INTRASPECIFIC RELATIONSHIP WITHIN THE GENUS *CONVOLVULUS* L. INFERRED BY *rbc*L GENE USING DIFFERENT PHYLOGENETIC APPROACHES

SHAZIA KOUSAR[1], SYEDA QAMARUNNISA[2], ANJUM PARVEEN[3*] AND ISHRAT JAMIL[3]

[1]*Department of Botany, University of Karachi, Karachi, Pakistan*
[2]*The Karachi Institute of Biotechnology and Genetic Engineering, KIBGE, University of Karachi, Karachi, Pakistan*
[3]*Centre for Plant Conservation, University of Karachi, Karachi, Pakistan*
[*]*Correspondence author's email: anjum_tahir@yahoo.com*

Abstract

A molecular systematics analysis was conducted using sequence data of chloroplast *rbc*L gene for the genus *Convolvulus* L., by distance and character based phylogenetic methods. Fifteen representative members from genus *Convolvulus* L., were included as ingroup whereas two members from a sister family Solanaceae were taken as outgroup to root the tree. Intraspecific relationships within *Convolvulus* were inferred by distance matrix, maximum parsimony and bayesian analysis. Transition/transversion ratio was also calculated and it was revealed that in the investigated *Convolvulus* species, transitional changes were more prevalent in *rbc*L gene. The nature of *rbc*L gene in the present study was observed to be conserved, as it does not show major variations between examined species. Distance matrix represented the minimal genetic variations between some species (*C. glomeratus* and *C. pyrrhotrichus*), thus exhibiting them as close relatives. The result of parsimonious and bayesian analysis revealed almost similar clades however maximum parsimony based tree was unable to establish relationship between some *Convolvulus* species. The bayesian inference method was found to be the method of choice for establishing intraspecific associations between *Convolvulus* species using *rbc*L data as it clearly defined the connections supported by posterior probability values.

Key words: *Convolvulus*, Intraspecific relationships, Phylogenetics, Genetic distance, Transition/transversion, *rbc*L gene.

Introduction

Convolvulus is a genus of flowering plant family Convolvulaceae and comprised of about 200 to 250 species worldwide (Carine & Lavinia, 2010; Parnell & Curtis, 2012). The species belonging to the genus *Convolvulus* are found across all or most of the world in appropriate habitats thus having a cosmopolitan distribution. The biodiversity hubs of *Convolvulus* are mainly found in western Asia, Mediterranean and Macronasia (Carine *et al*., 2004). In Pakistan, the genus *Convolvulus* is represented by 21 wild and cultivated species including one endemic species *Convolvulus scindicus* (Ghazanfar, 1979). The *Convolvulus* species are well known for their economic and medicinal value and they have long been used as ornamentals, laxatives and as a brain tonic (Bhowmik *et al*., 2012; Agarwal *et al*., 2014a; Sahu & Gupta, 2014; Agarwa *et al*., 2014b).

Many genera of family Convolvulaceae are difficult to delineate including genus *Convolvulus* (Wilson, 1960; Austin, 1975). The species origin is challenging in *Convolvulus* especially in those regions where this genus is reasonably diverse. All species included in the genus *Convolvulus*, form clusters where the boundaries of individual species are far from satisfactory and many that are adequately delineated although having close relatives. The placement of a number of intraspecific taxa has also been moved by different authors from one species to another has been a source of debate (Wood *et al*., 2015).

The high rate of variations in the phenotypic characteristics of *Convolvulus* made it difficult to classify at intraspecific level. Sometimes variations are found in same species in response to the environmental conditions. Previous reports did not provide stronger evidence in species delimitation and their association as few characters were taken into consideration for the classification (Stefenovic *et*

al., 2002; Khalik, 2008). The genus *Convolvulus* has been revised taxonomically from different areas due to overlapping characters and as a result some new species are also discovered by means of phenetic parameters (Alfarhan, 1993; Johnson, 2001; Aykurt & Sümbül, 2011; Mill, 2013). However, fewer studies of *Convolvulus* at molecular have been conducted in (Stefenovic *et al*., 2002; Carine *et al*., 2004). The plastid *rbc*L gene, which codes for the large subunit of ribulose- 1,5-bisphosphate carboxylase/oxygenase (RuBisCO or RuBPCase), is the extensively used marker for the phylogenetic investigation of higher plants (Schuettpelz *et al*., 2006; Bafeel *et al*., 2012; Zitouna *et al.,* 2014). Other non-coding markers are also used successfully in molecular systematic studies (Jamil *et al*., 2014). The present study was focused on establishing phylogenetic relationship between species of *Convolvulus* using *rbc*L data by distance method, maximum parsimony and bayesian approach.

