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Research Paper

Analysis of Phylogeny And Evolutionary Divergence of Mat K Sequence of Barleria acuminata Ness From Pachamalai Hills (A Part of Eastern Ghats, Tamil Nadu, India)

Botany

KEYWORDS : maturase K gene, Barleria acuminata, Pachamalai hills,

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ABSTRACT

Barleria acuminata Ness belongs to the family Acanthaceae which is endemic distribution. Presence of this plant in pachamalai hills a part of Eastern Ghats, located in Tiruchirappalli District of Tamil Nadu, India. The maturase K gene is widely used in systematic studies to resolve divergences at many taxonomic levels. The collected B. acuminata were subjected to sequence analysis of mat K gene and the same was deposited in Gene Bank and an accession number has been assigned for the same (Accession No. KR337262). The data was further analyzed for the construction of neighbor joining tree and to infer the evolutionary divergence among the maximum identical sequences retrieved from NCBI Gene Bank through BLAST search. The results of the search revealed that the mat K sequence of B. acuminata has not been sequenced earlier from any part of this world. It is obvious that the mat K sequence of B. acuminata may be used for the identification of this species reported from any part of the world through BLAST analysis if the identical sequences are submitted to Gene Bank in future.

1. INTRODUCTION

The large and primarily tropical plant family Acanthaceae contains at least 4000 species, placing it among the top 12 or so most diverse families of flowering plants. The group is part Of the order Lamiales, which includes familiar plants such as snapdragons (*Antirrhinum*, Plantaginaceae), mints (Lamiaceae), African violets (Gesneriaceae), and monkey flowers (*Mimulus*, Phyrmaceae). Notably, as a result of the largely tropical distribution of Acanthaceae, species-level diversity (as well as some generic diversity) remains poorly understood, and there is little doubt that many new species remain to be discovered. A wide variety of essential and medicinally active ingredients have been identified and extracted from some members of the family. Many scientists have worked on some members of the family and have discovered some very essential active ingredients present in them [1].

In DNA barcoding, a short DNA sequence is used as a molecular marker for identifying the diversity that exists among plant and animal species. An internal transcribed spacer (ITS) region of nuclear ribosomal cistron is the most commonly used sequence locus for plant molecular systematic investigations [2]. Many chloroplast, mitochondrial and nuclear genes have been utilized for studying sequence variation at genus level. Among these genes *rbcL* gene sequence have been analyzed by various workers to address plant systematics [3]. The *mat* K gene of chloroplast is 1500bp long, located within the intron of the *trn*K and codes for maturase like protein, which is involved in Group II intron splicing. The two exons of the *trn*K gene that flank the *mat* K were lost, leaving the gene intact in the event of splicing. The gene contains high substitution rates within the species and is emerging as potential candidate to study plant systematics and evolution [4]. The *mat* K-*trn*K gene complex is commonly used for plant evolution studies and addresses the solution for various taxonomic levels **[5]**. The *mat* K gene has ideal size, high rate of substitution, large proportion of variation at nucleic acid level at first and second codon position, low transition/transversion ratio and the presence of mutationally conserved sectors. These features of *mat* K gene are exploited to resolve family and species level relationships. Polymorphism of chloroplast DNA especially *trn*K, *mat* K and intergenic *trnL - trnF* regions has been used to study the phylogeny of various plants [6]. The position of *mat* K in the *trn*K gene was determined by comparing with a *mat* K sequence of *Trillium* [7]. This data was used to identify molecular markers, which was used for identifying species of these taxa and also to provide the valuable information for both conventional and molecular plant breeding studies [8].

