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## Research Paper

# ITS2 is an ideal barcode to infer the phylogenetic relationships in *Artemisia* spp.

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## Abstract

Molecular studies have shown that many biological species that have accumulated genetic divergence without accompanying morphological variations, cannot be identified using the morphological species concept. So the identification of such morphologically obscure species is a major problem to modern taxonomy. Some organisms undergo intricate developmental life cycles which consists of several morphologically distinct stages. These species have morphological keys that explain adult stages with reference to a single gender. Sometimes, specimens are damaged or incomplete, with only a small portion of tissue for identification which makes morphological determination difficult. In the case of plants, lack of vegetative states, ie. (periods lacking flowers or fruits) make identification difficult. DNA sequencing has been used to explain evolutionary relationships for more than 20 years in molecular systematics. Advancement in molecular biology and DNA sequencing techniques has helped to characterize the genomes of various organisms fastly. Analyses of the DNA sequences of various species provide valuable information about their taxonomy, gene makeup and utilizations. The present study focussed on the phylogenetic analysis of selected *Artemisia* spp. using the sequences retrieved from the GenBank. The phylogenetic tree were constructed using ITS2 gene sequences in Mega software in order to infer the relationships with other *Artemisia* species.

**Keywords:** phylogeny, ITS2, *Artemisia*, Gen bank, DNA sequencing, phylogeny.

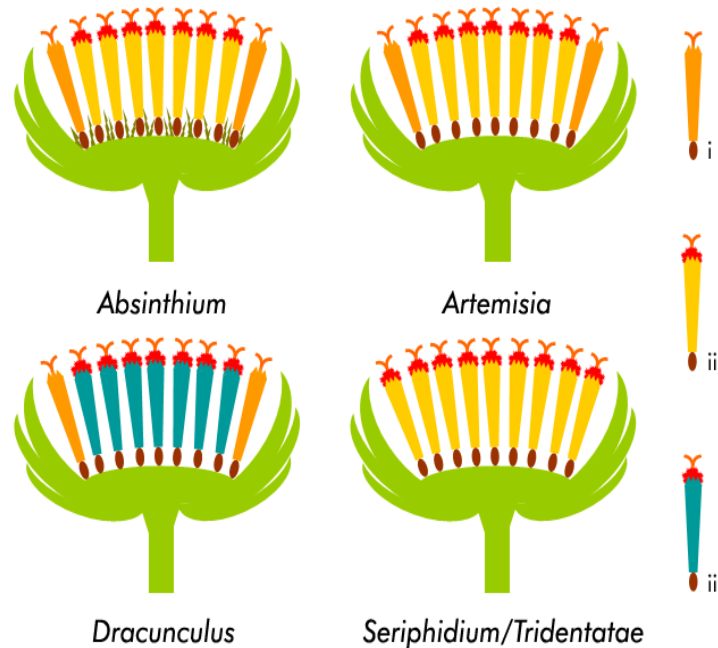
## Introduction

DNA barcoding is an increasingly attractive tool for identification of species in terms of cost, speed and objectivity. Standardized barcodes provide transparent and comparable results, which can be repeated easily, even by a non-taxonomist specialist. It also allows the analysis of poor or fragmented samples of different life stages, and is effective in species discovery<sup>[1,2]</sup>. The analysis of DNA sequences based on PCR amplification and sequencing of the selected molecular markers became common and widely accepted molecular technique for reconstructing phylogeny and taxonomy of plants<sup>[3]</sup>.

The classification of the genus *Artemisia* into various sections is based on the floral structure. The floral characters which differentiate each section is as follows (Figure 1)<sup>[4]</sup>.

- ***Absinthium***: Heterogamous capitula with outer florets female and central florets hermaphrodite and fertile, hairy receptacle.
- ***Artemisia* (= *Abrotanum*)**: Heterogamous capitula with outer florets female and central florets hermaphrodite and fertile, glabrous receptacle.
- ***Dracunculus***: Heterogamous capitula with outer florets female and central florets hermaphrodite but functionally male, glabrous receptacle.

