

## ASSESSMENT OF PHYLOGENETICS RELATIONSHIP AMONG THE SELECTED SPECIES OF FAMILY *LEGUMINOSAE* BASED ON CHLOROPLAST *RPS14* GENE

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### Abstract

The *Leguminosae* is a cosmopolitan family comprised of approximately 770 genera and 20,000 species mostly found in temperate and tropical regions of the world. This family is well known for its commercial, nutritional and medicinal importance. Twenty-six species of *Leguminosae* were collected from different geographical regions of Pakistan to resolve their controversial phylogenetic position using Ribosomal Protein Subunit (*rps14*) gene. Furthermore, in each species, the *rps14* gene was successfully amplified, purified, sequenced and the dendrogram was constructed using molecular evolutionary genetic analysis (MEGA7) tool which divided the species into two main clades showing a narrow genetic diversity of 0.5 with well supported bootstrap values. Moreover, the range of pairwise distance was found to be 0.02 to 0.40 with a mean value of 0.15. The phylogenetic analysis is in agreement with the work done by earlier phylogeneticists with the addition of new species, *Crotalaria medicaginea*, *Argyrolobium roseum*, *Vicia sativa* sub spp. *nigra*, *Vicia sepium*, *Medicago falcata* and *Mimosa himalayana* from Pakistan. The stereochemical analyses were evaluated and 3D protein structures were authenticated by Iterative Threading Assembly Refinement (I-TASSER) and Ramachandran plots. 3D protein models were quite good and can be reliably used for the application of different bioinformatics tools. Future studies can focus on the examination of morphology, anatomy, phylogenetics with multiple marker system and evolution of these and other variable characters within this diverse family.

**Key words:** *Leguminosae*, *rps14* gene, Genetic analysis, Phylogenetic studies, Ramachandran plots.

### Introduction

The third largest angiospermic family *Leguminosae* also known as "*Fabaceae*" is comprised of approximately 650 genera and 20,000 species, cosmopolitan in distribution, mostly found in temperate and tropical regions of the world (Yahara *et al.*, 2013; Rahman & Parvin, 2014, da Silva Gomes *et al.*, 2018, Zhang *et al.*, 2020). The family is mainly divided into three subfamilies (*Papilionoideae*, *Caesalpinioideae* and *Mimosoideae*) (Heywood, 2008; Group, 2013; Marinho *et al.*, 2018). Pakistan is hosting approximately 146 genera and 831 species, mostly found in Islamabad, Murree, Karachi, Azad Kashmir, Balakot, Gujarat, Rawalpindi and other ranges with several species still unexplored (Kanwal *et al.*, 2011; Shinwari & Qaiser, 2011). The family is famous for its significant importance in pharmaceutical industries acting as a source of various biological compounds like alkaloids, flavanoids, phenolics, carbohydrates, minerals, vitamins with therapeutic potential against diabetes, cancer, leprosy, dysuria, liver and heart diseases (Usman *et al.*, 2013; Joy & Gorge, 2014; Marinho *et al.*, 2018).

The taxonomy and systematic position of the *Leguminosae* has been controversial in the scientific community (Simon *et al.*, 2009). Complexity of habitat range, considerable variation in morphology and genetics has made the classification of *Leguminosae* difficult. The revised classification left family *Leguminosae* with three subfamilies, 35 tribes, 751 genera and approximately 20,000 species using coherent phylogenetic characters (Lewis *et al.*, 2005). Multiple molecular markers of plastid and nuclear gene regions have been used for genetics characterization of *Leguminosae* (Lewin, 2005; Simon *et al.*, 2009; Patel & Panchal, 2014). These existing studies provided some new insights into its classification, but because of the inclusion of new species and ecotypes from different parts of the world need further research studies.

