum* (Wakasugi *et al*., 1998). The *rps*14 gene had been used for phylogenetic analysis of *Plantago* species, *Mentha*, *Citrus* species and *Date palm* varieties (Saeed *et al*., 2011; Jabeen *et al*., 2012; Wali *et al*., 2013; Akhtar *et al*., 2014).

Verbenaceae is flowering plant family in the large asteroid order Lamiales (Refulio & Olmstead, 2014). The Verbenaceae included 35 genera and 830 species (Atkins, 2004; O'Leary *et al*., 2009, 2012; Thode *et al*., 2013). In Brazil Verbenaceae is represented by 300 species in 16 genera (Salimena & Mulgura, 2015). The Verbenaceae is primarily Neotropical in distribution and center of diversity spread in the arid region of southern South America (Marx *et al*., 2010; Múlgura *et al*., 2012). Verbenaceae is characterized by trees, shrubs, herbs and lianas. The morphological characters are opposite leaves, slightly bilateral symmetrical flowers and inflorescence is either terminal or axiliary (Atkins, 2004). The fruit of Verbenaceae is dry and divided in 2 or 4 segments that comprised of 2 or 4 seeds (O'Leary *et al*., 2012). The Verbenaceae has great economic importance as ornamental, forage resources and aromatic purposes (Atkins, 2004). The *Lantana camara* is grown as ornamental plant as well as medicinally useful because the decoction of *L. camara* is used for curing tetanus, malaria and rheumatism. The secretion of *L. camara* leaves protects against herbivores and pathogens for spreading of fruit and seeds and also attract pollinators (Fahn, 1979; Gottlieb & Salatino, 1987; Gershenzon & Croteau, 1991; Pare & Tumlinson, 1999). The objective of present study is phylogenetic relationship among Verbenaceae species on the basis of *rps*14 gene and structural validation of *rps*14 protein by Ramachandran plots.

Materials and Methods

Plant Material and Genomic DNA Extraction: The *Verbena tenuisecta, Citharexylum spinosum, Duranta erecta, V. officinalis, V. bonariensis, Verbena incisa*, *Petrea volubilis, Lantana indica* and *L. camara* were collected from Islamabad, Rawalpindi and Lahore (Pakistan). The plant material was washed, dried and then preserved at 4ºC for further use. The collected specimens were identified by observing morphological characterstics using Flora of Pakistan and the correct taxonomic placement of plant species through International Plant name Index the Royal Botanic Gardens Kew, UK (http://plantsoftheworldonline.org/). The voucher specimens, plant species and locality were shown in (Table 1). The genomic DNA was extracted using CTAB Method with some alterations (Richards, 1977). The quality of genomic DNA was checked on 1 % agarose gel and bands were seen in the gel documentation system.

Table 1. List of selected species of family Verbenaceae .

S. No.	Plant species	Locality	Voucher number
1.	*Verbena tenuisecta*	Islamabad	TMRK-12
2.	*Citharexylum spinosum*	Rawalpindi	TMRK-13
3.	*Duranta erecta*	Islamabad	TMRK-14
4.	*V. officinalis*	Islamabad	TMRK-15
5.	*V. bonariensis*	Islamabad	TMRK-16
6.	*V. incisa*	Islamabad	TMRK-17
7.	*Petrea volubilis*	Lahore	TMRK-18
8.	*Lantana indica*	Islamabad	TMRK-19
9.	*L. camara*	Islamabad	TMRK-20

Primer designing of *rps*14 gene: The Primers were designed using primer 3 (version 4.10). The *rps*14 gene was designed from cp genome of *Nicotiana tabacum* available in NCBI Genbank. The forward and reverse primer sequence was mentioned

*rps*14 F : 5′-ATGGCAAGGAAAAGTTTGATTC-3'

*rps*14 R : 5′-TTACCAACTTGATCTTGTTGCTCCT-3'. The reverse primer was 3' to 5′ direction from stop codon to start codon and Polymerase enzyme added nucleotide to 3' end of both primers. The direction of synthesis on both primers were from 5′ to 3' direction.

