Journal of
Drug Designing & Bioinformatics

JDDB, 1(1), 8-14, 2023
https://doi.org/10.00000/jddb
ISSN(Print):0000-0000

Research Article

Ascertaining the phylogenetic position of ethnomedicinally important genera *Clerodendrum* using DNA Barcoding

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Article Information:
Received: 19 November, 2023
Revised: 29 January, 2023
Accepted: 30 January, 2023

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Academic Editor:
Prof. Dr. Samir Chtita

Keywords:
Clerodendrum; matK; Rps16; TrnL-F; DNA barcode

ABSTRACT

Clerodendrum is a member of Lamiaceae family; mostly they are perennial shrubs and in Indian Ayurvedic system they are used in many of the herbal preparations. Species of *Clerodendrum* are distributed in tropical regions of Asia including India, Myanmar, Bangladesh, Malayasia, Indonesia, Thailand, Bhutan, Nepal and also in temperate Tibet. Moreover, the medicinal properties of the species are attributed to their phytochemical constituents that are believed to cure varied ailments. Although, ethnobotanically, they have diverse medicinal properties but there is no sufficient information illustrating the comprehensive genetic variation among the different medicinal species of *Clerodendrum*. Hence, an initiative was undertaken to discriminate the genetic variations among 11 important species of *Clerodendrum* through DNA barcoding techniques. The DNA barcode analysis by means of matK, Rps16 and TrnL-F clearly reflected that two subfamily Symphorematoideae and Nepetoideae very close to Ajugoideae which validates the traditional classification of Cronquist.

1. Introduction

In the year 1753, Linnaeus first described the genus *Clerodendrum*, with the identification of *C. infortunatum*. Adanson changed the Latin name "*Clerodendrum*" to its Greek form "*Clerodendron*" in the year 1763; and later Moldenke (1942), changed the Latinized name "*Clerodendrum*", which is currently used for the classification and description of the genus and species [1-4]. In Greek 'Klero' means chance and 'dendron' means tree [5].

Shrivastava and Patel [5] reported that, *Clerodendrum* is a very large and diverse genus with about 580 identified species is distributed throughout the world. But according to 'The Plant List', 701 plants are enlisted under *Clerodendrum* with 327 accepted, 345 synonym, 9 unplaced and 20 unresolved (http://www.theplantlist.org/browse/A/Lamiaceae/ Clerodendrum). Rajendran and Daniel [6] recorded 23 species in India of which 16 were recorded from Arunachal Pradesh by Srivastava and Choudhary [7]. There is some controversy of the genus *Clerodendrum* for its systematic position. Previously, Fletcher [8], Kochummen [9], Liang and Gilbert [10] and Munir [11] placed *Clerodendrum* in the family Verbenaceae but Cantino *et al*. [12] and Harley *et al*. [13] placed *Clerodendrum* under the family Lamiaceae using morphological and molecular phylogenic evidence.

Review of literature showed that there has no sufficient information illustrating the comprehensive genetic variation among the different medicinal species of *Clerodendrum*. Hence, an initiative step was carried out to explore the genetic variations of some species of *Clerodendrum* through DNA fingerprinting techniques.

Subsequently, a new modified molecular technique i.e. DNA barcoding was developed to explore the evolution, genetic relatedness and identification of unknown animal and plants species resolving various anomalies in the taxonomic levels by using a short stretch of DNA sequence [14]. Thus, the main objective of the work is to isolate and sequence the chloroplast matK, TrnL-TrnF and rps16 gene of *Clerodendrum* and the gene sequences were further used to evaluate the phylogenetic relationships of the plant comparing its sequence with the other sequences from subfamilies under Lamiaceae family i.e. Symphorematoideae, Ajugoideae, Prostantheroideae, Nepetoideae, Scutellarioideae and Lamioideae by using the chloroplast matK, TrnL-TrnF and rps16 gene.

2. Materials and methods

2.1. Plant material collection

Selected places covering the two districts in North Bengal and one district in Assam were visited for the collection of eleven different species of *Clerodendrum* (Fig. 1 and Table 1). The plant material was identified by plant taxonomist and the voucher specimen was deposited at the Herbarium of the Botany department.