The discovery of new and upto date markers provided an opportunity to study plant phylogeny at morphological, biochemical and molecular levels, but molecular markers are more reliable due to their stability, adaptability and less susceptibility to environmental changes, while others (morphological, biochemical) are potentially prone to developmental and environmental plasticity (Iqbal *et al.*, 2019; Zhang *et al.*, 2020). Thus, chloroplast DNA provides the most suitable solution for phylogenetic analysis due to its conserved nature and high genetic potential as compared to nuclear and mitochondrial DNA (Daniell, 2016; Zhu *et al.*, 2019). The *rps14* gene encodes ribosomal protein S-14 and was used in past studies for elucidation of phylogenetic relationship among different plant species due to its least substitution rate (Saeed *et al.*, 2011; Akhtar *et al.*, 2014; Wang *et al.*, 2017).

### Material and Methods

**Plants collection and DNA isolation:** Young fresh leaves of 26 different plant species of *Leguminosae* (20 genera) were collected from different geographical regions of Pakistan (Table 1). For molecular analysis, their DNA was extracted using Cetyltrimethylammonium bromide (CTAB) method (Richard, 1997).

**DNA amplification and sequencing of *rps14* gene region:** The amplification of targeted gene (*rps14*) was carried out using previously optimized protocol Akhtar *et al.*, (17). Annealing temperature was optimized in the range of 44.4 to 48.6°C for each specimen. The PCR products were purified (GeneJET PCR Purification kit, ThermoScientific), sequenced (Beijing Genomic Institute, Shenzhen, China) and the sequences were submitted to National center for biotechnology information (NCBI) for generating accession numbers (Table 2).

**Table 1. List of selected species along with their longitude, latitude and area of collection.**

S. No	Species name	Local name	Location	Geographic coordinates
1.	<i>Argyrolobium roseum</i> (Camb.)	Kauri booti	Islamabad	33.7444° North, 73.0417° East
2.	<i>Glycine max</i> (L.)	Soya	Islamabad	33.7444° North, 73.0417° East
3.	<i>Medicago polymorpha</i> (L.)	Jangli mausari	Islamabad	33.7444° North, 73.0417° East
4.	<i>Medicago minima</i> (L.)	Mausari	Murree	33.9078° North, 73.3915° East
5.	<i>Medicago falcata</i> (L.)	Mausari	Murree	33.9078° North, 73.3915° East
6.	<i>Medicago sativa</i> (L.)	Mausari	Islamabad	33.7444° North, 73.0417° East
7.	<i>Vicia sativa</i> var <i>nigra</i> (L.)	Paratha	Islamabad	33.7444° North, 73.0417° East
8.	<i>Vicia sepium</i> (L.)	Gol paratha	Islamabad	33.7444° North, 73.0417° East
9.	<i>Pisum sativum</i> (L.)	Mattar	Abbottabad	34.1688° North, 73.2215° East
10.	<i>Lens culinaris</i> (Medik.)	Masoor	Islamabad	33.7444° North, 73.0417° East
11.	<i>Vigna mungo</i> (L.)	Mash	Murree	33.9078° North, 73.3915° East
12.	<i>Vigna radiata</i> (L.)	Munga	Abbottabad	34.1688° North, 73.2215° East
13.	<i>Cicer aurientium</i> (L.)	Chola	Abbottabad	34.1688° North, 73.2215° East
14.	<i>Sesbania sesban</i> (L.)	Kandiala Sesban	Islamabad	33.7444° North, 73.0417° East
15.	<i>Cajanus cajan</i> (L.)	Arhar	Islamabad	33.7444° North, 73.0417° East
16.	<i>Millettia pinnata</i> (L.)	Sukhchain	Islamabad	33.7444° North, 73.0417° East
17.	<i>Crotolaria medicagana</i> (Lam.)	Phaliara	Islamabad	33.7444° North, 73.0417° East
18.	<i>Melilotus indicus</i> (L.)	Sinjahi	Murree	33.9078° North, 73.3915° East
19.	<i>Dalbergia sisso</i> (Roxb. ex DC.)	Tahli	Islamabad	33.7444° North, 73.0417° East
20.	<i>Cassia fistula</i> (L.)	Amaltas	Islamabad	33.7444° North, 73.0417° East
21.	<i>Senna occidentalis</i> (L.)	Amla	Islamabad	33.7444° North, 73.0417° East
22.	<i>Phanera variegata</i> (L.) Benth.	Koliar	Murree	33.9078° North, 73.3915° East
23.	<i>Mimosa himalayana</i> (Gamble.)	Kikaray	Islamabad	33.7444° North, 73.0417° East
24.	<i>Mimosa pudica</i> (L.)	Chuimui	Islamabad	33.7444° North, 73.0417° East
25.	<i>Vachellia nilotica</i> (L.)	Kikar	Islamabad	33.7444° North, 73.0417° East
26.	<i>Senegalia modesta</i> (Wall.) P.J.H. Hurter	Palosa	Islamabad	33.7444° North, 73.0417° East