PCR components and conditions: The *rps*14 gene was amplified in 25 µL total reaction mixtures that included 2.5 µL of *Taq* buffer, 16.2 µL of nanopure water, 1 µL of forward primer, 1 µL of reverse primer, 0.3 µL of *Taq* DNA polymerase, 1.5 µL of dNTPS, 1.5 µL of $MgCl_2$ and 1 µL of DNA template in PCR. The conditions for amplification were mentioned as initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing in the range of 45-51°C (Gradient) for 1 minute, extension at 72°C and final extension at 72°C for 20 minutes.

PCR product purification and sequencing: The quality of PCR product was analyzed on 1.5% agarose gel in Dolphin Doc Plus documentation system. The purification of PCR product was done through Gene JET PCR Purification Kit and sequencing was carried out in Beijing Genomic Institute, Shenzen China.

Sequence analysis: The sequences were aligned and assessed through various bioinformatics tools. The BLASTn was conducted to find out the homology with already reported sequences in the Genbank. The cleaning and alignment of nucleotide sequences were done through online software Just Bio. The EXPASY- Translate tool was used to translate nucleotide sequences in to amino acid sequences. The *rps*14 gene sequences from nine Verbenaceae species were submitted in to the Genbank for allotment of accession numbers (Table 2).

Molecular evolutionary genetics analysis (MEGA7): MEGA7 is used for estimation of evolutionary pattern at the molecular level based on DNA and protein sequences (Kumar *et al*., 1994; Tamura *et al*., 2011). The analysis of aligned sequences were done by MEGA7 to build phylogenetic tree by Neighbor-joining (NJ) method. The MEGA7 was used to determine the nucleotide composition, Tajima's Neutrality test, Pairwise distance and Substitution pattern.

I-TASSER: The 3D protein models were built by I-TASSER. The amino acid sequences of *rps*14 gene from nine species of Verbenaceae were pasted on I-TASSER and five 3D atomic models for each sequence were built.

RAMPAGE: The 3D protein model was validated by Ramachandran plots through pasted the Pdb files of 3D protein models in the RAMPAGE. The purpose of Ramachandran plots was conformation of dihedral angle φ and ψ of amino acid residues in the proteins and structural validation of 3D protein Structures.

Results

PCR amplification: The amplification of *rps*14 gene was done through forward and reverse primers. The best amplification was observed at different temperatures such as 45.0°C, 45.3°C, 47.0°C, 48.2°C, 48.6°C, 49.0°C, 49.3°C, 50.0°C and 51.0°C (Table 3). The amplification showed clear band of 303 bp for nine Verbenaceae species (Fig. 1).

Sequence analysis: The BLASTn of *rps*14 gene sequence in comparison with already reported sequences resulted in 97 % similarity with cp genome of *V. hastata* (Accession # KT178966.1).

Table 2. Accession number of *rps*14 gene from nine Verbenaceae species.

S. No.	Plant species	Accession numbers
1.	*Verbena tenuisecta*	MH382174
2.	*Citharexylum spinosum*	MH382175
3.	*Duranta erecta*	MH382177
4.	*V. officinalis*	MH382178
5.	*V. bonariensis*	MH382185
6.	*V. incisa*	MH382187
7.	*Petrea volubilis*	MH382189
8.	*Lantana indica*	MH382193
9.	*L. camara*	MH329774

Table 3. Amplification of *rps*14 gene from nine Verbenaceae species at different temperature.

Sample No.	PCR Row number	Temperature
A	1	45.0
B	2	45.3
C	3	47.0
D	4	48.2
E	5	48.6
F	6	49.0
G	7	49.3
H	8	50.0
I	9	51.0

Computation of pair wise distance: The genetic diversity was calculated through Pairwise distance that ranged from 0.092 to 0.017 and value of overall mean distance was 0.067. *Citharexylum spinosum* has highest genetic diversity of 0.092, while *Verbena officinalis* has lowest genetic diversity of 0.017. The low overall mean distance revealed that all species show close genetic relationship and low genetic diversity between them on the basis of *rps*14 gene analysis (Table 4).

