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DNA BARCODING AND MOLECULAR SYSTEMATICS OF SELECTED SPECIES OF FAMILY ACANTHACEAE

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Abstract

In the present study the phylogeny of the various selected genera of Acanthaceae was investigated based on macro-morphological characters, chloroplast DNA (cp-DNA) genes *rbc*L, *mat*K and intergenic spacer *trnH-psbA* sequences. About 34 macro-morphological characters of taxonomic importance were selected from the different floras of the world which were used to construct a phylogeny based on morphology. For morphological analysis selected species belonging to five different genera of Acanthaceae including *Ruellia, Justicia, Barleria, Dicliptera* and *Strobilanthes* were studied. The phylogenetic tree was constructed using Multivariate Statistical Package (MVSP 3.1) by the method of UPGMA (Unweighted Pair Group Method Analysis). Our results showed that *Ruellia brittoniana* seemed to be the closest relative of *Justicia* and *Barleria* species based on morphological analysis. The plastid gene regions ribulose-1,5-bisphosphatecarboxylase/oxygenase (*rbc*L), maturase kinase (*mat*K) and *trnH–psbA* intergenic spacer region were selected for taxonomic study on molecular level. These genes *rbc*L (1400 bp), *mat*K (800 bp) and *trnH–psbA* spacer (400 bp) were sequenced and the sequence data was aligned and used for the phylogenetic tree in combination with the other sequences retrieved from GenBank, National Centre of Biotechnology Information (NCBI). Molecular data based on these *mat*K, *rbc*L and *trnH–psbA* sequences and the BLAST result shows that genera *Barleria, Strobilanthes* and *Ruellia* are monophyletic but genus *Justicia* is non-monophyletic. Our results conclude that *rbc*L is more effective barcode; while combination of *mat*K and *trnH–psbA* can be used for identification and phylogenetic study of plants.

Key words: Acanthaceae, DNA barcoding, *rbc*L, *mat*K, *trnH-psbA*.

Introduction

Acanthaceae is taxonomically complex family with large number of medicinally important species. In Africa and Kenya some species of the family are used to feed goats and pigs (Burkill, 1985). Species of Acanthaceae can also be used as bio-indicators to proxy the spatial distribution of plant communities (Mabberley, 2008). Member of Acanthaceae are rich source of flavonoids. Different types of important and medicinally active ingredients have been identified and extracted from some species of the family. Leaves of *Hygrophila spinosa* have anti-inflammatory and antipyretic activities on rats. Root extract of *Hygrophila difformis* induced hepatotoxicity in rats (Patra *et al.,* 2009). Paste of *Blepharis maderaspatensis* leaves and onion bulbs are applied externally for wounds and cuts. Leaves of *Hygrophila auriculata* are used to relieve from cough. Juice of leaves of *Blepharis maderaspatensis* is used externally for wound healing (Sandhya *et al.,* 2015).

In biology identification and characterization of different biological specimens are the keystone therefore different methods and tools are used to identify and place different organisms into groups on the basis of their similarities and differences. Species identification and classification hinges upon the knowledge of taxonomists for whom it's very difficult to identify all the organisms' morphologically accurately requested by the non-taxonomists. To circumvent these difficulties and shortcomings of morphological identification, the project 'DNA Barcode of Life' which is inexpensive, standard and rapid method can be used by non-professionals to identify and characterize organisms (Channa *et al.,* 2018; Jamil *et al.,* 2014; Khan *et al.,* 2015; Shinwari & Shinwari, 2010; Shinwari *et al.,* 2014; Shinwari *et al.,* 2018; Zahra *et al.,* 2016).

DNA barcoding is a novel technique for identification, characterization and classification of biological specimens or species of organisms using a short conserved DNA sequence from either nuclear or organelle genome of an organism. These genetic barcodes can be stored in digital library and used to identify unknown species (Zahra *et al.,* 2016). With the beginning of DNA barcoding there has been a positive impact on identification and biodiversity classification (Hebert & Gregory, 2005). The basic concept behind the DNA barcoding is very simple, that is to discriminate the biological entities by the variation in the molecular markers. DNA barcoding analysis entails the distributions of inter and intra specific variations separated by a distance which is known as "DNA barcoding gap" (Meyer & Paulay, 2005). Initially, the concept was applied to metazoans using cytochrome oxidase 1 (*COX*1); a mitochondrial molecular marker, and later the idea expanded to flowering plants using standard barcode regions such as *rbc*L, *mat*K, *ITS* and *trnH-psbA* (Kress *et al.,* 2005; Hollingsworth *et al.,* 2011).

An ideal standard barcode sequence is still not discovered in plants. Therefore, combination of two or more plastid barcode loci is used for species discrimination. The molecular study of *mat*K and *rbc*L showed that it can identify about 70% of the tea ingredients (Frezal & Leblois, 2008). Following the debate and numerous laboratory researches, various plant barcode combinations were proposed by scientists which are, *rpoC1 + mat*K+ *trnH-psbA* or *rpoC1 + rpoB + mat*K, *rbc*L+ *trnH-psbA, mat*K + *atpF/H* + *trnH-psbA, mat*K + *atpF/H + psbK/I* (Kress & Erickson, 2007).