2.2. DNA extraction, amplification and sequencing

Genomic DNA was isolated according to the protocol developed by Doyle [15]. As phylogenetic markers such as matK, rps16 and trnL-F region were amplified using standard PCR protocols. Primers used for amplification and sequencing are given in Table 2. 25µl of PCR reaction mixture were prepared containing 12.5 µl PCR master Mix (2X), 1.25 µl of each forward and reverse primer (0.25 µM), 8 µl Pyrogen free (PF) water and 2 µl template DNA (25 ng/µl). The PCR reactions were performed on an Applied Biosystems Thermocycler 2720. The amplification cycle consisted of the following specifications: 4 min at 94°C, 30 sec at 48° C, 1 min at 72°C, 35 cycles of 1 min at 94°C, 30 sec at 48°C, 1 min at 72°C, and a final extension time of 7 min at 72°C for the matK intron;

4 min at 94°C, 30 sec at 56°C, 1 min at 72°C, 35 cycles of 45 sec at 94°C, 30 sec at 56°C, 1 min at 72°C, and a final extension time of 15 min at 72°C for the rps16;

5min at 95°C, 45 sec at 54°C, 2 min at 72°C, 35 cycles of 45 sec at 95°C, 45 sec at 54°C, 2 min at 72°C, and a final extension time of 7 min at 72°C for the trnL-F region. The PCR products were sequenced from Chromous

Fig. 1: Flowers and foliages of selected species of *Clerodendrum* used in the present study.

Table 1: Collection sites of 11 different *Clerodendrum* samples.

Name of the plant species	Sample ID	Accession No.	Collection Site	District (State)	Latitude	Longi-tude
Clerodendrum indicum (L.) Kuntze	CL-1	CIL/ NBU/09814	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum inerme (L.) Gaertn. (Syn. *Volkameria inermis* L.)	CL-2	VIL/ NBU/09815	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum japonicum (Thunb.) Sweet	CL-3	CJL/ NBU/09825	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum splendens G. Don	CL-4	CSPL/ NBU/09812	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum speciaosum Dombrain	CL-5	CSPEL/ NBU/09810	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum thomsoniae Balf. f.	CL-6	CTL/ NBU/09828	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum infortunatum L. (Syn. *Clerodendrum viscosum* Vent.)	CL-7	CINL/ NBU/09809	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum serratum (L.) Moon (Syn. *Rotheca serrata* (L.) Steane & Mabb.)	CL-8	CS/NBU/ ASM/1007	Azra, Gu-wahati	Kamrup (Assam)	26º18′ N	91º73′ E
Clerodendrum colebrookianum Walp.	CL-9	CCL/ NBU/09816	Azra, Gu-wahati	Kamrup (Assam)	26º18′ N	91º73′ E
Clerodendrum chinense (Osbeck) Mabb. (Syn. *Clerodendrum fragrans* Willd.)	CL-10	CCHL/ NBU/09855	NBU campus	Darjeeling (West Bengal)	26º42′ N	88º21′ E
Clerodendrum bracteatum Wall. ex Walp.	CL-11	CBL/NBU/ JAL/09877	Lataguri	Jalpaiguri (West Bengal)	26º7′ N	88º77′ E

Biotech Pvt. Ltd, Bangalore for both the forward and reverse primers individually.

Table 2: Primers used for amplification of matK, Rps16 and TrnL-F gene segments.

Primer name	Binding	Primer sequence (5′– 3′)
matK F	Forward	CGATCTATTCATTCAATATTTC
matK R	Reverse	TCATGCACACGAAAGTCGAAGT
Rps16F	Forward	GTGTGTAGAAAGCAAC-GTGCGACTT
Rps16R	Reverse	TCGGGATCGAACATCAATT-GCAAC
TabC	Forward	CGAAATCGGTAGACGCTACG
TabF	Reverse	ATTTGAACTGGTGACACGAG

2.3. Sequence submission in public domain

A total of 29 partial matK, rps16 and trnL-F sequences were submitted online to European Molecular Biology Laboratory (EMBL) nucleotide sequence database (http:\\ www.ebi.ac.uk/embl) with proper annotations and descriptions [definition of the sequence (i.e. the specific region of the genome), source of the sequence (chloroplast DNA in this case; name of the plant species along with its taxonomic position, date and place of collection, tissue type etc.)] after registering to the website.