Materials and Methods

Plant materials: Fifteen different *Convolvulus* species were collected from different locations of Sindh, Pakistan while two species from a sister family Solanaceae were also taken as out group. Some specimens were also taken from Karachi University Herbarium (KUH), Centre for Plant Conservation.

Identification of plants on morphological basis: The collected species were identified with the help of their morphological characters described in different floras, literature and authentic herbarium specimens. Herbarium sheets were prepared and deposited to Karachi University Herbarium (KUH), Centre for Plant Conservation. The plant materials, their location, herbarium voucher number and Genbank accession numbers are listed in Table 1.

Table 1. List of plant material used in this study along with their collection site (locality) voucher number and Genbank accession number.

S. #	Species	Locality	Specimen voucher No.	Genbank accession No.
1.	*Convolvulus arvensis*	Botanic garden (KU)	GH86574	KT596044
2.	*Convolvulus prostratus*	North Nazimabad	GH86568	KT596045
3.	*Convolvulus scindicus*	Thanobulla Khan	GH87724	KT596046
4.	*Convolvulus glomeratus*	North Nazimabad	GH86572	KT596047
5.	*Convolvulus spinosus*	Thatta	GH86624	KT596048
6.	*Convolvulus fruticosus*	Herbarium	GH58159	KT596049
7.	*Convolvulus virgatus*	Herbarium	GH61439	KT596050
8.	*Convolvulus pseudocantabricus*	Herbarium	GH61485	KT596051
9.	*Convolvulus cephalopodus*	Herbarium	GH46285	KT596052
10.	*Convolvulus rhyniospermus*	Herbarium	GH61370	KT596053
11.	*Convolvulus lineatus*	Herbarium	GH46811	KT596054
12.	*Convolvulus kotscyanus*	Herbarium	GH47622	KT596055
13.	*Convolvulus pyrrhotrichus*	Herbarium	GH45068	KT596056
14.	*Solanum forskalii*	Malir	GH88012	KT596057
15.	*Datura alba*	Karachi University	GH88010	KT596058

DNA extraction, PCR amplification and sequencing: DNA from fresh leave tissue was isolated by using cetyltrimethylammonium bromide (CTAB) method described by Doyle & Doyle (1987). Dried tissue from herbarium specimen was used to extract DNA by using Plant Direct PCR Kit (MOLEQULE-ON, NZ). The *rbc*L region was amplified by using genus specific primer; forward 5'GCTGCGGAATCTTCTACTGG3' reverse 5'CTAAAGTTCCTCCCCCGAAC3'. Total 30µl reaction mixture was made with 1X PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 1µM of each forward and reverse primer and 1U of DNA Taq polymerase (MOLEQULE-ON, NZ). The Thermal cycler profile set at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 35 sec, 72°C for 1 min and a final extension at 72°C for 5 min. The amplicons were checked on 1% agarose gel electrophoresis and after purification with PCR purification kit (MOLEQULE-ON, NZ) sent to direct sequencing to Bioneer Inc. (Korea).

Sequence editing and alignment: Each sequence was analyzed by using nucleotide specific Basic Local Alignment Search Tool (BLASTn) (Altschul *et al*., 1990). Sequences were edited manually to resolve any discrepancy. Multiple sequence alignment (MLA) was performed for all sequences using Multalin software (Corpet, 1988). Open reading frames and protein sequences were obtained by using NCBI ORF Finder (Wheeler *et al*., 2003) and Expasy translate tool (Gasteiger *et al*., 2003) respectively. The refined sequences were deposited to NCBI Genbank and accession numbers were recorded.