Systematics of Acanthaceae including species delimitation was carried out by Wood & Scotland (2003). Several different infra-familial classifications and groups have been proposed by different experts of Acanthaceae, but no taxonomic consensus has yet been reached. The Consortium for the Barcode of Life (CBOL) Plant Working Group (2009) officially announced the core barcodes for plant species identification (Anon., 2009). They agreed that chloroplast markers *rbc*L and *mat*K can serve as universal plant barcodes. There is still need to assess additional markers or marker combinations, as this 2 loci combination only has a 72% efficiency to identify plants at the species level (Group *et al*., 2009).

By noting these gaps, the present study aims to use conserved gene sequences of *rbc*L, *mat*K and *trnH-psbA* intergenic spacer to solve phylogenetic issues related to the family Acanthaceae. The previous investigations of Shinwari *et al.,* (2014), Zahra *et al.,* (2016) and Shinwari *et al.,* (2018) have reported the utility of these regions. To test whether the *rbc*L, *mat*K or *trnH-psbA* genes could be used to correctly identify the selected species of genera *Barleria, Strobilanthes, Ruellia, Justicia* and *Dicliptera* to study the evolutionary relationships among the selected species through morphological and molecular analysis, and to investigate the validity of taxonomically difficult and medicinally important plants from the wild.

Materials and Methods

Plant materials: Six plant species of family Acanthaceae were collected during their flowering season from the different regions of Islamabad and district upper Dir, Khyber Pakhtunkhwa (KP), Pakistan. The morphology was studied in detail in the light of available literature and species were identified by comparing with the Flora of Pakistan (Malik & Ghafoor, 1988). Herbarium sheets were prepared and the voucher samples were deposited to the herbarium of Molecular Systematics and Applied Ethnobotany Lab, Quaid-i-Azam University, Islamabad. The detail of the samples is provided in Table 1. The plant material was kept in sealed bags in ultra-freezer (-80°C).

Morphological study: On the basis of 34 macro-morphological characters; a dendrogram was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method by MVSP 3.1 (Multi Variate Statistical Package). These characters along with their key are indicated in Table 4. About 11 macroscopic characters of leaves were studied including quantitative traits like leaf blade width, length and size. Fifteen macroscopic characters of flower and four characters of seed were also studied.

Plant material processing: Genomic DNA was extracted from the freshly-collected plant material while the rest was stored in ultra-freezer. The leaf material was washed with distilled water followed by drying. The leaf material was gently fragmented into small pieces using an already sterilized scissor. 0.3 g of the leaf material was used for DNA extraction using CTAB method (Cetyltrimethylammonium bromide) (Richards *et al.,* 1997).

Polymerase chain reaction: For the amplification of the desired loci, PCR reaction was carried out in 0.5 ml PCR tubes. The PCR reaction for *rbc*L, *mat*K and *trnH-psbA* regions were carried out in PEQSTAR PCR using KAPA3G Plant PCR Kit (KapaBiosystems, Woburn, Massachusetts, USA) (Schori *et al.,* 2013; Shinwari *et al.,* 2014; Zahra *et al.,* 2016; Shinwari *etal.,* 2018). Each reaction contained the KAPA3G Plant PCR Buffer (1× final concentration, includes dNTPs at 0.2 mM each), $MgCl_2$ (1.5 mM final concentration), 1 unit KAPA3G Plant DNA polymerase, primers at a final concentration of 0.3 µM each, template DNA and PCR-grade water to bring the volume to 50 µL. The details of primers are given in Table 3. PCR program profile used for *rbc*L, *mat*K and *trnH-psbA* was inferred from previous work of Shinwari *et al.,* (2014), Zahra *et al.,* (2016) and Shinwari *et al.,* (2018).

PCR Product confirmation: For the confirmation of isolated DNA from all species, gel electrophoresis was performed. The PCR product was run for 35 minutes with a voltage maintaining at 90 volts. The gel was taken out and visualized in gel documentation system under UV light.

Sequencing: The purified amplified samples were sequenced commercially from Macrogen (South Korea). For the sequencing of *mat*K gene and *trnH-psbA*, the same primers were used as were for amplification while in case of the sequencing of *rbc*L gene, a pair of internal and external primers were used (Fay *et al.,* 1998).

Table 1. List and details of the species used in this study.

S. No.	Species	Sample source	Location	Voucher No
1.	*Ruellia brittoniana*	Fresh wild form	Islamabad	MoSAEL-414
2.	*Barleria* sp.	Fresh wild form	Islamabad	MoSAEL-415
3.	*Justicia adhatoda*	Fresh wild form	Islamabad	MoSAEL-416
4.	*Barleria cristata*	Fresh wild form	Islamabad	MoSAEL-417
5.	*Dicliptera roxburghiana*	Fresh wild form	Upper Dir	MoSAEL-418
6.	*Strobilanthes urticifolia*	Fresh wild form	Islamabad	MoSAEL-419

Table 2. Gene bank accession used in this study.