2.4. Construction of phylogenetic tree

The matK, rps16 and trnL-F region sequences of selected species generated in the present study and the other reference sequences (Table 3) of matK, rps16 and trnL-F regions of different families or subfamilies (six subfamily namely Symphorematoideae, Ajugoideae, Prostantheroideae, Nepetoideae, Scutellarioideae and Lamioideae from Lamiaceae, Acanthaceae) were retrieved from NCBI (National Center for Biotechnology Information) (http:// www.ncbi.nlm.nih.gov) and used to construct phylogenetic tree by means of Molecular Evolutionary Genetics Analysis (MEGA 4.0) [16] software version with neighbour joining (NJ) [17] and UPGMA (Unweighed Pair Group Mean Average) methods after the proper alignment of DNA sequences using CLUSTAL W2 (www.ebi.ac.uk/Tools/clustalw2) and T-Coffee (www.ebi.ac.uk/Tools/t-coffee) software. Parsimony analysis, various clades, transition/ transversion (ns/nv) ratio and variability in different regions were also determined by MEGA 4.0 [16].

Table 3: Taxa, specimens and GenBank accession numbers for sequences used in the present study.

Taxa	Sub-family	matK accession number	TrnL-F accession number	Rps16 accession number
Congea tomentosa	Symphore-matoideae	HQ384499	HQ412929	AJ505411
Clerodendrum serratum (Syn. *Rotheca serrata*)	Ajugoideae	LM651031*	LM651037*	LN832032*
Teucrium chamaedrys	Ajugoideae	KJ204543	KT006827	----------
Ajuga ciliata	Ajugoideae	AF477756	----------	----------
Clerodendrum indicum	Ajugoideae	LM651024*	LM651034*	LN832025*
Clerodendrum japonicum	Ajugoideae	LM651026*	FJ952043	LN832027*
Clerodendrum splendens	Ajugoideae	LM651027*	FJ952027	LN832028*
Clerodendrum speciosum	Ajugoideae	LM651028*	LM651035*	LN832029*
Clerodendrum thomsoniae	Ajugoideae	LM651029*	JN408588	LN832030*
Clerodendrum infortunatum (Syn. *Clerodendrum viscosum*)	Ajugoideae	LM651030*	LM651036*	LN832031*
Clerodendrum colebrookianum	Ajugoideae	LM651032*	LM651038*	LN832033*
Clerodendrum chinens (Syn. *Clerodendrum fragrans*)	Ajugoideae	LN832023*	LN823952*	LN832034*
Clerodendrum bracteatum	Ajugoideae	LN832024*	LN823953*	LN832035*
Clerodendrum inerme (Syn. *Volkameria inermis*)	Ajugoideae	LM651025*	FJ952058	LN832026*
Aegiphila panamensis	Ajugoideae	JQ588060	----------	----------
Westringia rigida	Prostanthe-roideae	HQ911373	HQ911707	HQ911569
Lavandula stoechas	Nepetoideae	JF357833	----------	JQ322781
Isodon melissoides	Nepetoideae	JF954204	FJ593441	FJ593321
Ocimum basilicum	Nepetoideae	KC571817	AY570462	FJ593338
Salvia brandegeei	Nepetoideae	KP852670	KP852896	KP852569
Mentha sp.	Nepetoideae	AY943530	FJ593456	FJ593336
Holmskioldia sanguinea	Scutellarioi-deae	HQ911382	HQ911720	HQ911581
Scutellaria lateriflora	Scutellarioi-deae	HQ593439	----------	----------
Lamium lycium	Lamioideae	KF055082	KF055028	----------
Leonurus japonicus	Lamioideae	EF395815	----------	----------
Pogostemon aquaticus	Lamioideae	KR608468	KR608655	KR608717
Outgroup				
Justicia adhatoda	Acanthaceae	JN228938	KF953921	KP744197

*Present Study (submitted to GeneBank)

3. Results and discussion

3.1. DNA barcoding analysis

DNA barcoding is a novel and innovative technique which can be used to explore the evolution, identification and genetic relatedness of unknown plants and animal species by using a short stretch of DNA sequence [14]. Chloroplast and mitochondrial genes are being recently used to study the sequence variation at generic and species level. The chloroplast genes such as matK, Rps16 and TrnL-F have been utilized by various workers to study the plant evolutionary pattern as well as to resolve various anomalies in the taxonomic levels.