- ***Seriphidium* and *Tridentatae***: Homogamous capitula with all florets hermaphrodite and fertile, glabrous receptacle.



**Figure 1: Diagrams of the flower head structure of each section in *Artemisia*. i) Female florets, ii) hermaphrodite fertile florets, iii) hermaphrodite but functionally male florets<sup>[4]</sup>**

Taxonomically the genus *Artemisia* is a single, large genus of about 500 species<sup>[5-10]</sup>. ITS sequence studies<sup>[9]</sup> for phylogenetic analysis which clearly supports the monophyly of the genus *Artemisia* in its classical broad sense, which includes five major groups, including “*Seriphidium*” and “*Tridentatae*”. *Seriphidium* and *Tridentatae* have different capitula (ie. homogamous), unique planological and carpological features, but their classification within *Artemisia* was supported by the molecular study<sup>[9]</sup>.

Studies<sup>[11]</sup> by the ITS sequencing explained that the structure of *Artemisia* and that of Artemisiinae are not satisfactorily determined. They mentioned that some groups clearly established their similarity within *Artemisia* that reflects the suggestion of an infrageneric classification. Biomolecular analysis<sup>[12]</sup> using ITS sequencing of the genus *Artemisia* gives a clear division in five groups (*Absinthium*, *Artemisia*, *Seriphidium*, *Dracunculus* and *Tridentatae*) based on morphological features.

Studies based on ITS and ETS sequences do not support traditional classification. The placement of some segregate within *Artemisia* denotes shortcomings of the traditional taxonomy and details of character evolution and the major relationships within *Artemisia*<sup>[13]</sup>. Studies based on nuclear ribosomal DNA ETS and ITS sequences showed the combined phylogeny of *Artemisia* and allies, which highly supports the monophyly of the genus *Artemisia* including six of the twelve segregated or related genera considered and also of the opinion that a subgenus *Artemisia*-type taxon (species with heterogamous capitula with all hermaphrodite florets fertile) should be the progenitor of the remaining lineages within *Artemisia*<sup>[14]</sup>. The studies re-investigated the phylogenetic relationships among *Artemisia* species, based on nuclear ITS and chloroplast trnH-psbA DNA markers and suggested the three sections of the genus *Artemisia* namely *Seriphidium*, *Dracunculus* and *Artemisia*<sup>[15]</sup>. They also mentioned that ITS and trnH-psbA sequence data is capable of systematic revision of problematic taxa at intra-genus level in plants. The present study focussed on the phylogenetic analysis using the barcode ITS2 to infer the relationships of the selected *Artemisia* spp. reported from Kerala, ie. *Artemisia nilagirica* and *Artemisia japonica* with the *Artemisia* spp. which are deposited in the Gen Bank.

## Materials and Methods

The fresh leaves from each plant sample were used for isolating genomic DNA. The DNA was isolated 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      | rbclLa_f    | Forward   | ATGTCACCACAAACAGAGACTAAAGC |
|           | rbcl724_rev | Reverse   | GTAATCAAGTCCACCCRCG        |
| ITS       | ITS-F5      | Forward   | AATGGTCCGGTGAAGTGTTTC      |
|           | ITS-R2      | Reverse   | CTCGCCGTTACTAGGGGAAT       |
| ITS2      | ITS-F3      | Forward   | CCGTGAACCATCGAGTCTTT       |
|           | ITS-R2      | Reverse   | CTCGCCGTTACTAGGGGAAT       |
| psbA-trnH | psbA3_f     | Forward   | GTTATGCATGAACGTAATGCTC     |
|           | trnHf_05    | Reverse   | CGCGCATGGTGGATTCAATCC      |