Accession of *mat*K	Accession of *rbc*L	Accession of *trnH-psbA*
GU135009.1	GU135172.1	GU135340.2
KR337262.1	JF265299.1	KR735937.1
KF890175.1	KF890169.1	KR735792.1
KR734969.1	JQ590037.1	KR735637.1
KF890173.1	KF890171.1	KR736015.1
KF890176.1	KF890172.1	KR735896.1
KR734372.1	JQ933303.1	KR735851.1
JF270654.1	L14401.1	KR735936.1
KR734933.1	JQ590035.1	KR736038.1
HQ384510.1	JQ590048.1	KR736025.1
KR735096.1	KF669390.1	KR735665.1
JQ586382.1	HM850082.1	KP271162.1
JQ586398.1	JQ590053.1	GU135437.2
HM850912.1	KF669389.1	
JQ586403.1	KJ594299.1	
KR735182.1	JQ590064.1	
JQ586412.1	KJ773606.1	
KR735174.1	KU569182.1	
JQ589977.1	KF669392.1	
KP093372.1	JQ594959.1	
KJ773100.1	GU135168.1	
JQ586423.1	KJ773849.1	
KR734471.1	JQ590074.1	
JQ586426.1	JQ590079.1	
GU135099.1	JQ590082.1	
KP093336.1	JQ590085.1	
KR735076.1	GU135266.1	
KR735162.1	KJ939250.1	
GU135004.1	JQ933455.1	
	KR337263.1	
	JF265300.1	
	KR861703.1	

Sequence analysis: Sequences were trimmed to create contigs and generation of consensus sequences was done using Geneious 6.1.6 (Biomatters Ltd., Auckland, New Zealand). Sequences were compared to already deposited data in the GenBank (http://www.ncbi.nlm.nih.gov/genbank) using the Mega blast parameter. The percent similarities were observed and the correct identification was related to the highest BLAST percent identity with the query sequence belonging to the expected genera. Ambiguous authentication means that the highest BLAST percent identity for a query sequence was found to match several genera of the expected family while incorrect identification means that the highest BLAST percent identity of the query sequence was not from the expected species, genera or family. Available sequences were downloaded from the GenBank (Table 2) and these selected accessions and our generated sequences were aligned using Clustal W (Thompson *et al.*, 1994). The final alignments were analyzed separately using Geneious 6.1.6 and final consensus phylogenetic trees were developed for *rbc*L, *mat*K and *trnH-psbA*.

Results

Macro-morphological study: A total of 34 characters of taxonomic importance were analyzed for ten species. These characters and their keys are tabulated in Table 4. Moreover, separate analysis on the basis of leaves, flowers and seed characters were performed.

Figure 1a shows the morphological analysis of ten Acanthaceae species. Two major clades are evident. Both the major clades are further divided into two clusters. One cluster separating *Strobilanthes glutinosus* and *Strobilanthes urticifolia* from the rest of the species. While, the other cluster is further divided into six sub clusters, separating *Dicliptera roxburghiana* from all the members of the same main cluster. High level of similarity was observed between *Justicia japonica* and *Justicia peploides*. Likewise, the species belonging to genus *Barleria* appeared to have taxonomic resemblance based on morphological characters. In addition, *Ruellia brittoniana* seemed to be the probable closest sister species of *Justicia adhatoda* and *Justicia brandegeeana* based on morphological study. Our results of leaves character suggest the possible ancestral relationship of *Ruellia brittoniana* to other species. One major cluster produced is further divided into two sub clusters separating *Strobilanthes glutinosus* and *Strobilanthes urticifolia* from the rest. *Justicia brandegeeana* and *Justicia japonica* seems to have high level of similarity based on seed characters. *Justicia adhatoda* seemed to be the probable ancestor of *Justicia brandegeeana* and *Justicia japonica* (Fig. 1b).

The dendrogram based on floral characters divided all studied species in two major clades. The floral characters based dendrogram results showed that *Dicliptera roxburghiana* grouped separately from the rest of the selected species. The second clade was divided into two sub clades. One sub clade was sub-clustered. One sub cluster had both the members of genus *Barleria* because these two had high level floral similarity. Other sub-cluster had both the members of genus *Strobilanthes* showing they were also sister species. The other sub cluster result was little surprising as they suggested that *Justiciapeploides and Justiciaadhatoda*were related species and *Justicia japonica* was evolved earlier from the other two species of genus *Justicia* based on the floral characters. An interesting observation is that *Ruelllia brittoniana* and *Justicia brandegeeana* have similar floral characteristics (Fig. 1c).

The dendrogram of seed characters divided selected species into two main clades. The seed characters based dendrogram results shows that *Justicia brandegeeana*, *Justicia adhatoda* and *Justicia peploides* are sisters species and *Ruellia brittoniana* and *Justicia japonica* evolved earlier from the same group. The second sub-clade contains two species of *Strobilanthes* because they have high level of resemblance (Fig. 1d). One sub clade shows that both the species belonging to genus *Barleria* are sister species, and *Dicliptera* evolved earlier from genus *Barleria* giving controversial results.