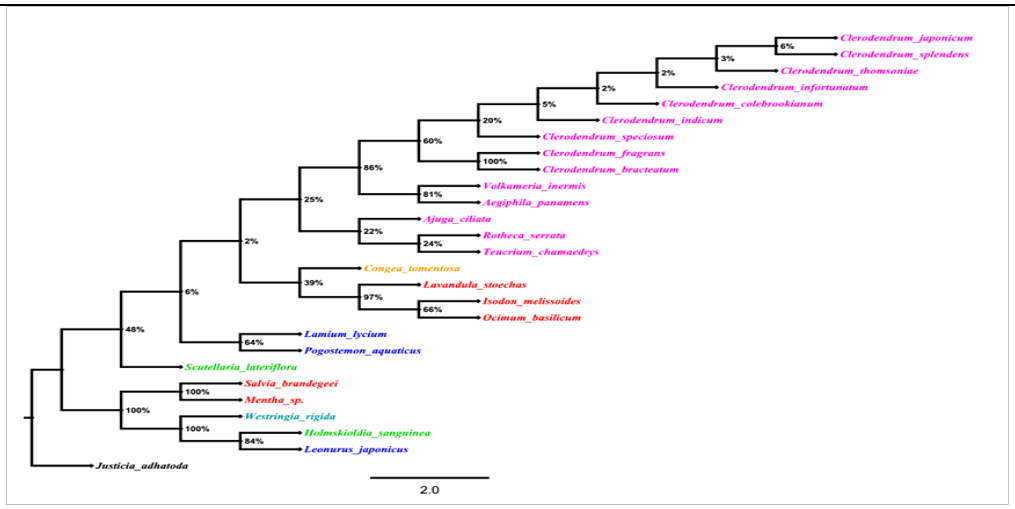

Fig. 2: Most parsimonious tree (neighbour joining method) showing the relationship of matK region of 26 different taxa. Numbers at nodes indicate the bootstrap values.

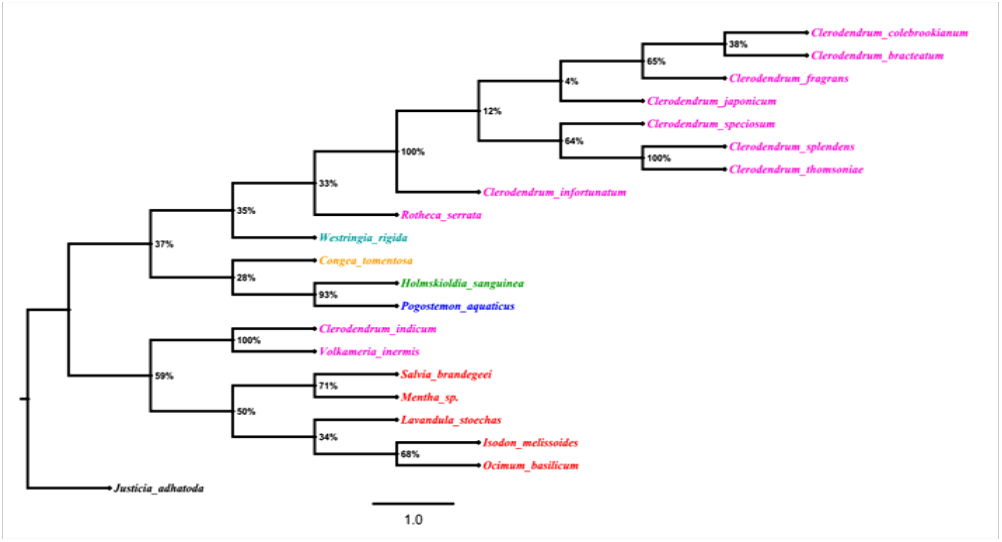

Fig. 3: Most parsimonious tree (neighbour joining method) showing the relationship of Rps16 region of 20 different taxa. Numbers at nodes indicate the bootstrap values.