**Table 2. Accession numbers of rps14 gene sequences amplified and sequenced from 26 Leguminosae species.**

S. No	Species name	AN	S/N	Species name	AN
1.	<i>Argyrolobium roseum</i>	KY053495	14	<i>Sesbania sesban</i>	KY073396
2.	<i>Medicago polymorpha</i>	KY026193	15	<i>Dalbergia sisso</i>	KY042004
3.	<i>Medicago falcata</i>	KY073392	16	<i>Millettia pinnata</i>	KY073402
4.	<i>Medicago minima</i>	KY073397	17	<i>Cajanus cajan</i>	KY042000
5.	<i>Medicago sativa</i>	KY041999	18	<i>Crotolaria medicaginea</i>	KY073401
6.	<i>Vicia sativa</i> sub sp. <i>Nigra</i>	KY041995	19	<i>Melilotus indicus</i>	KY073399
7.	<i>Vicia sepium</i>	KY041996	20	<i>Cassia fistula</i>	KY073394
8.	<i>Glycine max</i>	KY041997	21	<i>Senna occidentalis</i>	KY042002
9.	<i>Pisum sativum</i>	KY041994	22	<i>Bauhinia variegata</i>	KY073395
10.	<i>Lens culinaris</i>	KY041998	23	<i>Mimosa himalayana</i>	KY073398
11.	<i>Vigna mungo</i>	KY073393	24	<i>Mimosa pudica</i>	KY042003
12.	<i>Vigna radiata</i>	KY073400	25	<i>Acacia nilotica</i>	KY042006
13.	<i>Cicer arietinum</i>	KY042001	26	<i>Acacia modesta</i>	KY042005

AN= Accession numbers, S/N= Serial numbers

**Analysis of sequences of *rps14* gene region:** Moreover, phylogenetic trees were constructed through neighbor-joining tree making method using MEGA7 (Kumar *et al.*, 2016) with bootstrap replicates of 1000 along with Tajima's neutrality test for finding genetic diversity ( $\pi$ ). Nucleotide sequences were translated through online web server Translate tool (<http://web.expasy.org/translate/>). I-TASSER was used to predict 3D proteins models to elucidate the structure and function of proteins. Furthermore, 3D protein structures were validated using Ramachandran plot analysis and PROCHECK (<http://services.mbi.ucla.edu/SAVES/>) (Zhang, 2008, Munir *et al.*, 2013).

**Results**

**Phylogenetic analysis based on DNA sequences of *rps14* gene:** Molecular analysis based on *rps14* gene apparently revealed two main clades, having fourteen species in clade I and eleven species in clade II with the bootstrap values up to 98%, showing overall genetic diversity of 0.05%. Different species of the genera *Mimosa*, *Senegalia*, *Vachellia* (*Mimosoideae*) and *Phanera* and *Cassia*, *Senna* of (*Caesalpinioideae*) showed close genetic relationship with each other as shown in the cluster I of clade I (Fig. 1). However, *Sesbania sesban* has shown unique position between two species of *Mimosoideae*. The cluster II of clade I and clade II contained the selected genera of *Papilionoideae* showing close genetic relationship among the related species. *Medicago falcata* is forming an outgroup with rest of the species in clade I and clade II suggesting that it might be the possible ancestor of all the species. Moreover, the

value of genetic divergence was observed in the range of 0.02 to 0.47 with overall mean distance of 0.04 for *rps14* gene sequences. The lowest genetic diversity was noticed between *Mimosa pudica* and *Mimosa himalayana* and highest divergence was found between *Vicia sativa* sub spp. *nigra* and *Vicia sepium*.