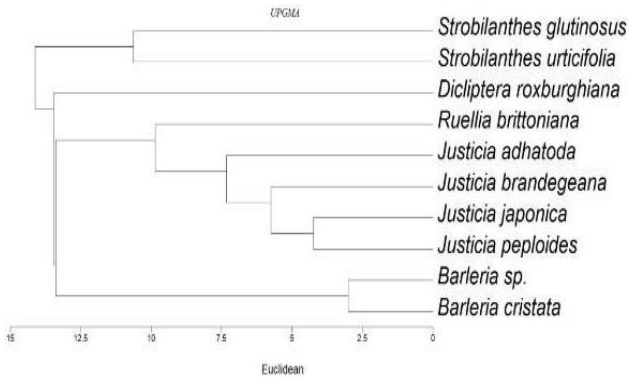

Fig. 1a. Morphology based phylogenetic tree for 34 characters of selected species of Acanthaceae constructed by UPGMA method.

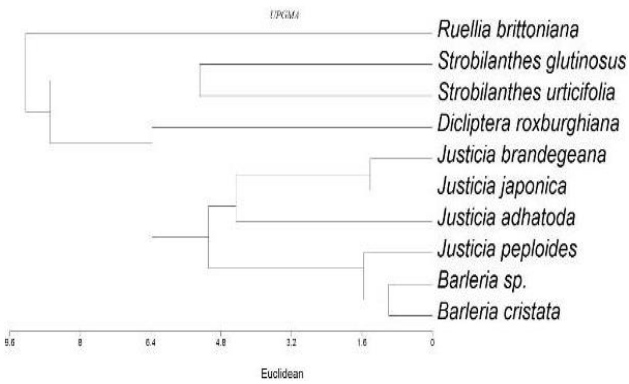

Fig. 1b. Dendrogram of selected species of Acanthaceae based on leaf characters.

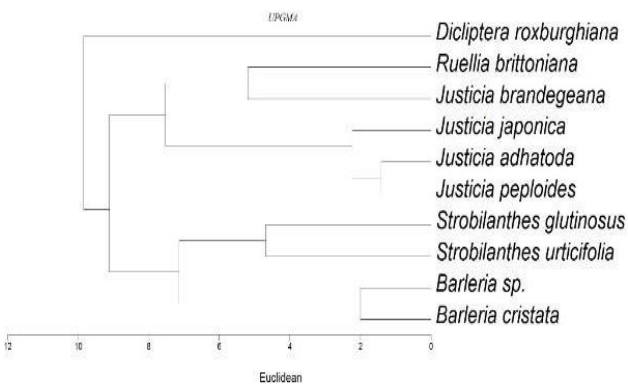

Fig. 1c. Dendrogram of selected species of Acanthaceae based on flowers characters.

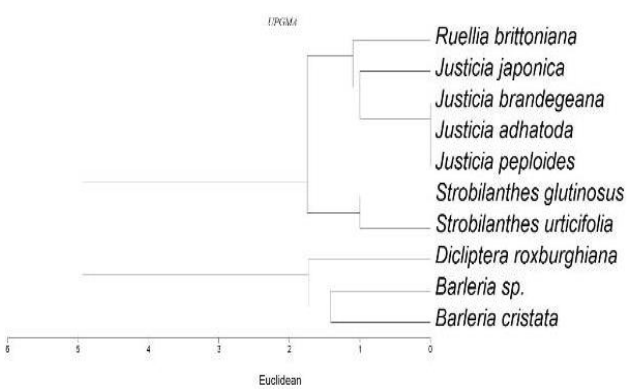

Fig. 1d. Dendrogram of selected species of Acanthaceae based on seed characters.

Molecular analysis: Genomic DNA was successfully isolated from the selected plant species using CTAB method and the clear bands were observed using gel electrophoresis. The genomic DNA was amplified successfully in 50 µL PCR reaction tubes. The expected product size for *rbc*L and *mat*K was found to be around 800 bp and that of *trnH-psbA*product size was 400 bp. The amplification pictures by Gel documentation system for *rbc*L, *mat*K and *trnH-psbA*are shown in Fig. 2a-c.

Sequencing: The purified PCR products were sent for sequencing and yielded good quality of sequences by MACROGEN. The *rbc*L, *mat*K and *trnH-psbA* sequencing of *Ruellia brittoniana, Justicia adhatoda, Barleria cristata, Dicliptera roxburghiana, Strobilanthes urticifolia* and *Barleria* sp. was performed.

Phylogenetic trees: In the present research, we have conducted separate studies on *mat*K, *rbc*L *and trnH-psbA.* On the basis of molecular data of *matK, rbc*L and *trnH-psbA* the phylogenetic trees were constructed using NJ (Neighbor Joining) method by Geneious 6.1.6. These phylogenetic trees are shown in Figs. 3-5. The accessions downloaded from GenBank are tabulated in Table 2.

***matK* analysis:** Figure 3 shows the molecular analysis of 27 *mat*K sequences (Three from the selected species and the rest downloaded from GenBank). Two major clades are evident from the tree. The tree shows that eleven species of genus *Justicia* including one of our selected species are distributed in same clade. *Justicia carnea* make a separate clade from the rest of *Justicia* species, which suggest that the possible ancestral relationship of *Justiciacarnea* to other species of *Justicia*. *Justicia adhatoda* and *Justicia anagalloides* grouped together supported by weak bootstrap (68.5). Similarly, *Diclipteraroxburghiana* and *Justicia diclipteroides* show similarity by a weak bootstrap (59). The second clade is further divided into two sub-clades include all *Barleria* species, while the lower sub-clade includes *Hygrophila polysperma, Strobilanthes dimorphotricha* and these two species of different genera are grouped together by weak bootstrap (79.5) while, all species of *Ruellia* genus are clustered in same sub clade. *Ruellia brittoniana* has close relationship with *Ruellia tweediana* with 83.5 bootstrap score.