2. MATERIALS AND METHODS

Pachaimalai hills is situated to the north of Thuraiyur taluk of Tiruchirappalli districts at latitudes 11 ° 09' 00 " to 11 ° 27' 00" N and longitudes 78 ° 28' 00" to 78 ° 49' 00" E and occupy an area of about 527.61 sq. Km. Climate is tropical with temperature ranging between 25 to 30 ° C and a minimum temperature range 12 to 18 ° C and annual rain fall of 800 – 900 mm in the altitude of 1015 MSL. It has dry mixed deciduous forests. The area is marked by the presence of crystalline rocks of the Archaean age comprising gneisses, charnokites and granites with little soil cover of red loamy and black. The crystalline terrain exhibits multispectral and poly metamorphic complexity. According to there are three types of sedimentary rocks in pachaimalai hills based on their period of origin [9]. For the present study Barleria acuminata belonging to Acanthaceae Family were collected. Keeping in view the criteria for the collection, fresh leaves were collected from similar environmental conditions for DNA isolation studies. Young leaves of the respective species were collected in sterile Ziploc bags and stored at -20 °C until further use.

DNA isolation using NucleoSpin® Plant II Kit (Macherey-Nagel) About 100 mg of the tissue is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 is added and vortexed for 1 minute. Ten microlitres of RNase A solution is added and inverted to mix. The homogenate is incubated at 65°C for 10 minutes. The lysate is transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid is collected and the filter is discarded. Four hundred and fifty microlitres of buffer PC is added and mixed well. The solution is transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid is discarded. Four hundred microlitre buffer PW1 is added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid is discarded. Then 700 µl PW2 is added, centrifuged at 11000 x g and flow through liquid is discarded. Finally 200 µl of PW2 is added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column is transferred to a new 1.7 ml tube and 50 µl of buffer PE is added and incubated at 65°C for 5 minutes. The column is then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4°C.

Agarose Gel Electrophoresis for DNA Quality check. The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the

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bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) (Figure 1) PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).Forward primer CGATCTATTCATTCAATATTTC and Reverse primer TCTAGCACACGAAAGTCGAAGT. PCR amplification profile for matK 98ºC, 98ºC, 50ºC, 72ºC, 72ºC, 4ºC

Agarose Gel electrophoresis of PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). Consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37ºC for 15 minutes followed by enzyme inactivation at 80ºC for 15 minutes [10].Sequencing using BigDye Terminator v3.1 sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) [11]. The PCR mix consisted of the following components: PCR Product (ExoSAP treated) - 10-20 ng, Primer - 3.2 pM (either Forward or Reverse) Sequencing Mix - 0.28 µl , 5x Reaction buffer -1.86 µl, Sterile distilled water- make up to 10µl. The sequencing PCR temperature profile consisted of a 1st cycle at 96ºC for 2 minutes followed by 30 cycles at 96ºC for 30 sec, 50ºC for 40 sec and 60ºC for 4 minutes for all the primers.

Post Sequencing PCR Clean up Make master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction. Add 12µl of master mix I to each reaction containing 10µl of reaction contents and are properly mixed. Make master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction. Add 52 µl of master mix II to each reaction. Contents are mixed by inverting. Incubate at room temperature for 30 minutes Spin at 14,000 rpm for 30 minutes decant the supernatant and add 100 µl of 70% ethanol Spin at 14,000 rpm for 20 minutes. Decant the supernatant and repeat 70% ethanol wash Decant the supernatant and air dry the pellet. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1. [12].The sequenced mat K of *B. acuminata* was deposited in Gene Bank (Accession Number: KR337262). Identical sequences of mat K were obtained from Gene Bank using BLAST algorithm and of them the sequences with maximum identity (whose Accession Numbers are: HQ384514, AJ429345, AJ429334, HQ384528, HQ384523, KR734766, KR734991, JF270653, JN114740, KR861702, KF890173, KF890175, JQ586383, JQ586384, F270654, KR734933, KR734783, KF890176, KF890174) Evolutionary relationships of taxa .The evolutionary history was inferred using the Neighbor-Joining method [13]. The optimal tree with the sum of branch length = 20.33665948 is shown. (Next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method [14] and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. There were a total of 697 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [15].