Phylogenetic tree reconstruction using molecular data: The relationship between different species of genus *Convolvulus* was studied by maximum parsimony (MP) method in PAUP* version 4.0b10 (Swofford, 2003) using heuristics search with 1000 replicates, random stepwise addition of sequence and tree- bisection-reconnection (TBR) branch swapping algorithm. Strength of clades was estimated using the 1000 bootstrap replicates (Felsenstein, 1985). *Datura alba* and *Solanum forskalii* from Solanaceae were used as

outgroup in this study. Kimura 2-parameter genetic distances and transition/transversion ratio were also calculated using PAUP* (Swofford, 2003). Bayesian Inference (BI) a character and model based approach, which estimates probability value was used for further corroboration by using BEAST (v1.8.0) (Drummond & Rambaut, 2007). Model selection for BI analysis was performed using default parameters of JModelTest (v 2.1.4) (Darriba *et al*., 2012). Final trees were visualized in FigTree (v1.4.0).

Results

The PCR for *rbc*L gene in all investigated species produced ~1050 bp monomorphic fragment. The analogue of the sequenced products was recognized using the BLAST on NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All the sequences showed 95 to 99% identity to available *Convolvulus* species. The average GC content in all investigated species found to be around 43% and the aligned sequences span around 1000 nucleotide. The ratio of transitions (changes between pyrimidines 'C↔T' or between purines 'A↔G') to transversions (changes between pyrimidines and purines 'A↔ C' 'A↔ T' 'G↔ C' 'G↔ T') was estimated using the treescores/likelihood option in PAUP*4 on the 13 *Convolvulus* ingroup species only. Kimura 2-parameter distance matrix is shown in Table 2 and the recorded data for transition/ transversion ratio is given in Table 3. The K2P genetic distance between the investigated species was found to be in the range of 0.009 to 0.029 with the lowest distance determined between the *C. glomeratus* and *C. pyrrhotrichus* while the greatest distance farther the *C. lineatus* from *C. arvensis*. The *Convolvulus rbc*L data set was measured to have a transition/transversion (ti/tv) ratio between 0.2 to 2.2. The low transition/transversion (ti/tv) rate (0.2) between the *C. virgatus* and *C. glomeratus* indicated more tranversional changes between the two species while C. *kotschyanus* and C. *rhyniospermus* accumulate more transitional changes (2.2) during the evolutionary course. The overall ti/tv rate between all investigated taxa gave 53.8% transitions, 38.5% transversions and 7.7% equal changes.

Table 2. Kimura 2-parameter distance matrix of *Convolvulus* species.

	1	2	3	4	5	6	7	8	9	10	11	12
2	0.0110	-										
3	0.0178	0.0181	-									
4	0.0199	0.0213	0.0169	-								
5	0.0250	0.0211	0.0179	0.0109	-							
6	0.0210	0.0202	0.0159	0.0159	0.0159	-						
7	0.0280	0.0223	0.0199	0.0240	0.0231	0.0230	-					
8	0.0290	0.0254	0.0238	0.0178	0.0189	0.0228	0.0168	-				
9	0.0245	0.0269	0.0203	0.0225	0.0227	0.0205	0.0255	0.0225	-			
10	0.0265	0.0290	0.0244	0.0245	0.0247	0.0225	0.0255	0.0244	0.0148	-		
11	0.0236	0.0250	0.0183	0.0122	0.0123	0.0164	0.0215	0.0122	0.009	0.0160	-	
12	0.0249	0.0212	0.0178	0.0190	0.0170	0.0139	0.0229	0.0230	0.0203	0.0245	0.0184	-
13	0.0281	0.0252	0.0220	0.0160	0.0119	0.0159	0.0261	0.0140	0.0215	0.0236	0.0113	0.0149

The numbers in the Table 2 represent the following species. **1.** *C. arvensis*, **2.** *C. fruticosus*, **3**. *C. spinosus*, **4**. *C. kotschyanus*, **5**. *C. virgatus*, **6**. *C. rhyniospermus*, **7**. *C. prostratus*, **8**. *C. lineatus*, **9**. *C. pyrrhotrichus,* **10**. *C. pseudocantabricus*, **11**. *C. glomeratus*, **12**. *C. scindicus*, **13**. *C. cephalopodus*

Table 3. Apparent transition/transversion ratio between all investigated species of *Convolvulus*.