***rbc*L analysis:** The molecular tree in figure 4 shows *rbc*L based analysis of thirty-seven sequences (six from the selected species and the rest downloaded from GenBank. Two major clades are produced and each of them is further divided into sub-clades and each sub-clade is further sub-clustered. Thirteen *Justicia* species occur together in same clade however; their monophyly is interrupted by *Peristrophecernua, Dicliptera obanensis* and *Dicliptera roxburghiana*. *Dicliptera obanensis* and *Dicliptera roxburghiana* form sister species pair with *Peristrophecernua* seeming to be the probable ancestor of the two. One sub-cluster grouped all the *Barleria* species including our selected species of the genus. *Petalidium oblongifolium* and *Hygrophila polysperma* form closely related pair while *Strobilanthes dimorphotricha* and *Strobilanthes urticifolia* form sister species pair with *Aechmanthera gossypina* with strong BS support seeming to be the probable closest relative of the two species of *Strobilanthes*. *Eranthemum pulchellum* form sister species relation with *Ruellia* species seeming to be the sister genus of *Ruellia* species.

Table 3. Sequences of primer used in this study.

S. No.	Primers	Sequence	Product size (bp)
1.	*rbc*L 1F	ATGTCACCACAAACAGAAAC	800
2.	*rbc*L724 R	TCGCATGTACCTGCAGTAGCATTCAAGT	800
3.	*mat*K 390F	CGATCTATTCATTCAATATTTC	800
4.	*mat*K 1326R	TCTAGCACACGAAAGTCGAAGT	800
5.	*trnH-psbA* F	GTTATGCATGAACGTAATGCTC	400
6.	*trnH-psbA*R	CGCGCATGGTGGATTCACAATCC	400

Table 4. Macro-morphological characters studied and characters key.

S. No.	Character	Character state and its (Code)
1.	Plant habit	Erect (0), Climbing (1)
2.	Plant nature	Herb (0), Shrub (1), Tree (2), Sub-shrub (3)
3.	Size of plant	≤ 5 cm (0), ≤ 15 cm (1), ≤ 25 cm (2), ≤ 35 cm (3), ≤ 50 cm (4), ≤ 70 cm (5), ≤ 85 cm (6), ≤ 1m (7), ≤ 3 m (8), ≤5 m (9)
4.	Habit	Perennial (0), Biennial (1), Annual (2)
5.	Leaf petiole	Petiolate (0), Sessile (1)
6.	Petiole size	Absent (0), ≤ 1cm (1), ≤ 3 cm (2), ≤ 5 cm (3), ≤ 9 cm (4)
7.	Lamina shape	Elliptic (0), ovate (1), Elliptic-ovate (2), Obvate (3), Elliptic-oblong (4), Linear (5), Lanceolate (6), Oblanceolate (7), Elliptic-lanceolate (8)
8.	Leaf composition	Lobed (0), Simple (1)
9.	Leaf arrangement	Alternate (0), opposite (1), whorled (2)
10.	Leaf margin	Entire (0), Spiny (1), Sinuate (2), Crenate (3), Undulate (4)
11.	Leaf apex	Acute (0), Acuminate (1), Obtuse (2), Acute-acuminate (3)
12.	Leaf indumentum	Glabrate (0), Hairy (1), Spiny (2), Soft (3)
13.	Leaf base	Cuneate (0), Rounded (1), Cordate (2), Truncate (3) Attenuate-obtuse (4)
14.	Leaf blade (Lamina) length	≤ 1 cm (0), ≤ 3 cm (1), ≤ 5 cm (2), ≤ 9 cm (3)
15.	Leaf blade width	≤ 5 mm (0), ≤ 1 cm (1), ≤ 3 cm (2), ≤ 5 cm (3), ≤ 7 cm (4),
16.	Inflorescence	Present (0), Absent (1)
17.	Types of inflorescence	Absent (0), Spike (1), Raceme (2), Panicle (3), Cyme (4)
18.	Bracts of inflorescence or flower	Present (0), Absent (1)
19.	Inflorescence or flower position	Axillary (0), Terminal (1), Both Axillary and Terminal (2)
20.	Flowers length	≤ 1 cm (0), ≤ 3 cm (1), ≤ 5 cm (2), ≤ 9 cm (3)
21.	Corolla color	White (0), Pinkish (1), Purple (2), Blue (3), Violet (4)
22.	Corolla shape	1-lipped (0), Bilabiate (1), Tube(2), Tube-cylindrical (3), Cylindrical below (4), Glandular-pubescent (5)
23.	Corolla tube length	Long (0), Short (1)
24.	Stamens number	Two (0), Four (1)
25.	Stamens length	Exerted (0), Included (1)
26.	Calyx (Sepal) shape	Lobed (0), Linear (1), Hairy (2), Glabrous (3) lobes-linear (4), Ovate-lanceolate (5)
27.	Calyx length	≤ 5 mm (0), ≤ 10 mm (1), ≤ 15 mm (2), ≤ 2 cm
28.	Ovary texture	Hairy (0), Glabrous (1)
29.	Stigma shape	Forked (0), Single (1), lobed (2), Bent (3), Entire (4)
30.	Ovary shape	Oblong (0), Oblong-globular (1), Oblong-conical (2), Bilocular (3), Glandular (4), Oblong-cylindrical (5)
31.	Length of style	≤ 5 mm (0), ≤ 1 cm (1), ≤ 2 cm (2), ≤ 4 cm (3), ≤ 6 cm (4)
32.	Seed shape	Orbiculate (0), Rugos(1), Ovate (2), Ovoid (3), Verrucose (4)
33.	Seed nature	Smooth (0), Rough (1)
34.	Seed texture	Hairy (0), Papillate (1)