Tajima's neutrality test: The Tajima's Neutrality Test was used to calculate the nucleotide diversity of Verbenaceae species using MEGA7. The nine numbers of sequences showed 47 segregation sites *(S)* with nucleotide diversity (π) of 0.063165. The low nucleotide diversity showed close genetic relationship between Verbenaceae species (Table 5).

Nucleotide composition: The nucleotide composition was calculated through MEGA7. The average of nucleotide number for Verbenaceae was T (U) =24.8, C= 18.6, A= 34.2 and G= 22.5. The highest average nucleotide number was observed in A that was 34.2. While the lowest average nucleotide number was found in C that was 18.6. The total average number of all nucleotides for *rps*14 gene was 303. The nucleotide composition is given in (Table 6).

Maximum likelihood (ML) calculation of substitution matrix: The substitutions pattern and rates were calculated using Tamura & Nei (1993) model. The relative values of instantaneous (*r*) were included when evaluating them, for easiness the sum of (r) values was made equal to 100. The values of transitional substitution of Verbenaceae species were higher than transversionsal substitution. In Verbenaceae the nucleotide frequencies were A = 34.18 %, T/U = 24.75 %, C = 18.59 %, and G = 22.48 %. The maximum Log likelihood for Verbenaceae was -878.437 (Table 7).

Phylogenetic tree of Verbenaceae : The phylogenetic tree was constructed using MEGA7 based on *rps*14 gene sequences (Kumar *et al*., 2016). In phylogram the Verbenaceae species show little genetic distance 0.0050 that revealed close genetic relationship between them and divided in to Cluster I and Cluster II. The Cluster I consists of four species such as *Lantana camara, L. indica, D. erecta* and *C. spinosum*. The Cluster I was divided in to group I in which *D. erecta* and *C. spinosum* depicted close phylogenetic relationship among them with BS value of 57 %. *Duranta erecta* shows recent evolution with branch length of 0.023 whereas *C. spinosum* were earliest evolved with branch length of 0.032. *L. camara* and *L. indica* showed close phylogenetic association among them in Cluster I. *L. camara* showed recent evolution having branch length of 0.020 whereas *L. indica* depicted earliest evolution with branch length of 0.023. The Cluster II consists of five species such as *Verbena tenuisecta*, *V. officinalis*, *V. bonariensis*, *V. incisa* and *Petrea volubilis*. In cluster II *V. tenuisecta, V. officinalis*, *V. bonariensis* and *V. incisa* were grouped together in the same cluster and showed close relationship among them with BS value of 67%. *V. bonariensis* had branch length of 0.014 and revealed recent evolution while *V. incisa* showed earliest evolution having branch length of 0.030. The Cluster II was further divided in to group II in which *V. tenuisecta* and *V. officinalis* were showed close phylogenetic association between them with BS value of 100%. *V. officinalis* showed recent evolution with branch length of 0.007 while *V. tenuisecta* depicted earliest evolution having branch length of 0.010. *P. volubilis* formed outgroup with members of Cluster II (Fig. 2).

I-TASSER: The 3D protein models were built by I-TASEER software. The top five models of 3D structure of protein were predicted. The confidence of each protein model was determined based on confidence score (C-score). The C- score values ranges from -5 to 2 on the basis of C-score through I-TASSER structural models of proteins and their function was predicted. The 3D protein model with higher C-score represented higher confidence and more reliable prediction. The 3D protein models with higher C-score is shown in (Fig. 3).

RAMPAGE: The Ramachandran plots were quadrant of different colors in which dark blue and dark orange area represented favored region. While light blue and light orange showed allowed region. The unshaded area showed outlier region (Fig. 4). The RAMPAGE results revealed that ≥78 to ≤ 93 amino acid residues occurred in favored region, ≥3 to ≤ 15 amino acid residues occurred in allowed region and ≤ 2 to ≥ 3 amino acid residues in outlier region. *V. tenuisecta, C. spinosum, V. bonariensis, V. incisa* and *P. volubilis* had good quality protein structural models having ≤ 2% amino acid residues occurring in the outlier region (Table 8).

Table 4. Pair wise distance of *rps*14 gene from nine Verbenaceae species.