	2	3	4	5	6	7	8	9	10	11	12	13
1	1.20	1.50	1.50	0.92	1.62	1.33	1.63	1.00	1.60	0.91	0.92	1.15
2	-	0.63	1.62	0.90	1.22	1.44	1.50	1.00	1.54	0.84	0.75	1.27
3		-	1.42	0.80	1.28	0.81	1.18	0.81	1.18	0.63	0.80	1.20
4			-	0.83	2.20	1.00	1.25	1.20	1.40	0.50	1.37	1.66
5				-	0.77	0.90	0.72	0.83	1.00	0.20	1.12	1.00
6					-	0.76	1.30	1.22	1.44	0.77	1.00	1.20
7						-	1.12	0.92	1.27	0.61	0.76	1.16
8							-	1.20	1.40	0.50	0.91	1.33
9								-	1.14	2.00	0.81	1.10
10									-	0.77	1.18	1.30
11										-	0.63	0.37
12											-	1.50

The numbers in the Table 3 represent the following species. **1**. *C. arvensis*, **2.** *C. fruticosus*, **3**. *C. spinosus*, **4**. *C. kotschyanus*, **5**. *C. virgatus*, **6**. *C. rhyniospermus*, **7**. *C. prostratus*, **8**. *C. lineatus*, **9**. *C. pyrrhotrichus,* **10**. *C. pseudocantabricus*, **11**. *C. glomeratus*, **12**. *C. scindicus*, **13**. *C. cephalopodus*

The maximum parsimony based phylogenetic tree was constructed based on the chloroplast *rbc*L gene sequence (Fig. 1). Total 1062 characters were used in this analysis, out of which 930 characters were constant. Eighty nine characters were considered informative for MP tree construction out of total 132 variable characters. The tree length was calculated as 222 while measure of homoplasy were determined as CI (0.72) and RI (0.58). The overall topology of MP tree was not fully resolved. Two major lineages with moderate support separate *C. rhyniospermus* from other species. The majority rule consensus tree gave weak support (51%) to a clade that represented three species; *C. arvensis, C. fruticosus* and *C. spinosus,* while within this clade the sister association between the first two species was strongly supported by 92% bootstrap value. A weak relation was also observed between *C. prostratus* and *C. lineatus*. A clade with moderate support (80%) established close association between *C. pyrrhotrichus, C. pseudocantabricus* and *C. glomeratus*.

