International Journal of Herbal Medicine
Available online at www.florajournal.com

I J H M International Journal of Herbal Medicine

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2016; 4(4): 38-42
Received: 02-05-2016
Accepted: 03-06-2016

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DNA Barcoding of the selected *Artemisia* spp. using the five universal barcodes

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Abstract
The morphology based identification methods are usually time consuming and may sometimes lead to misidentification and may not always provide resolution at the species level. The phenotypic variability of the taxa may lead to misidentifications. In the case of plants, lack of vegetative states, make identification difficult. DNA sequencing has been used to explain evolutionary relationships for more than 20 years in molecular systematics. The aims of DNA barcoding include species identification of known specimens and discovery of unknown species for enhancing taxonomy for the good of science and society. Here, the study focussed on the DNA barcoding of the two species of *Artemisia*, which is reported from Kerala, namely, *Artemisia nilagirica* (C.B. Clarke) Pampan. and *Artemisia japonica* Thunb. using the five universal barcodes namely rbcL, matK, ITS, ITS2 and trnH-psbA. All the five barcodes yield good quality sequences.

Keywords: DNA Barcoding, rbcL, matK, ITS, ITS2, trnH-psbA

1. Introduction
The term ‘‘DNA barcode’’ wasfirst coined by Paul Hebert of University of Guelph in 2003 and they suggested that the 5’ end of cytochrome c oxidase 1 (CO1 or cox I) from the mitochondrial genome was sufficient to generate DNA barcodes for the identification of animals [1]. The DNA barcode is similar to the black stripes of the Universal Product Code that is used to differentiate marketable products. The International Barcode of Life Consortium (http://ibol.org/) was established in 2004, which is an International initiative devoted to develop DNA barcoding as a global standard for the identification of biological species. The aims of DNA barcoding include species identification of known specimens and discovery of unknown species for enhancing taxonomy for the good of science and society [2]. A species can be better identified using DNA barcoding from a small amount of tissue, from seeds or from sterile, juvenile or fragmentary materials, where morphological identification is a failure [3]. DNA barcodes will help in the detection, monitoring and management of biodiversity, which is currently affected by climate change and other man made impacts [4]. A gene region as a DNA barcode, must satisfy three criteria: (*i*) contain significant species-level genetic variability and divergence, (*ii*) possess conserved flanking sites for developing universal PCR primers for wide taxonomic application and (*iii*) have a short sequence length so as to facilitate current capabilities of DNA extraction and amplification [2]. In the case of animals and even in some fungal species, including those of the groups Ascomycota, Basidiomycota and Chytridiomycota, the mitochondrial gene encoding cytochrome C oxidase subunit 1 (CO1), is used as a barcode, because of its variability and universality [5]. The 600bp portion of this gene has sequence divergence among species averaging nearly 11% and provides unambiguous species identification in more than 95% of cases for most of the major animal clades [1, 6]. But the mitochondrial cytochrome oxidase subunit 1(CO1) and other mitochondrial genes is not suitable as DNA barcodes in plants because of their low mutation rate, rapidly changing structure of this genome [7, 8, 9], their low nucleotide substitution rates and shows intra-molecular recombination [10]. In addition, the evolutionary processes such as hybridization and polyploidy are common in plants, so it is difficult to define species boundaries [11, 12]. Thus, screening for single or multiple regions appropriate for DNA barcodingstudies in nuclear and plastid genomes in plants has been animportant focus of research.

2. Materials and Methods
The fresh leaves of *Artemisia nilagirica* and *Artemisia japonica* were used for isolating genomic DNA. The DNA was isolation by using GenElute Plant Genomic DNA Miniprep Kit

(Sigma). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems), using the primers of rbcL, matK, ITS, ITS2 and trnH-psbA. The primer details were given in table 1 and the PCR amplification conditions are given in table 2.

