

## Molecular profiling of some *Barleria* species using *rbcL*, *matK* gene sequences and RAPD markers

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### Abstract

Many species of *Barleria* genus are used in folkore medicine and the biological effects of their compounds have been validated. Molecular identification of *Barleria* would be a valuable tool for its identification. In the present study, partial gene sequences of *rbcL* and *matK* were amplified and sequenced for four *Barleria* species namely *B. cristata*, *B. prattaensis*, *B. cuspidata* and *B. prionitis*. The divergence patterns of *rbcL*, *matK* gene sequences and their 2-locus combinations revealed discrimination from genera to subgenera level. Further, Randomly Amplified Polymorphic DNA (RAPD) markers were used to assess genetic relationships between the species. The banding pattern and dendrograms constructed showed divergence in all the four species.

**Key words:** *Barleria*, molecular marker, *rbcL*, *matK*, RAPD

### Introduction

*Barleria* is the third largest genus in the family Acanthaceae with 300 species worldwide. The genus has been classified into two subgenera (*Barleria* and *Prionitis*) and 7 sections namely *Barleria*, *Chrysothrix*, *Caviostrata*, *Fissimura*, *Stellatohirta*, *Somalia* and *Prionitis* (Balkwill and Balkwill, 1998). Besides their ornamental value, many *Barleria* species used in folkore medicine have been validated to contain compounds possessing biological effects such as anti-inflammatory, antihepatic (Singh *et al.*, 2004), analgesic, antileukemic, antitumor, antihyperglycemic, anti-amoebic, virucidal (Chen *et al.*, 1998), antioxidant (Ata *et al.*, 2009) and antibiotic activities (Johnson *et al.*, 1996; Kanchanapoom *et al.*, 2004; Kosmulalage *et al.*, 2007).

It is difficult to distinguish *Barleria* species based solely on their vegetative morphologies. Exposing the genetic diversity to augment the morphological traits, make the use of molecular markers an attractive tool for identification. They also have the advantage of being unaffected by environment and detectable in all stages of development.

Comparative analysis of DNA sequences is becoming increasingly important in plant systematics. The plastid-encoded *rbcL* (Chase *et al.*, 1993; Donoghue *et al.*, 1993) and *matK* (Hilu *et al.*, 1997; Johnson *et al.*, 1996) are typical genes used in plant phylogenetic analysis. Recently, the CBoL Plant Working Group recommended a standard plant barcode suggesting the 2-locus combination of *rbcL* and *matK* (Hollingsworth *et al.*, 2011; Li *et al.*, 2011). The combination of *rbcL* and *matK* loci as a barcode was chosen based on the straight forward recovery of the *rbcL* locus and the discriminatory aspect of the *matK* locus while RAPD generates distinguished DNA fingerprints which are widely applied in taxonomy, identification of related species, construction of genetic maps and analysis of genetic relationships among species / populations. RAPD markers are appealing as they reflect both coding and non-coding regions of the genome (Vanijajiva *et al.*, 2005), discriminate at species level, obviate radioactive analysis (Williams *et al.*, 1990) and are comparatively economical.

This background coupled with scanty literature on the genetic analysis of *Barleria* prompted the analysis of *rbcL*, *matK* genes and RAPD for the determination of genetic variation in the four species viz., *B. cristata*, *B. prattensis*, *B. prionitis*, *B. cuspidata* in this study.

### Materials and methods

**Plant materials:** Leaves of *B. cristata*, *B. prattensis*, *B. cuspidata* and *B. prionitis* were collected from different parts of Tamil Nadu and were dried in silica gel. DNA was extracted from dried leaves of all the four species by the method described by Li *et al.* (2010).