Another tree using same data was reconstructed by Bayesian inference. This method relies on best nucleotide substitution model and for this dataset it was found to be Hasegawa-Kishino-Yano with gamma distribution (HKY+G). The tree topology was much clear compared to parsimony approach. A monophyletic clade of genus *Convolvulus* was observed against two outgroups from the genera *Datura* and *Solanum* (Fig. 2). The *Convolvulus* clade diverges into two lineages. The basal lineage with strong support of posterior probability (PP) value combines three species of *Convolvulus* (*C. glomeratus, C. pyrrhotrichus* and *C. pseudocantabricus*), while the upper lineage with weak supportive value further separated into two nodes. The top nodal branch represents the sister association between *C. lineatus* and *C. prostratus* with 0.86 PP. The second nodal branch further splits into two groups with weak PP support. The first group exhibits two nodes, one showing strongly supported sister relationship between *C. scindicus* and *C. rhyniospermus*, while the other moderately support close association between *C. cephalopodus*, *C. virgatus* and *C. kotschyanus*. The second group clustered three species *C. fruticosus, C. arvensis* and *C. spinosus*, in which the association between the first two is strongly supported.
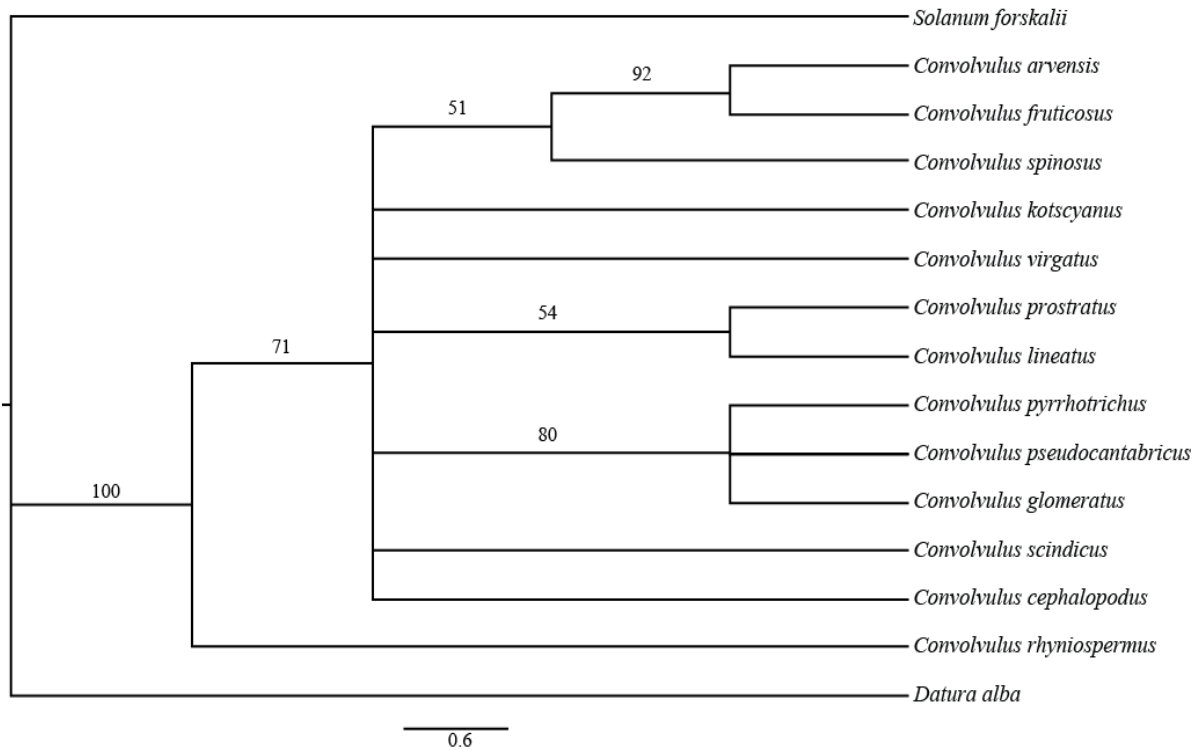
Discussion

Fig. 1. Majority rule consensus parsimonious tree resulting from the analysis of *rbc*L gene sequence for *Convolvulus*. Bootstrap supports in percentages based on 1000 replication analysis are shown in the nodes.
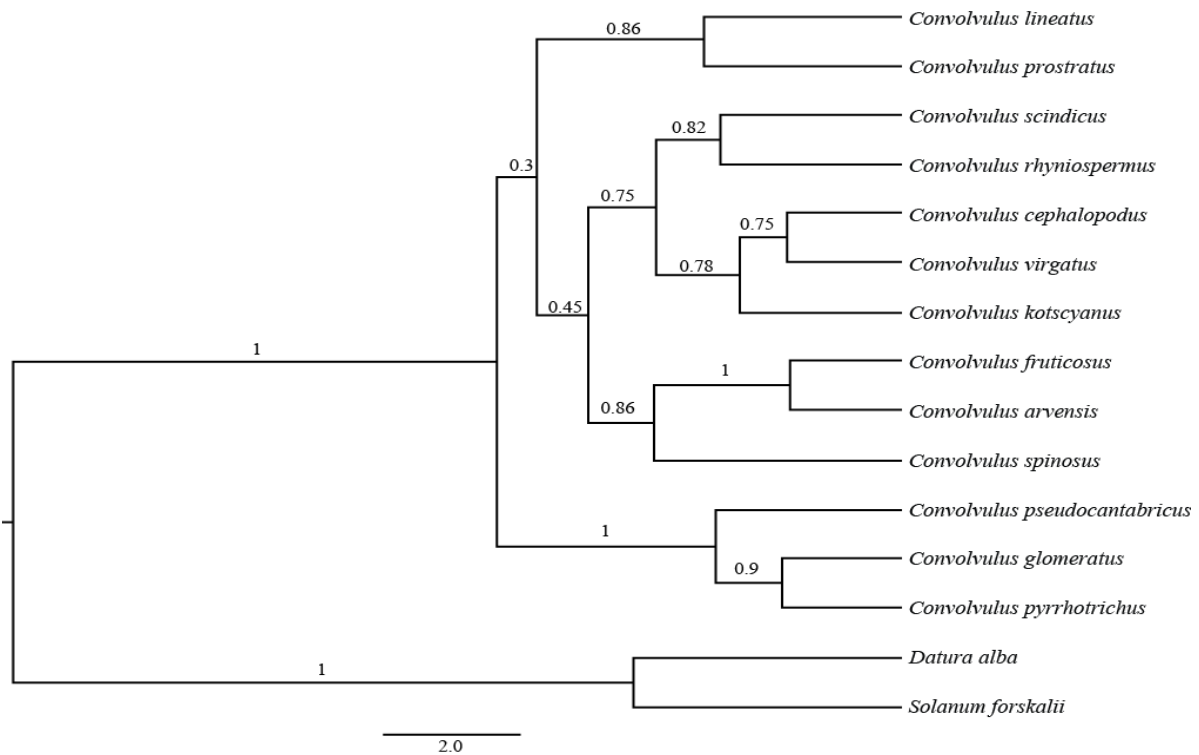