Table 1: The universal primers of rbcL, matK, ITS, ITS2 and trnH-psbA and their sequences

Target	Primer Name	Direction	Sequence (5' → 3')
matK	matK_xf	Forward	TAATTTACGATCAATTCATTC
	matK_MALPR1	Reverse	ACAAGAAAGTCGAAGTAT
rbcL	rbcLa_f	Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL724_rev	Reverse	GTAAAATCAAGTCCACCRCG
ITS	ITS-F5	Forward	AATGGTCCGGTGAAGTGTTC
	ITS-R2	Reverse	CTCGCCGTTACTAGGGGAAT
ITS2	ITS-F3	Forward	CCGTGAACCATCGAGTCTTT
	ITS-R2	Reverse	CTCGCCGTTACTAGGGGAAT
psbA-trnH	psbA3_f	Forward	GTTATGCATGAACGTAATGCTC
	trnHf_05	Reverse	CGCGCATGGTGGATTCACAATCC

Table 2: PCR amplification profiles

***mat*K** 95 °C-5.00 min 95 °C -0.30 min 45 °C - 0.40 min } 10 cycles 72 °C -1.00 min 95 °C-0.30 min 51 °C -0.40 min } 30 cycles 72 °C -1.00 min 72 °C - 7.00 min 4 °C - ∞	***rbc*L** 94 °C -5.00 min 94 °C-0.30 min 55 °C - 0.30 min } 40 cycles 72 °C-0.30 min 72 °C-5.00 min 4 °C -∞
ITS & ITS2 95°C -5.00 min 95°C -0.30 min 58°C -0.30 min } 40 cycles 72 °C -0.30 min 72 °C - 5.00 min 4 °C -∞	***psbA-trnH*** 95°C -5.00 min 95°C -0.30 min 57°C -0.30 min } 40 cycles 72 °C -0.30 min 72 °C -5.00 min 4 °C -∞

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA). 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 v5.6 [13]. The DNA sequences of *Artemisia* spp. under study were subjected to BLAST analysis for better identification at the species level. Sequences obtained were aligned and compared using Multiple Sequence Alignment software program of BioEdit Sequence Alignment Editor [14], CLUSTAL W Multiple Alignment [15]. The dendrogram was constructed using UPGMA method [16], using the five DNA barcodes namely rbcL, matK, ITS, ITS2 and trnH-psbA by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using MEGA 6.0 [17], and a tree was constructed using a combination of rbcL, matK, ITS, ITS2 and trnH-psbA. The evolutionary distances were computed using the number of differences method [18] and are in the units of the number of base differences per sequence. All positions containing gaps were eliminated. All the dendrogram were constructed using the same methodology. Sequences were combined using Geneious software version 8.1.6 [19]. Bar diagrams were constructed to show the sequence variation of different barcodes (rbcL, matK, ITS, ITS2 and trnH-psbA) in plant taxa under study by using pair wise alignment-Global alignment in BioEdit software.

3. Results and Discussion

The accession numbers of the DNA sequences submitted and size of the sequences are given in the table 3. The tree constructed by UPGMA method is given in the fig.1.

Table 3: Table showing the accession number of sequences submitted in the Gen Bank

Plant samples	Place of Collection	rbcL Accession No.	matK Accession No.	ITS Accession No.	ITS2 Accession No.	trnH-psbA Accession No.
A. nilagirica	Munnar	KF589298	KF604887	KP690132	KP856180	KP885707
A. nilagirica	Palakkad	KF639960	KF648716	KP747686	KP856181	KP885708
A. nilagirica	Wayanad	KF664584	KF664585	KP751380	KP856182	KP885709
A. japonica	Munnar	KF476063	KF530805	KP856178	KP856183	KP885710

Table 4: Table showing the size of the sequences

Plant samples	Gene	Size (bp)	Adenine (A) (bp)	Thiamine (T) bp	Guanine (G) bp	Cytosine (C) bp	Others
A. nilagirica- Munnar	rbcL	697	196	212	159	130	-
A. nilagirica-Palakkad		697	196	212	159	130	-
A. nilagirica-Wayanad		697	196	212	159	130	-
A. japonica		697	196	211	160	130	-
A. nilagirica- Munnar	matK	873	257	317	142	157	-
A. nliagirica-Palakkad		873	257	316	143	157	-
A. nilagirica-Wayanad		857	252	311	139	155	-
A. japonica		849	250	307	137	155	-
A. nilagirica- Munnar	ITS	787	175	182	220	210	Y- 5
A. nilagirica-Palakkad		782	182	180	212	208	W-1
A. nilagirica-Wayanad		821	189	187	226	219	W-1 M-1
A. japonica		775	174	177	213	211	Y-3
A. nilagirica- Munnar	ITS2	348	77	78	101	92	Y-4
A. nilagirica-Palakkad		296	61	69	86	80	W-1 M-1
A. nilagirica-Wayanad		351	77	78	101	95	Y-1 R-1
A. japonica		352	72	75	105	100	Y-1
A. nilagirica- Munnar	trnH-psbA	465	144	191	72	58	-
A. nilagirica-Palakkad		485	152	197	76	60	-
A. nilagirica-Wayanad		465	144	190	73	58	-
A. japonica		465	142	192	73	58	-