**Table 2: PCR amplification profiles**

|   |   |
|---|---|
| <b><i>matK</i></b>  | <b><i>rbcl</i></b>  |
| 95 °C-5.00 min<br>95 °C -0.30 min<br>45 °C - 0.40 min<br>72 °C -1.00 min<br>} 10 cycles                             | 94 °C -5.00 min<br>94 °C-0.30 min<br>55 °C - 0.30 min<br>72 °C -0.30 min<br>72 °C -5.00 min<br>4 °C -∞<br>} 40 cycles |
| 95 °C-0.30 min<br>51 °C -0.40 min<br>72 °C -1.00 min<br>72 °C - 7.00 min<br>4 °C - ∞<br>} 30 cycles                 |   |
| <b>ITS &amp; ITS2</b>   | <b><i>psbA-trnH</i></b>   |
| 95°C -5.00 min<br>95°C -0.30 min<br>58°C -0.30 min<br>72 °C -0.30 min<br>72 °C - 5.00 min<br>4 °C -∞<br>} 40 cycles | 95°C -5.00 min<br>95°C -0.30 min<br>57°C -0.30 min<br>72 °C -0.30 min<br>72 °C -5.00 min<br>4 °C -∞<br>} 40 cycles    |

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<sup>[16]</sup>. 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<sup>[17]</sup>, CLUSTAL W Multiple Alignment<sup>[18]</sup>. The plant species which represent each sections of the genus *Artemisia* was taken and three outgroups (*Tanacetum vulgare* L., *Achillea millefolium* L. and *Anthemis arvensis* L.) were taken from Anthemideae tribe in order to correctly resolve the phylogenetic tree. The sequences were compared and aligned using CLUSTAL W and edited using BioEdit and gaps were manually adjusted to improve the alignment. The phylogenetic tree was constructed using the Neighbour joining method<sup>[19]</sup> using MEGA 6.0<sup>[20]</sup>. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the

branches <sup>[21]</sup>. The evolutionary distances were computed using the Kimura 2-parameter method <sup>[22]</sup> and are in the units of the number of base substitutions per site.

## Results and Discussion

The accession numbers of the DNA sequences submitted and size of the sequences are given in the table 3 and the details of plant species taken from Gen Bank is given in table 4.

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

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

**Table 4: Details of plant species taken from Gen Bank**

| S. No.                            | Taxon   | Distribution  | Gen Bank<br>Accession<br>No. | Reference |
|-----------------------------------|---|---|------------------------------|-----------|
| <b>Section <i>Artemisia</i></b>   |   |   |                              |           |
| 1                                 | <i>Artemisia vulgaris</i> L.  | Northern hemisphere   | AY180199                     | [12]      |
| 2                                 | <i>Artemisia princeps</i> Pampan.                                     | Japan, Korea  | AY180195                     | [12]      |
| 3                                 | <i>Artemisia genipi</i> Weber.  | Mountains oof Europe  | AY180187                     | [12]      |
| 4                                 | <i>Artemisia verlotiorum</i><br>Lamotte.                              | Eastern Asia, naturalised<br>in Europe  | AF079939                     | [9]       |
| 5                                 | <i>Artemisia annua</i> L.   | Danube Basin, Caucasus,<br>central Asia, eastern<br>Europe and naturalised in<br>western Europe | AF079935                     | [9]       |
| <b>Section <i>Absinthium</i></b>  |   |   |                              |           |
| 6                                 | <i>Artemisia absinthium</i> L.  | Europe, naturalised on<br>North America   | AY180179                     | [12]      |
| 7                                 | <i>Artemisia pedemontana</i><br>Balbis.                               | Southern Europe   | AY180193                     | [12]      |
| 8                                 | <i>Artemisia umbelliformis</i> Lam.                                   | Alps, Appennines and<br>Pyrenees  | AY180197                     | [12]      |
| 9                                 | <i>Artemisia arborescens</i> L.                                       | Mediterranean arca  | AY180181                     | [12]      |
| 10                                | <i>Artemisia glacialis</i> L.   | Western Alps  | AY180189                     | [12]      |
| 11                                | <i>Artemisia rupestris</i> L.   | Europe  | AF061391                     | [7]       |
| <b>Section <i>Dracunculus</i></b> |   |   |                              |           |
| 12                                | <i>Artemisia campestris</i> L.<br>ssp. <i>campestris</i> Briq. & Cav. | Europe, northern and<br>central Asia and North<br>Africa  | AY180183                     | [12]      |
| 13                                | <i>Artemisia japonica</i> Thunb.                                      | South east Asia   | AY180191                     | [12]      |
| 14                                | <i>Artemisia dracunculus</i> L.                                       | Southern and eastern<br>Russia, northern and<br>Central Asia                                    | AF061392                     | [7]       |
| 15                                | <i>Artemisia scoparia</i> Waldst.<br>Kit.                             | Europe, Russia and North<br>Africa  | AF079953                     | [9]       |
| 16                                | <i>Artemisia crithmifolia</i> L.                                      | West coast of Europe  | AF079962                     | [9]       |
| 17                                | <i>Artemisia monosperma</i><br>Delile.                                | North Africa  | AF079951                     | [9]       |