**Phylogenetic analysis based on protein sequences of *rps14* gene:** For inferring genetic characterization protein data was also used (Fig. 2). Protein based phylogram revealed two clades with bootstrap value of 98% and overall genetic diversity of 0.05%. The species of selected genera of subfamily *Caesalpinioideae* and *Mimosoideae* are in same cluster (i.e. cluster I) of clade I. Similarly, the genera of *Papilionoideae* showed the same genetic relationship as in the nucleotide based phylogenetic tree. However, *Medicago sativa* and *Milletia pinnata* are forming outgroup indicating that these might be possible ancestor of species in clade II.

**Stereochemical analysis of protein sequences of *rps14* gene:** The function of proteins were also predicted based on 3D protein models of *rps14* with best Confidence score (C score) of I-TASSER (Fig. 3). Furthermore, residue by residue and overall geometry of protein structures were predicted by Ramachandran plots (Fig. 4). The most allowed regions were displayed by red patches yellow areas indicate allowed regions. Considering Ramachandran plots,  $\geq 81\%$  to  $\geq 91.8\%$  residues were found in the most allowed region and  $\leq 1\%$  to  $\leq 6\%$  were found in disallowed region. The detail of Ramachandran score of models for all 26 sequences predicted by PROCHECK is given in Table 3.