3. RESULTS AND DISCUSSION

The plant *Barleria acuminata* Ness belongs to the family Acanthaceae collected from pachamalai hills. Approximately 700bp DNA was isolated during the quality check through agarose gel electrophoresis method. The gene amplification adopted in the present study yielded enough quantity of DNA for further sequence analysis of mat K. The mat K gene had 898bp and the same sequence had been deposited in the Gene Bank with the Accession Number KR337262. This study provided an opportunity to utilize mat K sequence for identification of this species in future. During the BLAST search no sequence matches for this gene could be identified from databases on plant. Hence it may be concluded that the mat K sequence of *B. acuminata* was a first record for Gene Bank. The estimated value of the shape parameter for the discrete Gamma Distribution is 200.0000. Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (+G) [16]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*]). Mean evolutionary rates in these categories were 0.90, 0.96, 1.00, 1.04, 1.10 substitutions per site. The nucleotide frequencies are A = 28.98%, T/U = 36.66%, C = 17.47%, and G = 16.89%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -14365.642. The analysis involved 20 nucleotide sequences. Clustered sequences showed 125 conserved sites, 1627 variable sites, 1442 parsiminfo sites, 184 singleton sites of 529bp. Further the results revealed that there were 849 zero-fold sites, 70 two-fold sites and 28 four-fold sites. Of the 529bp sites, 697 sites showed 100 % coverage.

The results of NJ analysis of 898bp fragment of the mat K gene belonged to *B. acuminata* with the twenty sequences obtained through BLAST showed different branch lengths in the Phenogram. Maximum identical sequences were not available for *B. acuminata* in this NJ analysis (Fig. 1). Mat K is one of the most rapidly evolving coding regions in the plastid genome but unfortunately poses difficulty in PCR amplification with already existing universal primer sets especially in non-angiosperms contrary to another barcode region ribulose-bisphosphate carboxylase (rbcl) gene which is easy to amplify, sequence and align despite having modest discriminatory power [17]. The evolutionary history was inferred using the NJ method.

The optimal tree with the sum of branch length was 20.33. The tree was drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [18] and were in the units of the number of base substitutions per site. A detailed estimate of evolutionary divergence of mat K sequence of with their similar sequences through BLAST search is provided in Table 1. The results on the distance analysis indicated that the overall average for all species *B. acuminate* was 3.05.The maximum evolutionary distance observed between *Barleria prionitis* and *B. acuminata* was 3.865 (Table 1). Therefore, it is concluded that mat K sequence of *B. acuminata* may be used for the identification of this species reported from any part of the world through BLAST analysis if the identical sequences are submitted to Gene Bank in future.

Fig. 1: Neighbor-Joining (NJ) analysis of mat K sequence of *B. acuminata* with their similar sequences through BLAST search.