Fig. 2. Phylogenetic tree with mean branch lengths from a representative Bayesian analysis of Chloroplast *rbc*L gene data. Numbers on branches represent their respective posterior probabilities.

The current study emphasized on different phylogenetic approaches to establish intraspecific relationships between *Convolvulus* species. The species concept has long been a crucial issue in systematic biology and different phenetic, cluster and phylogenetic approaches were adopted to resolve the relationships between plant species. Moreover, the use of DNA sequence data for species delimitation is also an innovative approach in infraspecific classification (Williams *et al*., 2014). The genus *Convolvulus* has been studied in the present work by using different phylogenetic approaches and by distance matrix. The genetic distance between species are generally calculated by dividing number of variable sites from total number of sites available. Kimura two parameter (K2P) model assume that transitions and transversions happen at different rates and all nucleotides occur at the same frequency (Kimura, 1980), whereas other models such as Felsenstein (F84) and Hasegawa-Kishono-Yano (HKY85) allow the four nucleotides present in different frequencies and transitions and transversions occur at different rates (Felsenstein, 1981; Hasegawa *et al*., 1985). In the present study, K2P and HKY models were used to delimit the relations between *Convolvulus* species. However, both models almost established the same relationships between species. HKY model was used in Bayesian analysis, resulting in a resolved phylogenetic tree displaying different clades of sister species. It showed close associations between *C. glomeratus*, C. *pyrrhotrichus* and *C. pseudocantabricus* supported by full probability (1) for their occurrence in one clade. The consensus maximum parsimony tree also supports the close relationship of stated three species with strong bootstrap value. Morphologically, *Convolvulus glomeratus* is non-spiny herb while *Convolvulus pyrrhotrichus*, and *C. pseudocantabricus* are non-spiny shrubs. The three species share some common characters for instance their style and ovary are glabrous, seeds are brown, rough and inflorescence is axillary cymose (Ghazanfar, 1979). The genetic distance between *C. glomeratus, C. pyrrhotrichus* was estimated to be lowest (0.009) indicating not much difference at nucleotide level while between C. *pyrrhotrichus* and *C. pseudocantabricus* and *C. glomeratus* and *C. pseudocantabricus* is 0.014 and 0.016 respectively which is also support that there is little variations between the above mentioned species. The transition/transversion (ti/tv) ratio between *C. glomeratus*, C. *pyrrhotrichus* is found to be at higher side (2.00) demonstrating more transitional substitutions between these species. Because there are four possible transition and eight possible transversion states therefore transversions are expected to occur twice as frequently as transitions, assuming equal probabilities of all nucleotide substitutions. However, in almost all genomic DNA sequences, transitions have been noted to occur at higher frequencies than transversions (Soltis & Doyle, 2012). Our result also corroborates with the previous findings and determine that in all investigated species of *Convolvulus* transitions occur most frequently (53.8%) than transversions (38.5%). It has been observed that a negative correlation exists between the genetic distance and the observed transition/transversion (ti/tv) ratio, at low levels of genetic divergence, transition/transversion (ti/tv) appear to be high, and at high level of genetic divergence, transition/transversion (ti/tv) appears to be low (Yang & Yoder, 1999). This finding also seems to be true in our outcome that at low genetic distances, transition/transversion (ti/tv) ratio appear to be at higher site. The model-based tree exhibited the strong association between *C. lineatus* and *C. prostratus* with strong probability value. The presented parsimonious tree also demonstrates their relationship. From morphological point of view, these two species are non-spiny perennial green herbs with procumbent or ascending pubescent stem. They also share many other common characters (Ghazanfar, 1979). The genetic distance between *C. lineatus* and *C. prostrates* also show less variable sites (0.016) while the transition/transversion (ti/tv) ratio indicates more accumulation of transition mutations. Another clade was observed which support the associations between *C. fruticosus, C. arvensis* and *C. spinosus*, in which the association between the *C. fruticosus* and *C. arvensis* was strongly supported by both bayesian and parsimony based phylogenetic trees. Furthermore, the genetic distance also suggests the connection between *C. fruticosus* and *C. arvensis* by presenting low variations (0.011) while the genetic distance between the two species with *C. spinosus* is found to be 0.018. *Convolvulus arvensis* is non-spiny while *C. fruticosus* and *C. spinosus* both are perennial spiny shrubs.