Plant species	1	2	3	4	5	6	7	8	9
Lantana camara	0.00								
L. indica	0.041								
Verbena tenuisecta	0.077	0.070							
V. officinalis	0.074	0.062	**0.017**						
V. bonarensis	0.059	0.041	0.041	0.037					
V. incisa	0.066	0.070	0.059	0.055	0.048				
Duranta erecta	0.044	0.073	0.080	0.080	0.062	0.084			
Citharexylum spinosum	0.062	0.066	**0.092**	0.085	0.073	0.089	0.055		
Petrea volubilis	0.077	0.085	0.081	0.081	0.077	0.085	0.070	0.085	

Table 5. Tajima's neutrality test of *rps*14 gene of Verbenaceae species through MEGA7.

No. of sequences "*m*"	Number of segregating sites "*S*"	*Ps*	Θ	Nucleotide diversity "π"	Tajima's test statistics "D"
9	47	0.155116	0.057073	0.063165	0.543318

Table 6. Nucleotide composition of *rps*14 gene from nine species of family Verbenaceae .

S. No.	Plant species	T (U)	C	A	G	Total
1.	*Lantana camara*	24.8	18.8	34.0	22.4	303.0
2.	*L. indica*	24.4	18.5	34.7	22.4	303.0
3.	*Verbena tenuisecta*	25.1	18.5	34.7	21.8	303.0
4.	*V. officinalis*	25.1	18.5	34.3	22.1	303.0
5.	*V. bonarensis*	24.4	18.2	34.3	23.1	303.0
6.	*V. incisa*	25.4	18.8	33.7	22.1	303.0
7.	*Duranta erecta*	24.8	18.5	34.3	22.4	303.0
8.	*Citharexylum spinosum*	24.8	18.5	34.3	22.4	303.0
9.	*Petrea volubilis*	24.1	19.1	33.3	23.4	303.0
	Average	**24.8**	**18.6**	**34.2**	**22.5**	**303.0**

Table 7. Maximum likelihood values of *rps*14 gene from nine Verbenaceae species through MEGA7.

	A	T/U	C	G
A	-	*5.15*	*3.87*	**16.58**
T/U	*7.11*	-	7.12	*4.68*
C	*7.11*	**9.48**	-	4.68
G	**25.21**	*5.15*	*3.87*	-

The nucleotide frequencies was **A** = 34.18 %, **T/U** = 24.75 %, **C** = 18.59 %, and **G** = 22.48 %. The maximum Log likelihood was -878.437

Table 8. Ramachandran scores of *rps*14 protein model of Verbenaceae by RAMPAGE.

S. No.	Plant species	Favored region (%)	Allowed region %	Outlier region %
1.	*Lantana camara*	92	3	3
2.	*Verbena tenuisecta*	**93**	**4**	**1**
3.	*Duranta erecta*	89	7	2
4.	*Citharexylum spinosum*	**89**	**8**	**1**
5.	*V. officinalis*	87	8	3
6.	*V. bonariensis*	**91**	**7**	**0**
7.	*V. incisa*	**91**	**6**	**1**
8.	*Petrea volubilis*	**83**	**14**	**1**
9.	*L. indica*	78	15	5

Discussion

In past various molecular markers have been used for phylogeny of Verbenaceae such as Pentatricopeptide repeat genes PPR gene (Yuan *et al*., 2010), *ndhF* and *trnL– trnF* (Thode *et al*., 2013) *trnL-F, matK, rbcL, rpoC2, rps*3, *ccsA* (Marx *et al*., 2010), *trnT-L, rpI*32-*trnL* and *trnQ-rps*16 (Lu-Irving & Olmstead, 2013), *trnT-F, trnS-fM, trnS-G, trnD-T* and waxy (7F- 13R) (Yuan & Olmstead, 2008), *PHOT* gene duplicates (Yuan & Olmstead, 2008), *trnD-trnT*, *trnS-trnG*, *trnS-trnfM*, *trnT-trnL*, *trnG*, *trnL* and *trnL-trnF* and the nuclear ITS and ETS regions (O'Leary *et al*., 2009).