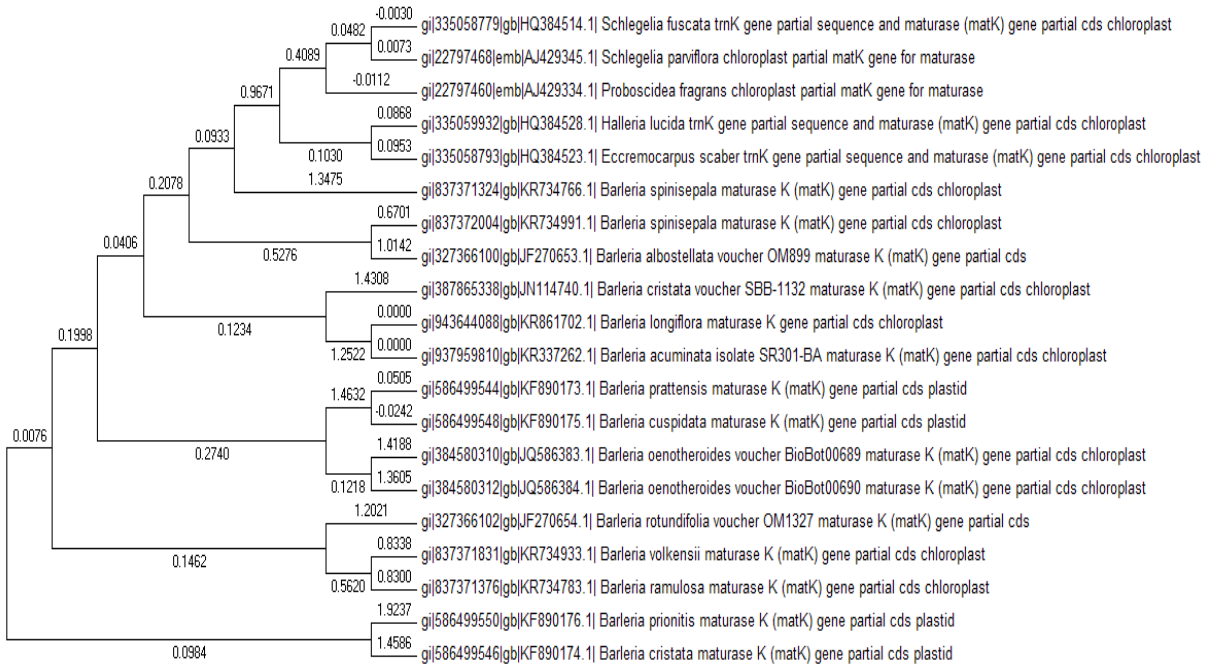

Table 1: Estimate of Evolutionary Divergence of mat K sequence of *B. acuminata* with their similar sequences through BLAST search.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*Barleria logiflora* (KR861702)																				
2	*Barleria acuminate* (KR337262)	0.000																			
3	*Barleria prionitis* (KF890176)	3.865	3.865																		
4	*Barleria prattensis* (KF890173)	2.706	2.706	4.757																	
5	*Barleria cristata* (KF890174)	3.314	3.314	3.382	3.185																
6	*Barleria cuspidate* (KF890175)	2.663	2.663	3.863	0.026	3.260															
7	*Barleria rotundifolia* (JF270654)	3.060	3.060	3.249	3.480	2.800	3.578														
8	*Barleria spinisepala* (KR734991)	2.822	2.822	3.782	2.856	2.911	2.929	3.392													
9	*Barleria albostellata* (JF270653)	2.895	2.895	3.594	4.793	3.872	3.934	3.481	1.684												
10	*Barleria oenotheroides* (JQ586383)	3.320	3.320	3.280	2.915	3.679	3.031	3.414	2.716	3.758											
11	*Barleria volkensii* (KR734933)	2.750	2.750	4.854	3.149	3.005	3.116	1.675	3.189	5.033	4.779										
12	*Barleria ramulosa* (KR734783)	2.949	2.949	2.909	2.736	3.065	2.704	3.516	5.1113	3.396	4.489	1.664									
13	*Barleria cristata* (JN114740)	2.683	2.683	3.612	3.751	3.288	3.785	3.442	2.674	3.403	3.545	2.674	3.433								
14	*Barleria oenotheroides* (JQ586384)	3.573	3.573	4.885	2.981	2.874	3.024	3.061	3.091	2.625	2.779	3.674	3.553	3.029							

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15	*Barleria spinisepala* (KR734766)	3.503	3.503	3.824	3.483	3.345	3.892	2.882	2.854	2.730	3.288	3.399	2.916	2.955	3.442						
16	*Schlegelia fuscata* (HQ384514)	3.667	3.667	3.701	3.410	3.748	3.435	2.913	2.496	2.898	3.515	3.372	3.395	3.181	3.674	2.536					
17	*Proboscidea fragrans* (AJ429334)	3.407	3.407	3.628	3.502	3.725	3.526	2.745	2.663	2.977	3.488	3.345	3.225	2.967	3.533	2.459	0.038				
18	*Schbegelia parviflora* (AJ429345)	3.667	3.667	3.774	3.427	3.748	3.452	2.913	2.551	2.898	3.516	3.388	3.325	3.181	3.662	2.622	0.004	0.040			
19	*Halleria lucida* (HQ384528)	2.676	2.676	3.661	2.928	3.529	2.905	3.117	2.813	2.757	2.729	2.560	2.638	3.506	3.564	2.755	0.487	0.482	0.488		
20	*Eccremocarpus scaber* (HQ384523)	2.243	2.243	3.489	3.642	3.663	3.602	2.595	2.771	2.673	3.073	2.382	2.675	3.286	3.833	2.710	0.758	0.765	0.754	0.182	

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