**Generation of partial gene sequences of *rbcL* and *matK*:** Experiments of *rbcL* and *matK* were conducted in triplicates. PCR amplifications were carried out by using mastercycler gradient thermal cycler (Eppendorf). Amplification of *rbcL* gene was carried out in 10  $\mu$ L reaction volume containing 1X PCR buffer, 2.5 mM Mg Cl<sub>2</sub>, 200  $\mu$ M each dNTP, 10% trehalose, 10  $\mu$ M of each primer (Kress *et al.*, 2007), 1U Taq polymerase and 50 ng of template DNA. PCR thermocycling parameters were as follows: initial duration at 94°C for 5 min followed by 40 cycles. Each cycle consisted of denaturation at 94°C for 30 sec, primer annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec, with final extension at 72 °C for 7 min.

Amplification of *matK* gene was carried out in 10  $\mu$ L reaction volume containing 1X PCR buffer, 200  $\mu$ M each dNTP, 0.25  $\mu$ g/ $\mu$ L BSA, 10  $\mu$ M of each primer (Tochika *et al.*, 2001; Um *et al.*, 2001), 1U Taq polymerase and 50 ng of template DNA. PCR thermocycling parameters were 94 °C for 4 min; 10 cycles of 94 °C for 30 secs, 52 °C for 30 secs, 72 °C for 1 min; 25 cycles of 88 °C for 30 secs, 48 °C for 30 secs, 72 °C for 1 min, with final extension at 72 °C for 10 min.

The PCR products were separated on 1.5% agarose gel in 1X TBE buffer using ethidium bromide staining and sequenced using an ABI 3730 capillary sequencer. The generated *rbcL* and *matK* sequences were submitted to Gene Bank under the accession numbers KF890169 – KF890176.

Sequences were aligned using ClustalW. Pair-wise evolutionary distance among generated gene sequences was determined by the Kimura 2-Parameter method using the software program MEGA 3.117. Maximum Likelihood (ML) tree was constructed and bootstrap analysis was carried out using 1000 pseudoreplications. *Hibiscus rosa-sinensis* sequences were used as outgroup and they were obtained from NCBI GenBank (Acc.Nos: JX887624 and JQ412251).

**RAPD Analysis:** A set of 37 random decamer primers from various Operon kits (Operon Technologies Inc., Alameda, CA, USA) were used for RAPD amplification. Amplification reactions (10 µL final volume) contained 50 ng of genomic DNA, 0.5 µM 10-mer primer, 10 mM Tris- HCl pH 9.0, 150 mM KCl, 2.5 mM MgCl<sub>2</sub>, 2.5 µM dNTPs and 0.5 units Taq polymerase. Amplification was performed in a programmable thermocycler (Model-PTC100: MJ research Inc., Waltham MA, USA) with 45 cycles of 94 °C for 30 sec, 35 °C for 1 min, and 72 °C for 2 min with an initial denaturation at 95 °C for 5 min and a final extension at 72 °C for 5 min. Then the amplified products were separated electrophoretically in a 1.5% (w/v) agarose gel. The gels were viewed and photographed on UV transilluminator. Reproducible DNA fragments were scored as discrete variables, using 1 for the presence of bands and 0 for the absence of bands. The scored RAPD markers were converted into a binomial (0/1) matrix. All experiments were conducted in triplicate. POPGEN v32 tool (Yeh *et al.*, 1997) was used to calculate Nei and Li's genetic distance and to construct a dendrogram using UPGMA method.

## Results

### Analysis using rbcL, matK and rbcL + matK sequences:

Pair-wise evolutionary distances, using all the 3 markers rbcL, matK genes and 2 locus combination of rbcL + matK, were generated (Table 1-3). Insertions, deletions or stop codons were not observed in any sequence under study. Conserved, Variable and Parsimony informative sites, overall mean divergence,

Table 1. Genetic distances of *Barleria* species based on rbcL gene sequences

	1	2	3	4
1. <i>B. cristata</i>				
2. <i>B. prattensis</i>	0.000			
3. <i>B. cuspidata</i>	0.009	0.009		
4. <i>B. prionitis</i>	0.009	0.009	0.000	
5. <i>Hibiscus rosa-sinensis</i>	0.097	0.097	0.095	0.095