The close phylogenetic relationship of *Verbena* species based on *rps*14 gene was supported by Yuan & Olmstead, (2008) study in which *Verbena* species were grouped together in the single clade and showed close relationship among them based on *PHOT1* and *PHOT2* gene sequences. The close phylogenetic relationship among *Verbena* species was also resolved by Yuan *et al*., (2010) based on PPR genes. Marx *et al*., (2010) also studied the close relationship among *Verbena* species based on *ndhF* ,*trnL-F* ,*matK, rbcL, rpo*C2, *rps*3 and *ccsA* markers. The close relationship among *Verbena* species was also demonstrated by Yuan & Olmstead, (2008) on the basis of *trnT-F, trnS-fM, trnS-G, trnD-T* and waxy (7F- 13R). *Verbena* species depicted close phylogenetic association based on plastid markers *ndhF* and *trnL– trnF* by Thode *et al*., (2013). O'Leary *et al*., (2009) also studied the relationship of *Verbena* species on the basis of chloroplast *trnD-trnT*, *trnS-trnG, trnS-trnfM, trnT-trnL, trnG, trnL, trnL-trnF* and the nuclear ITS and ETS regions.

In the present study *Lantana* species showed close relationship based on *rps*14 gene. The close relationship of *Lantana* was also supported by Lu-Irving & Olmstead, (2013) based on both nuclear PPR (11, 81 and 123), ETS region and chloroplast regions *trnT-L, rpl*32-*trnL* and *trnQ-rps*16. *Lantana* species depicted a close phylogenetic relationship by grouping in the same clade on the basis of *ccsA, matK, ndhF, rbcL*, *rpoC*2, *rps*3 and *trnL-F* markers (Marx *et al*., 2010). Lu-Irving *et al*., (2014) also studied the close phylogenetic relationship of *Lantana* species based on the chloroplast regions *trnT-trnL*, *rpl*32-*trnL* and *trnQ-rps*16 and nuclear regions ETS, PPR 123 and PPR 81. The close relationship of *Lantana* was also studied by Thode *et al*., (2013) on the basis of chloroplast markers *ndhF* and *trnL– trnF*.

Duranta showed close phylogenetic relationship with *Citharexylum* on the basis of *rps*14 gene. This result was in congruent with the findings of (Yuan *et al*., 2010) in which *Duranta* depicted close relationship with *Citharexylum* on the basis of PPR gene. The close relationship of *Duranta* with *Citharexylum* was also studied by Thode *et al*., (2013) based on *ndhF* and *trnL–trnF* chloroplast markers. Marx *et al*., (2010) also studied the close relationship of *Duranta* with *Citharexylum* based on *ndhF, trnL-F, matK*, *rbcL, rpoC*2, *rps*3 and *ccsA* markers.

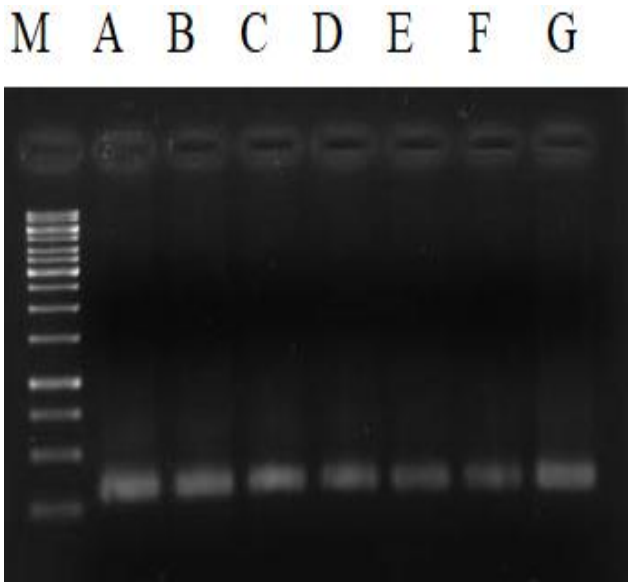

Fig. 1. Amplification of *rps*14 gene from selected Verbenaceae species.