The HKY with gamma distribution based bayesian tree represents close relationship between *C. scindicus* and *C. rhyniospermus*, while the moderately support is given to establish close association between *C. cephalopodus*, *C. virgatus* and *C. kotschyanus*. This association between five species is not resolved in MP based tree. These five species belong to a non-spiny group at morphological level.

Daughter species gradually separate with time, but they do not simultaneously acquire different phenotypic properties which define them as diverse species (Vanderpoorten & Shaw, 2014). In the present study, at the species level, molecular data (*rbc*L) supports the close association between different *Convolvulus* species and the model based bayesian inference approach appear to be the method of choice for establishing intraspecific relationships in genus *Convolvulus*. Further work is needed to accurately correlate these intraspecific groupings with morphology or geography.

References

Agarwal, P., B. Sharma, A. Fatima and S.K. Jain. 2014a. An update on Ayurvedic herb *Convolvulus pluricaulis* Choisy. *Asian Pac. J. Trop. Med.,* 4(3): 245-252.

Agarwal, P., B. Sharma, S.K. Jain, A. Fatima and S. Alok. 2014b. Effect of *Convolvulus pluricaulis* chois. On blood glucose and lipid profile in streptozocin induced diabetic rats. *Int. J. Pharm. Sci. Res.,* 5(1): 213-219.

Alfarhan, A.H. 1993. A new species of *Convolvulus* (Convolvulaceae) from Saudi Arabia. *Brittonia,* 45(2): 169-171.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.,* 215(3): 403-410.

Austin, D.F. 1975. Convolvulaceae. Flora of Panama. *Ann. Missouri Bot. Gard.,* 62: 157-224.

Aykurt, C. and H. Sumbul. 2011. New natural hybrids of *Convolvulus* (Convolvulaceae) from Turkey. *Nord. J. Bot.,* 29(4): 408-416.

Bafeel, S.O., I.A. Arif, A.A. Al Homaidan, A.H. AlFarhan and H.A. Khan. 2012. DNA barcoding of arid wild plants using rbcL gene sequences. *Genet. Mol. Res.,* 11(3): 1934-41.

Bhowmik, D., K.PS. Kumar, S. Paswan, S. Srivatava, A. pd. Yadav and A. Dutta. 2012. Traditional Indian herbs *Convolvulus Pluricaulis* and its medicinal importance. *J. Pharmacogn. Phytochem.,* 1(1):44-51.

Carine, M.A. and R. Lavinia. 2010. Taxonomy and evolution of the *Convolvulus sabatius* complex (Convolvulaceae). *Phytotaxa,* 14: 1-21

Carine, M.A., S.J. Russell, A. Santos-Guerra and J. Francisco-Ortega. 2004. Relationships of island and continental floras: molecular evidence for multiple colonisations into Macaronesia and subsequent back-colonisation of the continent in *Convolvulus* L. *Am. J. Bot.,* 91: 1070-1085.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.,* 16: 10881-10890.

Darriba, D., G.L. Taboada, R. Doallo and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods,* 9(8): 772-772.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.,* 19: 11-15.