Table 2. Genetic distances of *Barleria* species based on matK gene sequences

	1	2	3	4
1. <i>B. cristata</i>				
2. <i>B. prattensis</i>	0.001			
3. <i>B. cuspidata</i>	0.034	0.033		
4. <i>B. prionitis</i>	0.034	0.033	0.000	
5. <i>Hibiscus rosa-sinensis</i>	0.660	0.664	0.641	0.641

Table 3. Genetic distances of *Barleria* species based on 2- locus (rbcL+matK) combination

	1	2	3	4
1. <i>B. cristata</i>				
2. <i>B. prattensis</i>	0.001			
3. <i>B. cuspidata</i>	0.025	0.027		
4. <i>B. prionitis</i>	0.025	0.027	0.000	
5. <i>Hibiscus rosa-sinensis</i>	1.916	1.914	1.872	1.872

transition/transversion bias and G+C content are shown in Table 4. The transition/transversion ratios and G+C content showed a ranking order correlation in all the three markers, rbcL, matK and their 2 locus combination, respectively. The majority of changes were transitional changes. The genetic distance analysis of all the three markers have not shown divergence at species level but revealed a good variation at subgenera level.

The cluster analysis using these 3 markers indicated that the four *Barleria* species in the present study were grouped into 2 major clusters (Fig. 1-3). First cluster included *B. cristata* and *B. prattensis* and the second cluster *B. cuspidata* and *B. prionitis*. All the clusters were supported by high bootstrap values.

**RAPD analysis:** Operon primers (37 random primers) were used to probe genetic relationship of *B. cristata*, *B. prattensis*, *B. prionitis*, *B. cuspidata* of which 6 primers namely OP-AO18, OP-E1, OP-E4, OP-J19, OP-J20, OP-F12 yielded good and reproducible polymorphic bands. These 6 primers produced a total of 27 DNA fragments of which 26 (96.30%) fragments showed polymorphisms in one or more genotypes (Table 5 and Fig. 4).

The total number of bands for each primer ranged from 2 (OP-E1, OP-AO18) to 6 (OP-J19, OP-F12). The highest number of polymorphisms was observed with primers OP-J19 and OP-F12. The molecular phylogeny tree based on Nei and Li genetic distance (Table 6) of RAPD markers was constructed using UPGMA method (Fig. 5).

Table 4. Overall observations made after the alignment and pairwise genetic analysis of rbcL, matK genes and 2 locus combination of rbcL + matK

Results obtained after alignment and pair wise genetic analysis	rbcL	matK	rbcL+matK combination
Total consensus length	695	825	1562
Conserved sites	643 (92.51 %)	648 (78.54 %)	1295 (82.90%)
Variable sites	52 (7.48 %)	176 (21.33 %)	228 (14.59 %)
Parsimony informative sites	5 (0.71 %)	22 (2.6 %)	27 (1.7 %)
Overall mean divergence	4.2%	76.8%	27.4%
Transition/transversion bias	1.83	1.28	1.36
G+C content	43.786 %	35.4 %	39.076 %