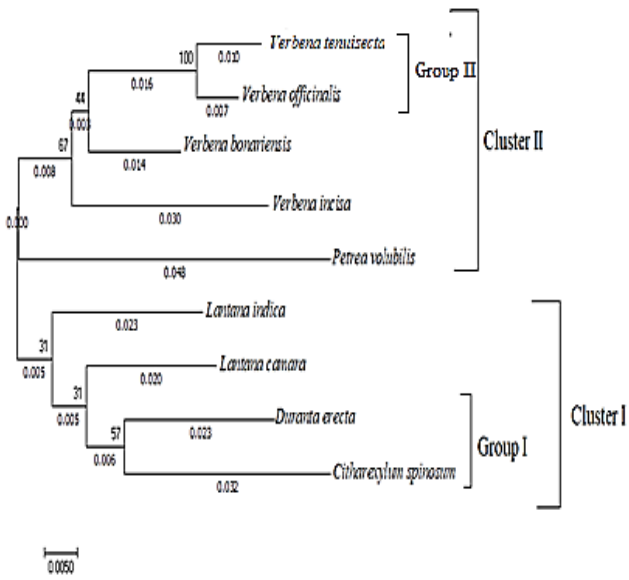

Fig. 2. Phylogenetic tree of Verbenaceae species through MEGA7 based on *rps*14 gene.

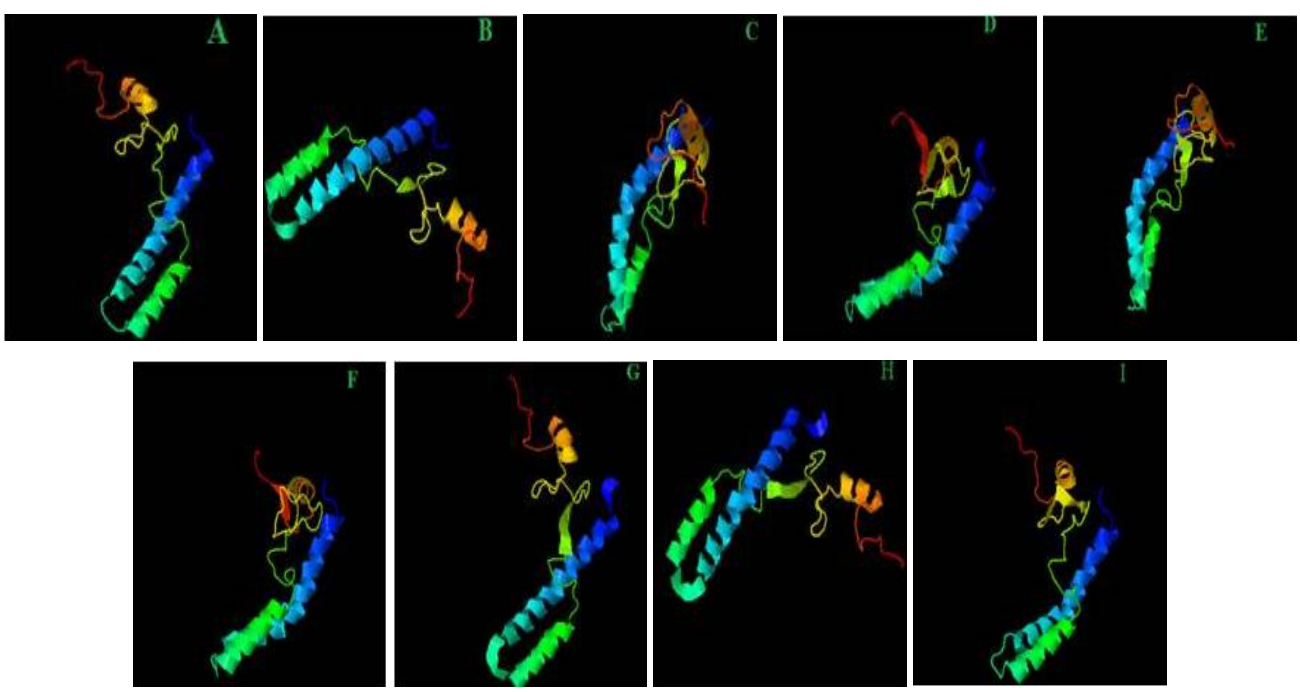

Fig. 3. The higher c- score of structural 3D protein models of *rps*14 protein of Verbenaceae by I TASSER. A: *Lantana camara,* B: *Verbena tenuisecta,* C: *Duranta erecta D: Citharexylum spinosum* E: *V. officinalis* F: *V. bonariensis* G: *V. incisa* H: *Petrea volubilis* I: *L. indica.*