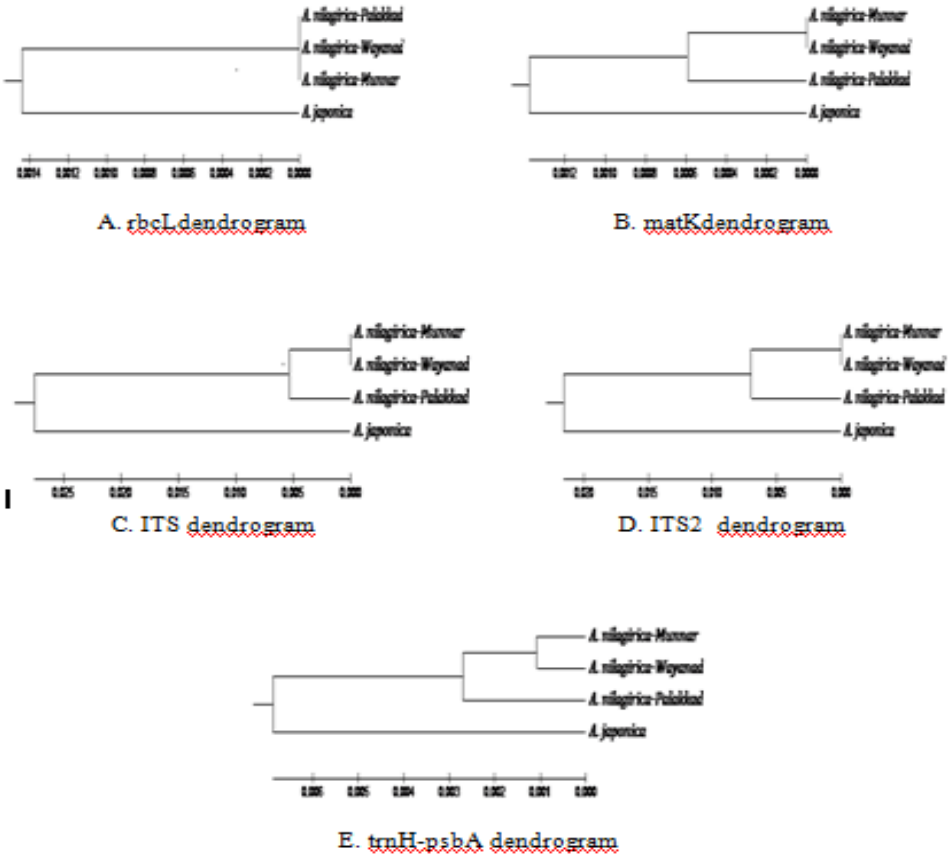

Fig 1: Dendrograms constructed using the five barcodes.

The dendrograms of rbcL, matK, ITS, ITS2 and trnH-psbA clearly showed that *A.nilagirica*- Munnar, Palakkad and Wayanad were in the same clade and *A. japonica* is seen as a distinct clade.