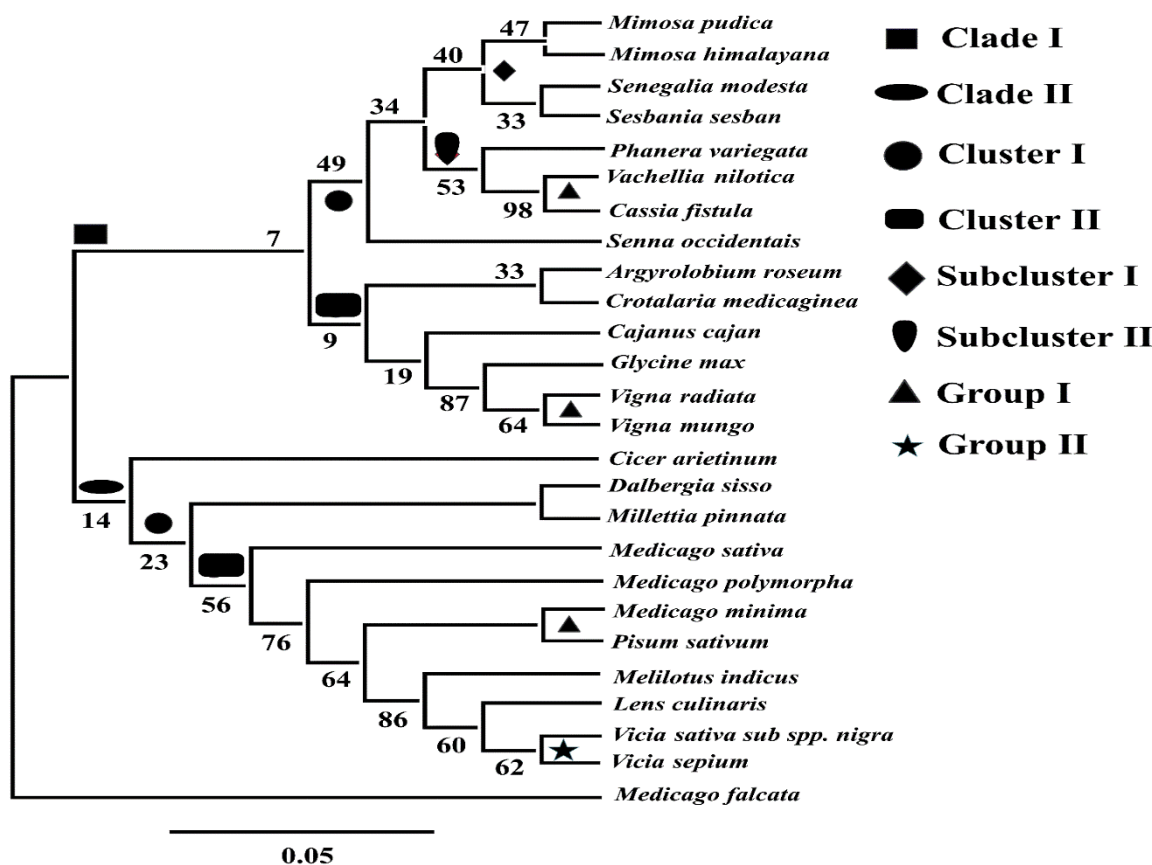


Fig. 1. Phylogenetic analysis of 26 selected species of family Leguminosae based on *rps14* gene sequences using MEGA7 software.

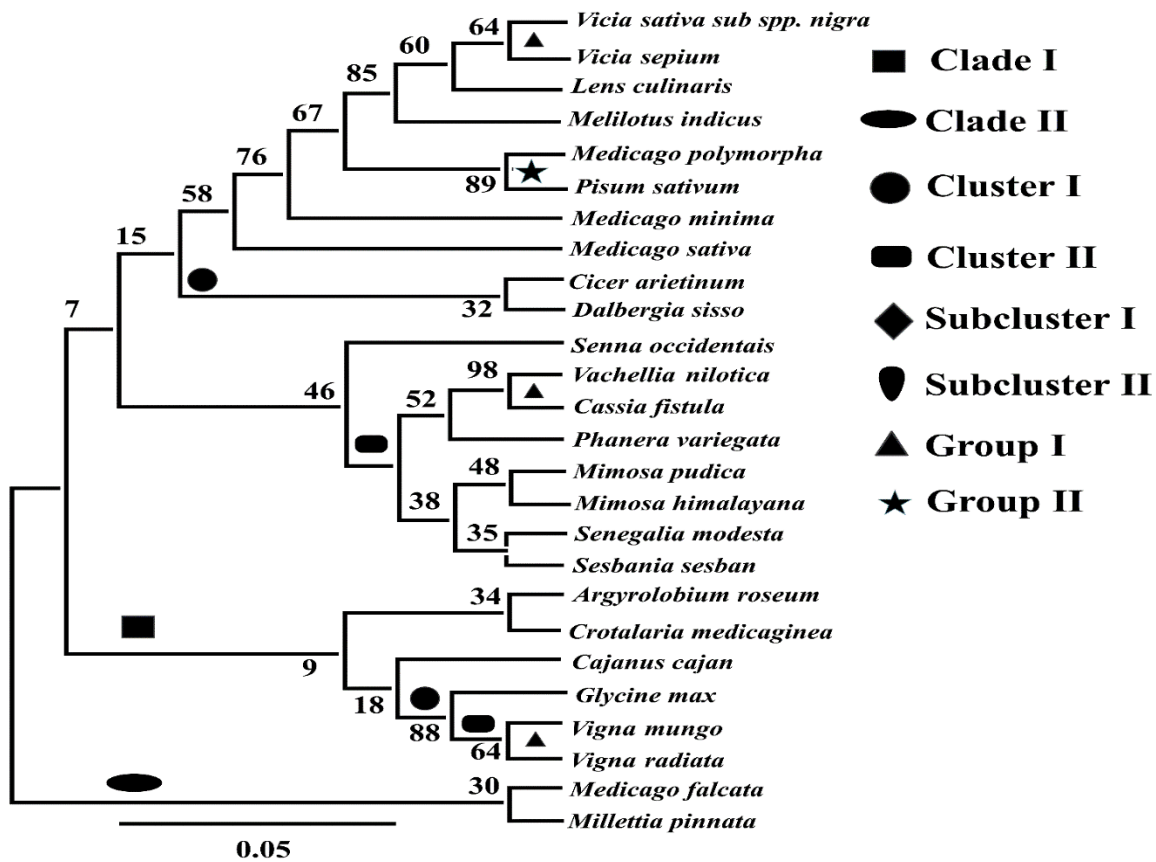


Fig. 2. Phylogenetic tree of 26 selected species of family *Leguminosae* species based on amino acid sequences of *rps14* gene using MEGA7 software.