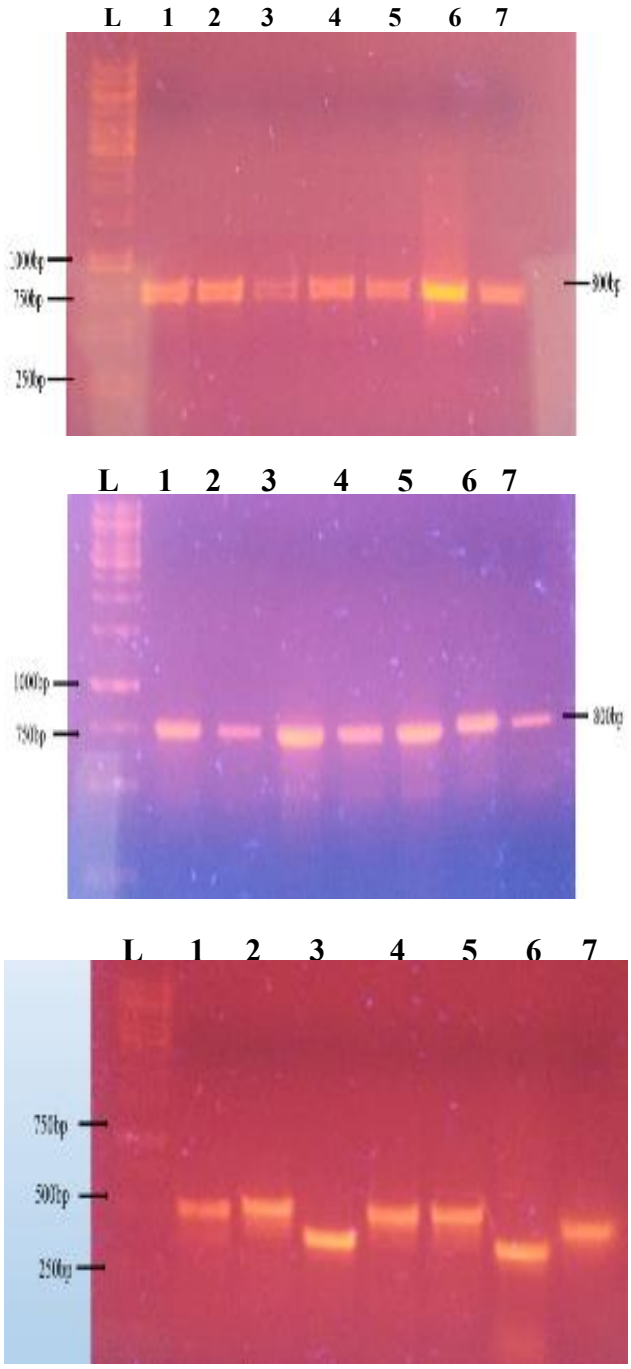

Fig. 2(a-c). PCR products of *rbc*L gene sequences, (b) PCR products of *mat*K gene sequences, (c) PCR products of *trnH-psbA* gene sequences; 1: *Ruellia brittoniana*, 2: *Justiciapeploides*, 3: *Justicia adhatoda*, 4: *Barleria cristata*, 5: *Dicliptera roxburghiana*, 6: *Strobilanthes urticifolia*, 7: *Barleria* sp. Ladder (1kb).

***trnH-psbA* analysis:** In case of *trnH-psbA* phylogenetic tree two major clades are evident. One clade grouped all *Barleria* species together by strong BS score. The other clade is further divided into three sub-clades. *Justicia* species occur together in same sub-clade however their monophyly is interrupted by *Dicliptera roxburghiana*. Molecular analysis of *Barleria* and *Ruellia* based on *trnH-psbA*, showed that both genera are not monophyletic probably because *trnH-psbA* is a spacer region whose proper alignment is a difficult to impossible task which eventually results in such tree shapes (Fig. 5).