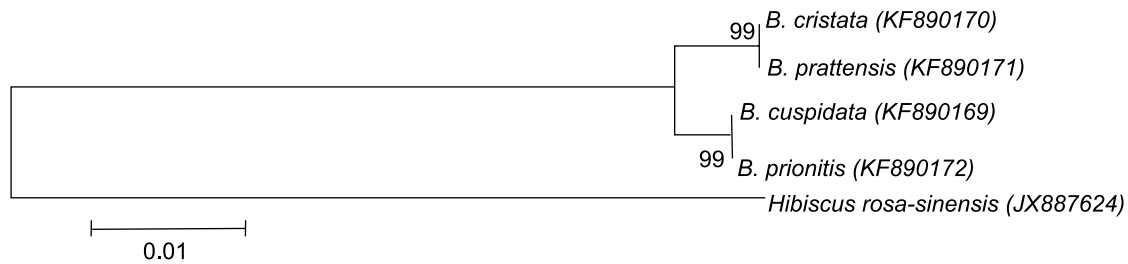
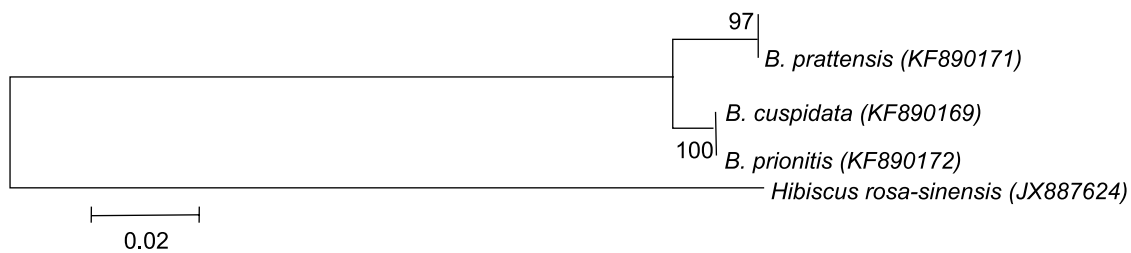
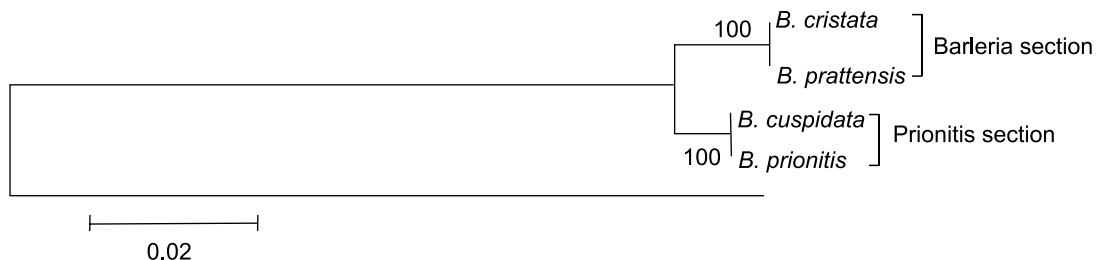
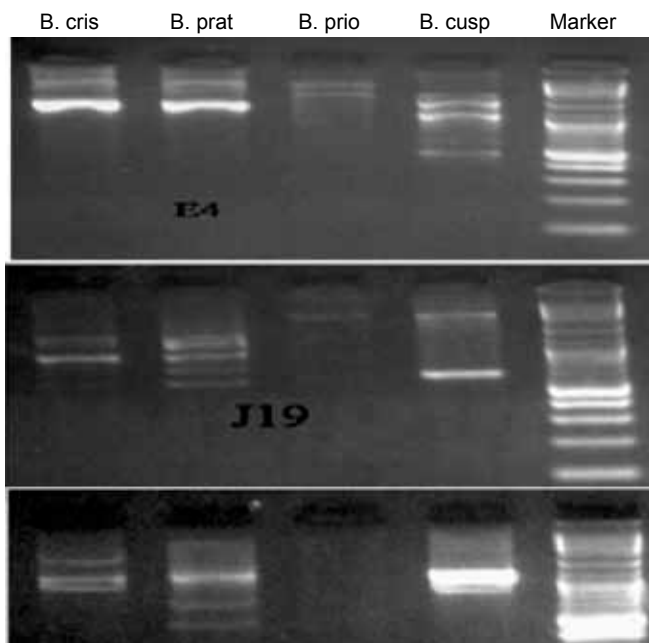
Table 5. Details of the primers producing polymorphic bands.