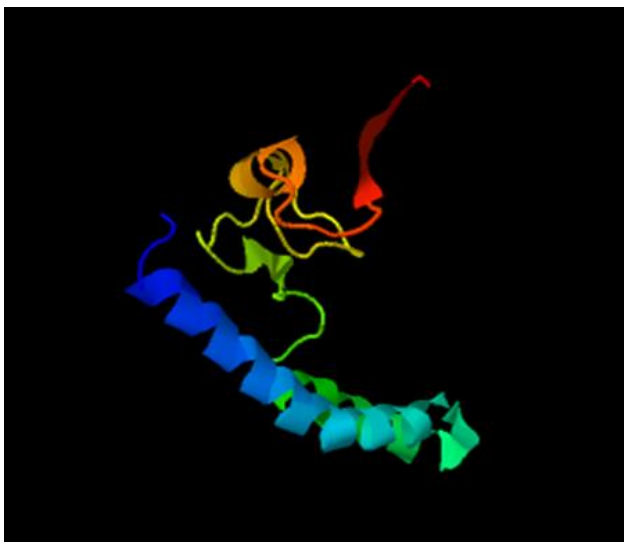


Fig. 3. The representative 3D protein model of *rps14* having the highest C-score build by I-TASSER for *Medicago polymorpha*.

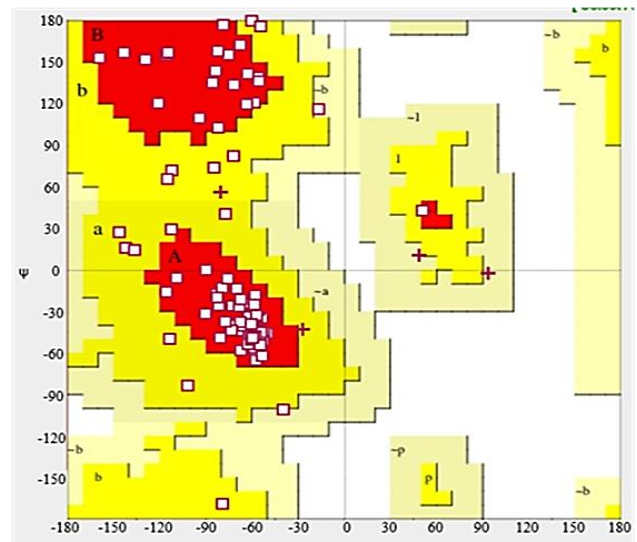


Fig. 4. The representative Ramachandran plot built by SAVES for *rps14* protein for *Medicago polymorpha*.

**Discussion**

Molecular analysis (based on gene and protein sequences) revealed narrow genetic background of 0.05 showing higher similarities among the selected species showing close genetic relationship. Previously, *Leguminosae* was studied based on molecular markers of plastid gene regions (*rbcL*, *matK*, *atpβ*, *trnL*, *trnK* and *trnI-F*), nuclear gene regions (*PHYC*, *ITS*, *ITS1*, *ITS2* of

*nrDNA* and *psbA-trnH* spacer) and different micro- and macro morphological characters (Di *et al.*, 2015; Group, 2013; Compton *et al.*, 2019; Zhao *et al.*, 2020) but no earlier study was performed on *Leguminosae* using *rps14* gene. Clade I was largely occupied by genera of *Mimosoideae*, *Caesalpinioideae* and very few from *Papilionoideae*, while Clade II was occupied by genera of *Papilionoideae* representing their similar origin and close evolutionary relationship. *Medicago polymorpha*,