Comparison of *matK, rbc*L and *trnH-psbA* analysis: On the basis of *matK* data our analysis indicates that *Justicia adhatoda* and *Justicia anagalloides* are sister species supported by 68.5 BS score while stronger BS support (76) was obtained for *rbc*L based analysis for the same species with *Justicia ventricosa* and incase of *trnH-psbA* based analysis *Justicia adhatoda* form close relationship with *Dicliptera roxburghiana* through weak (58.5) BS support. Our *mat*K analysis shows that *Dicliptera roxburghiana* is positioned in the same clade with *Justicia diclipteroides* having a weak BS value of 59 however, the *rbc*L analysis showed that *Dicliptera roxburghiana* forms a separate sub-clade with *Dicliptera obanensis* supported by a higher BS score of 82, but *trnH-psbA* comparisons did not show the same relationship and *Dicliptera roxburghiana* form cluster with *Justicia* species by 58.5 BS score. *Ruellia brittoniana* and *Ruellia tweediana* showed close relationship to each other when observed for *matK* and *trnH-psbA* but in case of *rbc*L *Ruellia brittoniana* show similarity with *Ruellia geminiflora* by 61 BS value. *matK* analysis of our selected *Barleria* speciesshowed them as sister species but result obtained in case of *trnH-psbA* shows that *Barleria cristata* form a separate cluster with *Ruellia patula* and *Strobilanthes urticifolia*, the possible reason is that *trnH-psbA* is a spacer region and its proper alignment is a difficult task which eventually form an unexpected tree shape. *Strobilanthes urticifolia* and *Strobilanthes dimorphotricha* were strongly supported as sister species and possessed high BS score of 98.5.

Discussion

The present study revolves around two types of phylogenetic analysis of selected species of tribe *Ruellieae* of Acanthaceae. One was based on the macroscopic morphological characters of selected species while other type of study was conducted on the basis of three standard plant DNA barcode regions i.e., *matK*, *rbc*L and *trnH-psbA*. The morphological tree for the selected species was constructed for 34 macro morphological traits by UPGMA method of MVSP. The UPGMA analysis of the selected species shows the evidence of two major clades. One clade represents that *Ruellia brittoniana*, *Justicia adhatoda* and *Barleria cristata* shares a close morphological state and *Dicliptera roxburghiana*seems to be the closest relative of all these species. While the second clade separating *Strobilanthes glutinosus* and *Strobilanthes urticifolia*from the rest of the species because these two showing a close relationship with each other. Interesting relationship has been investigated on the basis of morphology for *Ruellia* and *Justicia*. On the basis of 34 characters' phylogenetic tree; *Ruellia* and *Justicia*are grouped together in a single clade which is also justified from the phylogeny obtained on the basis of flowers and seed characters separately. However, these results are contradicted by the phylogenies obtained through the leaves characters. In Addition, *Ruellia brittoniana* seemed to be the closest ally of *Justicia species* based on 34 combined, flowers and seed morphological characters.

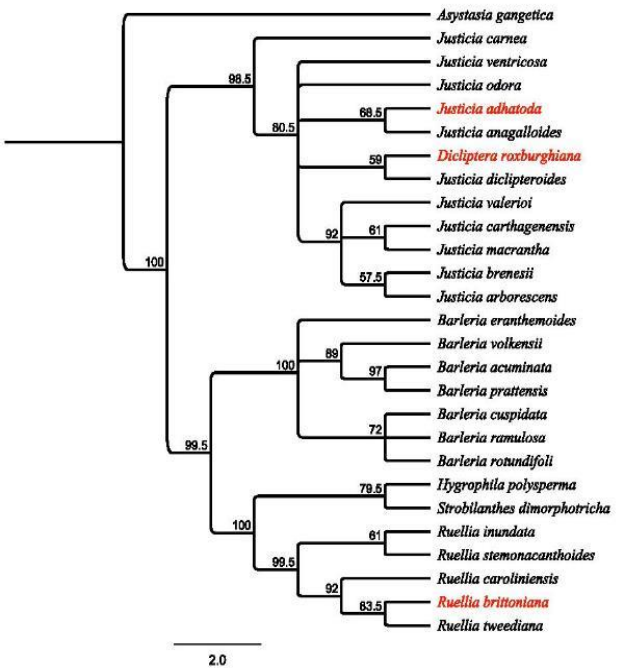

Fig. 3. Neighbor joining tree generated in the present study based on *mat*K gene by using Geneious v6.1. *Asystasia gangetica*is taken as an out group.

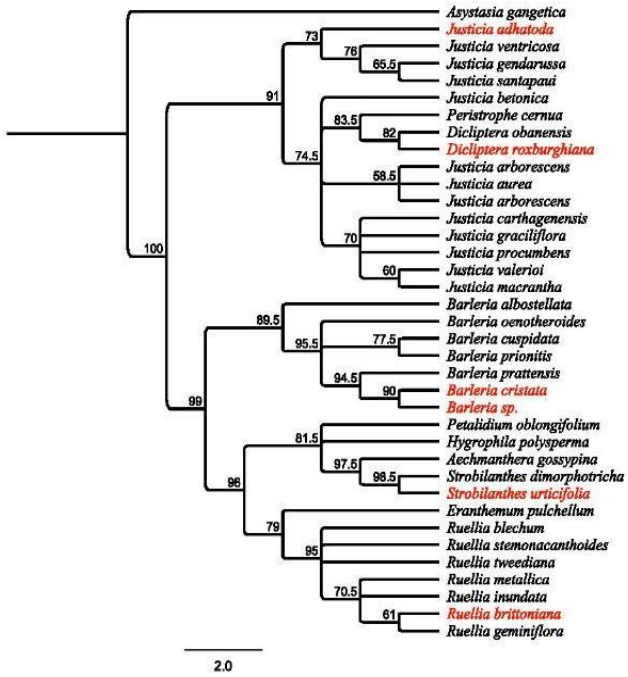

Fig. 4. Neighbor joining tree generated in the present study based on *rbc*L gene by using Geneious v6.1. *Asystasia gangetica*is taken as an out group.