Sl.No	Primer	Total number of bands	Polymorphic bands
1	OP-AO18	3 (11.11%)	3 (11.53%)
2	OP-E1	2 (7.40%)	2 (7.69%)
3	OP-E4	6 (22.22%)	6 (23.07%)
4	OP-J19	6 (22.22%)	6 (23.07%)
5	OP-J20	4 (14.81%)	3 (11.53%)
6	OP-F12	6 (22.22%)	6 (23.07%)

Table 6. Nei and Li genetic distance

	<i>B. cristata</i>	<i>B. prattensis</i>	<i>B. prionitis</i>	<i>B. cuspidata</i>
<i>B. cristata</i>	****	0.6296	0.5185	0.4815
<i>B. prattensis</i>	0.4626	****	0.3704	0.2593
<i>B. prionitis</i>	0.6568	0.9933	****	0.5185
<i>B. cuspidata</i>	0.7309	1.3499	0.6568	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Fig. 1. Maximum Likelihood Tree constructed using *rbcL* sequencesFig. 2. Maximum Likelihood Tree constructed using *matK* sequencesFig. 3. Maximum Likelihood Tree constructed using 2-locus (*rbcL*+*matK*) combinationFig. 4. RAPD products obtained by PCR amplification of DNA isolated from *B. cristata*, *B. prattensis*, *B. prionitis*, *B. cuspidata*, respectively using random primers OP-E4, OP-J19, OP-F12.

*B. cris* = *B. cristata*; *B. prat* = *B. prattensis*; *B. prio* = *B. prionitis*; *B. cusP* = *B. cuspidata*, Marker = 100bp marker

The value of genetic distance ranged from 0.4626 to 1.3499 while genetic identity ranged from 0.2593 and 0.5185. The cluster analysis grouped the four *Barleria* species into 2 major clusters; *B. cristata* and *B. prattensis* were in one cluster, *B. prionitis* and *B. cuspidata* were in another cluster. Nei's genetic distance of

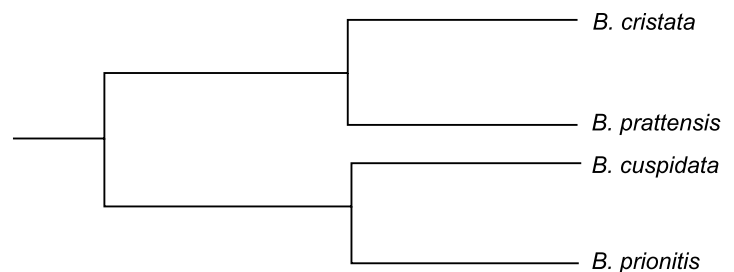


Fig. 5. Dendrogram based on Nei's genetic distance based on UPGMA method

the two sections: *B. cristata* and *B. prattensis* (Barleria section), *B. cuspidata* and *B. prionitis* (Prionitis section) were observed to be 0.4626 and 0.6568, respectively.

## Discussion

This is the first attempt to use molecular markers to investigate genetic relationships of *Barleria* species. The *rbcL*, *matK* and their combinations as markers discriminated *Barleria* to its subgenera level albeit at very low pairwise percentages. The combination of two loci promises a higher universality and the greatest ability to differentiate species rather than the single locus barcode.

Additionally, transition/transversion ratio is high in *rbcL* gene due to high number of transitions and it is less in *matK* gene due to high number of transversions (Oluwatoyin *et al.*, 2009) when compared to *rbcL* gene. This ratio obtained in the present

investigation falls between the expected value of 2.0 which indicates relatively recently diverged sequences but exceeds the value of 0.4 which denotes a highly substitution-saturated sequences (Holmquist, 1983).

The RAPD analysis also confirmed the results as obtained by rbcL, matK and their combination markers. Furthermore, discrimination at species level was also possible using RAPD marker. Thus the RAPD technique is a competent method for identification of *Barleria* species just as successfully as in other medicinal and ornamental plants (De Benedetti *et al.*, 2001; Shinde *et al.*, 2007). Hence the present study may serve as a reference point for future examinations of the genetic variation within populations of *Barleria* species.

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