*Medicago sativa* and *Medicago minima* showed higher similarity. However, *Medicago falcata* is forming an outgroup indicating that it might be the possible ancestor of all the species. Likewise, same position of *Vicia sativa* sub spp. *nigra*, *Vicia sepium*, *Pisum sativum*, *Lens culinaris* and *Cicer arietinum* was reported (Jansen *et al.*, 2008; Snafi, 2016; Steele *et al.*, 2010). In present research study *Cajanus cajan* and *Glycine max* are forming single cluster which are in agreement with the previous studies (Kass & Wink, 1997; Doyle & Luckow 2003). The distant position of *Crotalaria medicaginea* and *Argyrobium roseum* is in accordance to the findings of Subramaniam *et al.*, (2013) on genus *Crotalaria* and *Argyrobium*. The core findings based on molecular characteristics is that *Melilotus* fall close to *Medicago*, these two genera were marked as sister genus by Di *et al.*, (2015). A very close relationship was observed in *Vigna mungo* and *Vigna radiata* which is congruent to the studies of Wang *et al.*, (2008) based on gene-derived markers.

Further, molecular data based phylograms showed that *Vachellia nilotica* and *Senegalia modesta* are different from each other, which is similar to the earlier studies (Luckow *et al.*, 2003; Terra *et al.*, 2017). In the present study interesting monophyletic relationship was observed in

genus *Mimosa* where *Mimosa pudica* and *Mimosa himalayana* are in same group. Simon *et al.*, (2011) also reported close relationship between different species of genus *Mimosa*. The diverse relationship between *Cassia fistula* and *Senna occidentalis* was also observed by Tripathi (Tripathi, 2011). Recently, Nithaniyal *et al.*, (2014) reported a very close relationship between *Dalbergia sisso* and *Millettia pinnata* which is similar to our results. Furthermore, the diverse relationship between *Cassia fistula* and *Senna occidentalis* was also observed by Tripathi (2011). The position of *Phanera variegata*, *Senna occidentalis* and *Cassia fistula* in same cluster was also studied by Doyle & Luckow, (2003). *Sesbania sesban* fall close to *Senegalia modesta* but, very low bootstrap value showed distant relationship between these two species which is in accordance with the earlier work of Kass & Wink, (1997). Process of speciation, tree construction tools, use of different molecular markers and small sample size may be the reasons for unjustified position of species. Moreover, according to the best of our knowledge no earlier research studies have been reported on the molecular phylogenetics of *Crotalaria medicaginea*, *Argyrobium roseum*, *Vicia sativa* sub spp. *nigra*, *Vicia sepium*, *Medicago falcata* and *Mimosa himalayana*.

**Table 3. Ramachandran score of models for the 26 sequences of *rps14* gene representing selected species of family *Leguminosae* predicted by PROCHECK.**

S/N	Protein	Most allowed region	Allowed region	Disallowed region
1.	<i>Medicago polymorpha</i>	84.7	12.2	3.1
2.	<i>Pisum sativum</i>	88.8	7.10	4.1
3.	<i>Vicia sativa</i> sub sp. <i>nigra</i>	88.8	9.20	2.0
4.	<i>Vicia sepium</i>	89.8	8.20	2.0
5.	<i>Glycine max</i>	81.6	14.3	4.0
6.	<i>Lens culinaris</i>	89.8	8.20	2.0
7.	<i>Medicago sativa</i>	85.7	13.3	1.0
8.	<i>Cajanus cajan</i>	87.8	9.20	3.1
9.	<i>Cicer arietinum</i>	89.8	8.20	2.0
10.	<i>Argyrobium roseum</i>	82.7	12.2	5.0
11.	<i>Senna occidentalis</i>	82.7	15.3	2.0
12.	<i>Mimosa pudica</i>	84.7	13.3	2.0
13.	<i>Dalbergia sisso</i>	80.6	14.3	5.1
14.	<i>Acacia modesta</i>	80.6	13.3	6.1
15.	<i>Vachellia nilotica</i>	87.8	11.2	1.0
16.	<i>Medicago falcata</i>	88.8	8.20	3.1
17.	<i>Vigna mungo</i>	84.7	12.2	3.1
18.	<i>Cassia fistula</i>	84.7	11.2	4.1
19.	<i>Phanera variegata</i>	86.7	11.2	2.0
20.	<i>Millettia pinnata</i>	84.7	10.2	5.1
21.	<i>Sesbania sesban</i>	89.8	6.00	4.1
22.	<i>Crotalaria medicaginea</i>	87.8	11.2	1.0
23.	<i>Vigna radiata</i>	91.8	7.10	1.0
24.	<i>Medicago minima</i>	83.7	11.2	5.1
25.	<i>Medicago indicus</i>	92.6	5.10	1.0
26.	<i>Mimosa himalayana</i>	93.6	4.10	1.0