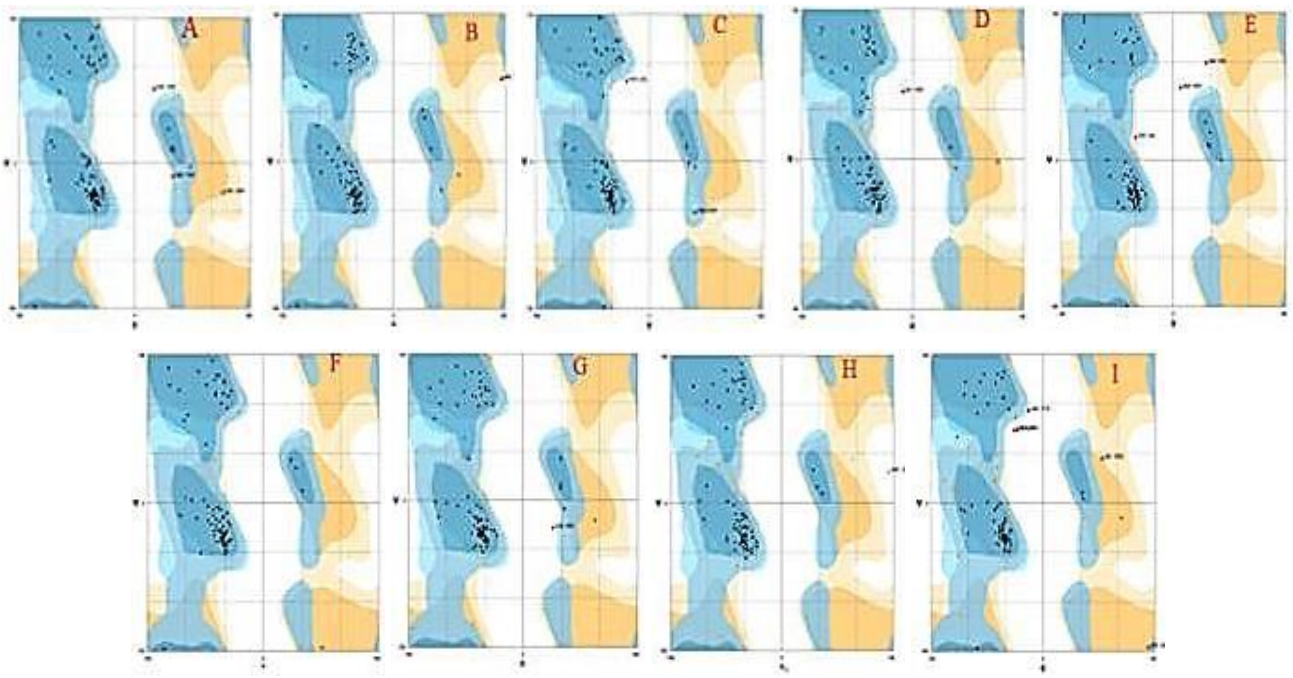

Fig. 4. Ramachandran plots of nine selected species of Verbenaceae by RAMPAGE.
A: *Lantana camara* B: *Verbena tenuisecta* C: *Duranta erecta* D: *Citharexylum spinosum* E: *V. officinalis* F: *V. bonariensis* G: *V. incisa* H: *Petrea volubilis* I: *L. indica*.

Conclusion

This study shows close genetic relationship and low genetic diversity among Verbenaceae species. The low nucleotide diversity also revealed close relationship among Verbenaceae species. The phylogenetic tree based on *rps*14 gene show close relationship among Verbenaceae species with well supported BS values. Moreover validation of *rps*14 protein by RAMPAGE revealed the best quality protein structural models of Verbenaceae species. This study signifies the chloroplast *rps*14 gene as useful marker for assessment of phylogenetic studies.

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