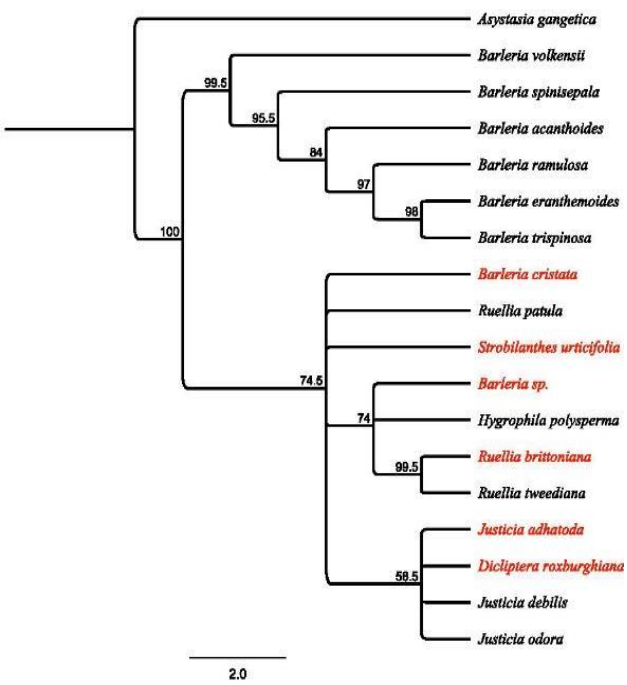

Fig. 5. Neighbor joining tree generated in the present study based on*trnH-psbA*intergenic region by using Geneious v6.1. *Asystasia gangetica*is taken as an out group.

From our study (*mat*K and *rbc*L) *Barleria, Justicia* and *Ruellia* genera are concluded as from same tribe. A more advanced classification of Acanthaceae based on molecular and phylogenetic studies seems to be emerging out. There have been some molecular systematic studies specifically addressing the higher level systematics of Acanthaceae. The genes used for these studies were *rbc*L, *mat*K, *trnH-psbA*, *ndh*F, *trn*L-*trn*F, and *ITS* separately as well as in different combinations (Moylan *et al.,* 2004). The molecular characterizing of medicinally important plant families plays a key role in the identification of new species (Shinwari, 2002; Shinwari, 1998).

Our study for *matK, rbc*L and *trnH-psbA* strongly support the findings of Potkar *et al*., (2013) based on *ITS* sequences and McDade*et al*., (2000) study was based on *trnL-trnF* chloroplast region that *Ruellia* had close relationship with *Hygrophila* supported by 79.5, 96 and 74.5 BS value respectively. Based on *trnH-psbA* sequence data our analysis indicates that *Ruellia brittoniana* and *Ruellia tweediana* are sister species having BS value of 99.5 which is also supported by our *mat*K sequence data having BS (83.5) and *rbc*L sequence data having BS (95).

Our analysis of the *rbcL* gene indicates that both of our selected *Barleria* members are sister species with strong BS score of 90. Which also corroborates with our morphology based tree (Fig. 3). The *rbc*L sequence of these species has no nucleotide difference but this analysis contradicts to *trnH-psbA* analysis where *Barleria cristata* showed its relationship with *Rubellia patula* and *Strobilanthes urticifolia* supported by 74.5 BS value.

According to our *rbc*L and *mat*K trees, genus *Barleria* and *Ruellia* are monophyletic but genus *Justicia* is not monophyletic because *Dicliptera* has been clearly placed among *Justicia* in *rbc*L and *mat*K and *trnH-psbA* based trees which also correlates with the findings of (McDade *et al.,* 2000). In case of *Barleria* and *Ruellia* in *trnH-psbA,* they are not appearing monophyletic probably because *trnH-psbA* is a spacer region whose proper alignment is a difficult to impossible task which eventually results in such tree shapes. The systematic position of *Strobilanthes urticifolia* in our present analysis is ambiguous as it forms strong relationship with *Strobilanthes dimorphotricha* on the basis of *rbc*L sequence data by 98.5 BS score as indicated by (Moylan

et al., 2004) on the basis of *ITS*, *trnL-F* and morphology in which he showed that *Strobilanthinae* are a monophyletic group but our *trnH-psbA*analysis indicates *Strobilanthes* as a possible ancestor of *Rubellia* and *Barleria* supported by 74.5 BS score.

The molecular and morphological systematics techniques bear their own advantages and disadvantages. Phylogenetic studies cannot be dependent just on molecular or morphological investigations but both should be considered in a phylogenetic study. The conflict and in congruence in morphological and molecular studies can be real or spurious. Spurious conflicts might be result of different methods of analysis and differences in assumptions about the evolutionary process. However, if the methods of analysis as well the assumptions are accurate and still there is a conflict, then the conflict is considered as real. There are conflicts observed in our morphological and molecular phylogenies which can be attributed to various factors (Hillis, 1987).

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