Generally, genetic distance in both phylograms (gene and protein based) were same i.e., 0.05 and bootstraps values were up to 98% depicting consistency in both trees. However, in protein based tree *Millettia pinnata* and *Medicago falcata* are forming outgroup indicating the fact that they might be the possible ancestor of all the species and genera like *Vicia*, *Lens*, *Melilotus*, *Medicago*, *Pisum*, *Cicer* and *Dalbergia* showed much closer relationship than rest of the members of *Papilionoideae*. The main reason of diversity in a protein sequence may be due to rare evolutionary pressure to arrest silent mutation so, protein homology is not much affected by mutation as DNA sequences are comprised of four nucleotides whereas twenty amino acids constitute protein. To attain statistically notable alignment by differentiating considerable characters is simple as these have very low liability to get an accidental match.

**3D structure analysis of protein and structure validation:** On average protein models for all the species predicted by I-TASSER were quiet good that can reliably be used for further applications of bioinformatics tool because they have  $\geq 81\%$  to  $\geq 91.8\%$  residues that lie in the most allowed region and  $\leq 1\%$  to  $\leq 6\%$  residues that lie in the disallowed region. But the protein models of *rps14* of *Vicia sativa* sub sp. *nigra*, *Vicia sepium*, *Lens culinaris*, *Medicago sativa*, *Senna occidentalis*, *Mimosa pudica*, *Cicer arietinum*, *Vachellia nilotica*, *Phanera variegata*, *Sesbania sesban*, *Crotalaria medicaginea*, *Vigna radiata*, *Melilotus indicus* and *Mimosa himalayana* were found to be the best quality structural models, as they have  $\leq 2\%$  residues that lie in the disallowed region. Previously Munir *et al.*, (2013) evaluated 3D protein structure and Ramachandran plot analysis of four different varieties of tomato. The authentication results from Ramachandran plot analysis indicated that *rps14* protein structure models of 26 species of *Leguminosae* have good quality structure model. To the best of our knowledge this is the first report on description of 3D protein structure and Ramachandran plot analysis for *rps14* in the family *Leguminosae*.

## Conclusion

The present study is the first to investigate the phylogenetic relationship among the selected species of *Leguminosae* from Pakistan using *rps14* gene. Many species, particularly *Crotalaria medicaginea*, *Vicia sativa* sub spp. *nigra*, *Vicia sepium*, *Argyrolobium roseum*, *Mimosa himalayana* and *Medicago falcata* are endemic to Pakistan, but to date, *Leguminosae* members have been poorly sampled in the evolutionary studies. This study concludes, that family *Leguminosae* may be monophyletic as well as polyphyletic. There are so many genera which show monophyletic relationship like genus *Vicia* and *Vigna*. In addition, certain genera have shown polyphyletic relationship such as genus *Medicago*. Moreover, the interspecific variation in the nucleotide sequence appears to be more prevalent in *rps14* gene sequence. Therefore, it may be suggested that *rps14* gene should be used as a potential barcode in the identification of plant species.

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