Drummond, A.J. and A. Rambaut. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC. Evol. Biol.,* 7: 214-222.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.,* 17(6): 368-376.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution,* 39(4): 783-791.

Gasteiger, E., A.Gattiker, C. Hoogland, I. Ivanyi, RD. Appel and A. Bairoch. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.,* 31(13): 3784-3788.

Ghazanfar, S. 1979. Convolvulaceae. In: *Flora of Pakistan*, (Eds.): Nasir, E. and S.I. Ali. 126: 1-64. National Herbarium, Islamabad.

Hasegawa, M., H. Kishino and T.Yano. 1985. Dating of human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.*, 22(2): 160-174.

Jamil, I., S. Qamarunnisa, A. Azhar, Z.K. Shinwari, S.I. Ali and M. Qaiser. 2014. Subfamilial relationships within Solanaceae as inferred from *Atpβ-rbcL* intergenic spacer. *Pak. J. Bot.,* 46: 585-590.

Johnson, R.W. 2001. A taxonomic revision of *Convolvulus* L. (Convolvulaceae) in Australia. *Austrobaileya,* 6(1):1-39.

Khalik, K.A. 2008. A phenetic study of Convolvulaceae from Egypt based on analysis of morphological characters. *Fl. Medit.,* 18: 65-87.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* 16(2): 111-120.

Mill, R.R. 2013. New taxa of *Convolvulus* (Convolvulaceae) from the Arabian Peninsula. *Edinb. J. Bot.*, 70: 367-379.

Parnell, J. and T. Curtis. 2012. *Webb's An Irish Flora*. Cork University Press. ISBN 978-185918-4783.

Sahu, P.K. and S. Gupta. 2014. Medicinal plants of Morning glory: Convolvulaceae Juss. of Central India (Madhya Pradesh and Chhattishgarh). *Biolife,* 2(2): 463-469.

Schuettpelz, E., P. Korall and K.M. Pryer. 2006. Plastid at pA data provide improved support for deep relationships among ferns. *Taxon,* 55(4): 897-906.

Soltis, P. and J.J. Doyle. 2012. Molecular systematics of plants II: DNA sequencing. Kluwer Academic Press, New York.

Stefanovic, S., L. Krueger and R.G. Olmstead. 2002. Monophyly of the Convolvulaceae and circumscription of their major lineages based on DNA sequences of multiple chloroplast loci. *Am. J. Bot.,* 89(9): 1510-1522.

Swofford, D.L. 2003. PAUP. Phylogenetic analysis using parsimony and other methods. Version 4. Sinauer associates.

Vanderpoorten, A. and A.J. Shaw. 2014. The application of molecular data to the phylogenetic delimitation of species in bryophytes: a note of caution. *Phytotaxa,* 9: 229-237.

Wheeler, D.L., D.M. Church, S. Federhen, A.E. Lash, T.L. Madden, J.U. Pontius and L. Wagner. 2003. Database resources of the National Center for Biotechnology. *Nucleic Acids Res.,* 31(1): 28-33.

Williams, B.R.M., T.C. Mitchell, J.R.I. Wood, D.J. Harri, R.W. Scotland and M.A. Carine. 2014. Integrating DNA barcode data in a monograph of *Convolvulus* L. *Taxon*, 63: 1287-1306.

Wilson, K.A. 1960. The genera of Convolvulaceae in the Southeastern United States. *J. Arnold. Arbor.,* 41: 298-317.

Wood, J.R., B.R. Williams, T.C. Mitchell, M.A. Carine, D.J. Harris and R.W. Scotland. 2015. A foundation monograph of *Convolvulus* L. (Convolvulaceae). *Phyto Keys,* 18(51): 1-282.

Yang, Z. and A.D. Yoder. 1999. Estimation of the transition/transversion rate bias and species sampling. *J. Mol. Evol.,* 48: 274-283.

Zitouna, N., M. Gharbi, H.B. Rhouma, A. Touati, A. Haddioui, N. Trifi-Farah and S. Marghali. 2014. The evolution of *rbc*L: A methodology to follow the evolution patterns of Medicago and Sulla (Fabaceae) genera. *Biochem. Sys. Ecol.,* 57: 33-39.