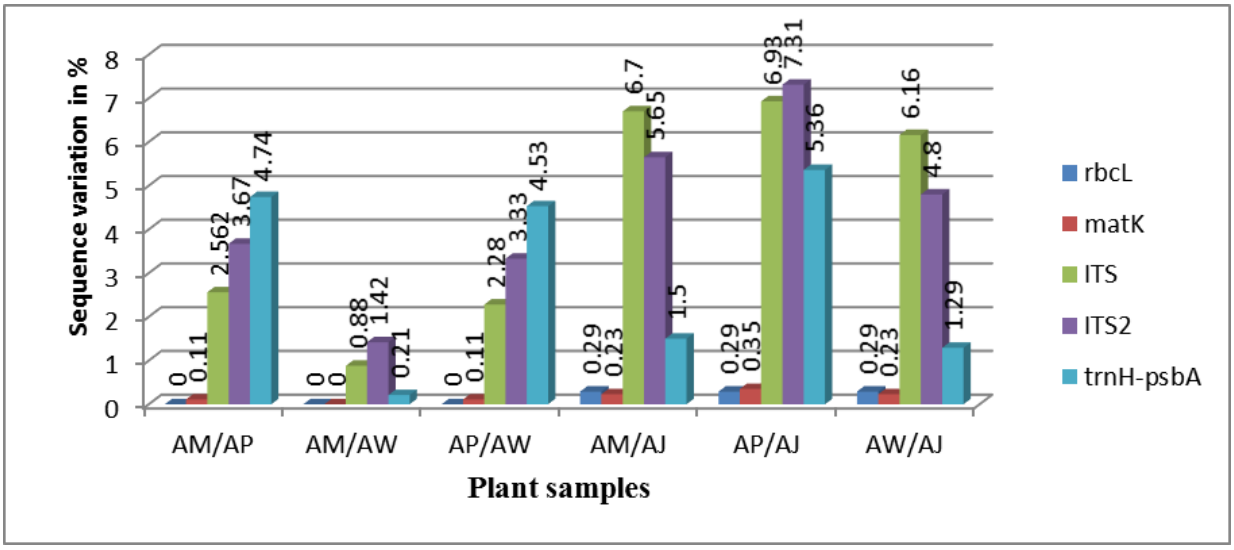

Fig 2: Sequence Variation of rbcL, matK, ITS, ITS2 and trnH-psbA between *Artemisia spp.* AM-*A.nilagirica*-Munnar, AP-*A. nilagirica*-Palakkad, AW-*A. nilagirica*-Wayanad, AJ- *A. japonica*

Bar diagrams (Fig.2) constructed by calculating the pairwise alignment showed variations in sequences based on different DNA markers. The results showed that there is no variation in rbcL gene sequences among *A. nilagirica* from Munnar, Palakkad and Wayanad, whereas, there are slight variations ie. 0.29% with *A. nilagirica* and *A.japonica*. The highest sequence variation in *A. nilagirica* from Munnar, Palakkad and Wayanad is given by trnH-psbAgene, whereas, ITS and ITS2 gene gives good resolution between *A. nilagirica* and *A. japonica*.

trnH-psbA gene gives good sequence variation between *A. nilagirica* and *A. japonica*.

ITS2 is reported [20] to be the most useful barcode in terms of universality, sequence variation and identification capability in Asteraceae family. Out of the five DNA barcodes examined by them, namely rbcL, matK, trnH-psbA, ITS and ITS2, ITS2 is proven as a valuable DNA marker for authenticating the species of the family Asteraceae.

From the results of the dendrogram, ITS2 clearly discriminates *A. nilagirica* from *A. japonica*. Internal Transcribed Spacer (ITS) sequences of nuclear ribosomal DNA (nrDNA) is uesd for phylogenetic study among plant species [21, 22], molecular identification of medicnal plants [23] and DNA barcoding [24]. The nrDNA-ITS region has frequent insertions/ deletions which make them phylogenetically informative [25]. The phylogenetic analysis of 8 different *Artemisia* species based on chloroplast gene RPS11 have been studied [26]. All the seven *Artemisia* species grouped into one cluster, except *A. vulgaris* which was parallel to the other species ie. *A. brevifolia, A. japonica, A. tangutica, A. tournifortiana, A. roxburghiana, A. dubia* and *A. persica.*

5. Acknowledgement

The authors thank Kerala State Council for Science, Technology and Environment for providing research fellowship for the current work.

6. Conclusion

The results of DNA barcoding showed that all barcodes produced good quality sequences. The highest sequence variation is shown by ITS, which is reported to be a feasible barcode in the family Asteraceae. Further studies are required to infer phylogenetic relationships between the species.

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