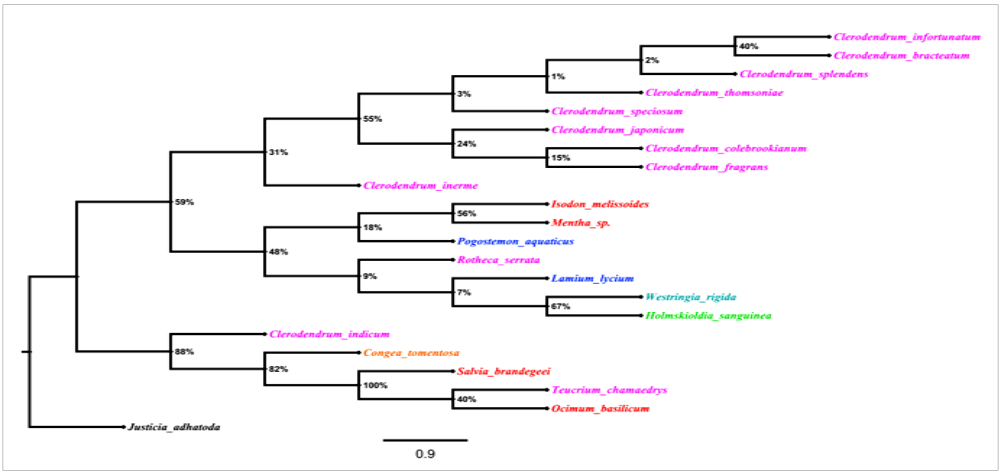

Fig. 4: Most parsimonious tree (neighbour joining method) showing the relationship of TrnL-F region of 21 different taxa. Numbers at nodes indicate the bootstrap values.

3.2. Sequencing of PCR-product and Submission to GenBank

A total of 29 samples (11 matK, 11 Rps16 and 7 TrnL-TrnF) were sequenced from Chromous Biotech Pvt. Ltd, Bangalore for both the forward and reverse primers individually. The sequencing resulted in an average of 810 bp for each reaction. In the present study, the nucleotide BLAST was performed for each of the sequence obtained to find out the homology with the sequences already present in the GenBank. The nucleotide BLAST showed 95 to 100% identity with the Clerodendrum sequence already available in the GenBank.

3.3. Data analysis

DNA barcoding is another kind of taxonomic method that has become a rational approach for identifying million species of plants and animals, based on the analysis of short, standardized and universal DNA regions. Molecular documentation of different taxa and their validated systematic position in the respective family of plant kingdom had always been a challenging task. Chloroplast gene like matK, Rps16 and IGS region like TrnL-F could be essential to resolve this problem. In the present study, a few selected species under the family Lamiaceae (Table 3) were employed to explore inter-generic and intra-generic differences using matK, Rps16 and TrnL-F locus. The phylogenetic analysis (Fig. 2, 3 and 4) of the matK, Rps16 and TrnL-F region revealed a close relationship among the selected taxa. Interestingly, Fig. 2 revealed all the fourteen genera of the subfamily Ajugoideae were appeared together, whereas, it has been found that the two subfamily Symphorematoideae and Nepetoideae very close to Ajugoideae as found in traditional classification [18]. Interestingly, Fig. 3 discloses that out of eleven genera from the subfamily Ajugoideae nine genera were clubbed together and two genera separated out, whereas, five genera of the subfamily Nepetoideae were appeared together and shared more similarities with each other. A similar trend was also observed in Fig. 4 Hence, from the above illustration, it may conclude that DNA barcode serve a reliable genetical approach to place the morphologically similar or dissimilar or disputed taxa into its appropriate systematic position [19,20].

4. Conclusions

In the present study, DNA barcode analysis by means of matK, Rps16 and TrnL-F clearly reflected that two subfamily Symphorematoideae and Nepetoideae very close to Ajugoideae which validates the traditional classification of Cronquist.

Acknowledgments

The author is thankful to Chromous Biotech Pvt. Ltd, Bangalore.

Funding

The present study was not supported by any external funding agencies.

Conflicts of Interest

The authors declare that they have no competing interests.

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