Table continued.....

| S. No.                     | Taxon  | Distribution                          | GenBank Accession No. | Reference |
|----------------------------|--|---------------------------------------|-----------------------|-----------|
| <b>Section Seriphidium</b> |  |                                       |                       |           |
| 18                         | <i>Artemisia caerulescens</i> L.<br>ssp. <i>cretacea</i> (Fiori) Br.-<br>Catt. & gubell. | Italy: Tuscany,<br>Romagna and Marche | AY180185              | [12]      |
| 19                         | <i>Artemisia barrelieri</i> Besser.  | Spain                                 | AF079961              | [9]       |
| 20                         | <i>Artemisia caerulscens</i> L.<br>ssp. <i>caerulescens</i>                              | Mediterranean area of<br>Europe       | AF079960              | [9]       |
| 21                         | <i>Artemisia araxina</i> Takht.  | Armenia                               | AF079959              | [9]       |
| 22                         | <i>Artemisia inculta</i> Delile.   | Egypt                                 | AF079956              | [9]       |
| <b>Section Tridentatae</b> |  |                                       |                       |           |
| 23                         | <i>Artemisia cana</i> Pursch.  | North America                         | AF061377              | [7]       |
| 24                         | <i>Artemisia nova</i> Nelson.  | North America                         | AF079964              | [9]       |
| 25                         | <i>Artemisia tridentata</i> Nutt.  | North America                         | AF061376              | [7]       |
| <b>Outgroups</b>           |  |                                       |                       |           |
| 26                         | <i>Achillea millefolium</i> L.   |                                       | AF155302              | [21]      |
| 27                         | <i>Anthemis arvensis</i> L.  |                                       | AF155303              | [21]      |
| 28                         | <i>Tanacetum vulgare</i> L.  |                                       | AF155279              | [21]      |

The phylogenetic analysis of the ITS2 sequences (Figure 2) constructed by Neighbour joining method revealed six main clades, one of which includes all the out groups (*Tanacetum vulgare*, *Achillea millefolium* and *Anthemis arvensis*), thus clearly separated the various sections of the genus. This phylogenetic analysis reveals the monophyly of *Artemisia* spp.

Of the five clades which represents the sections of the genus *Artemisia*, the first clade consists of the species of section *Artemisia*. This clade include *A. nilagirica*, collected from Munnar and Wayanad.

The second clade consists of the species of the section *Seriphidium*. *A. nilagirica* collected from Munnar and Wayanad is seen close to each other, whereas, *A. nilagirica* from Palakkad is seen as a sister clade to the next section *Tridentatae*. It seems that *A. nilagirica* have a polyphyletic nature which is seen in two clusters. The location may be an influential factor. Studies<sup>(22)</sup> on the Western Ghats revealed that, the Palaghat gap, which is a major low elevation break in the Western Ghats caused intraspecific variation. So further studies are required on *A. nilagirica*-Palakkad for the differences exhibited by them in molecular analysis. *A. annua* (Chinese medicinal herb), a member of the section *Artemisia* is grouped as a separate branch within the section *Seriphidium*, whose members are distributed in Africa and Europe.

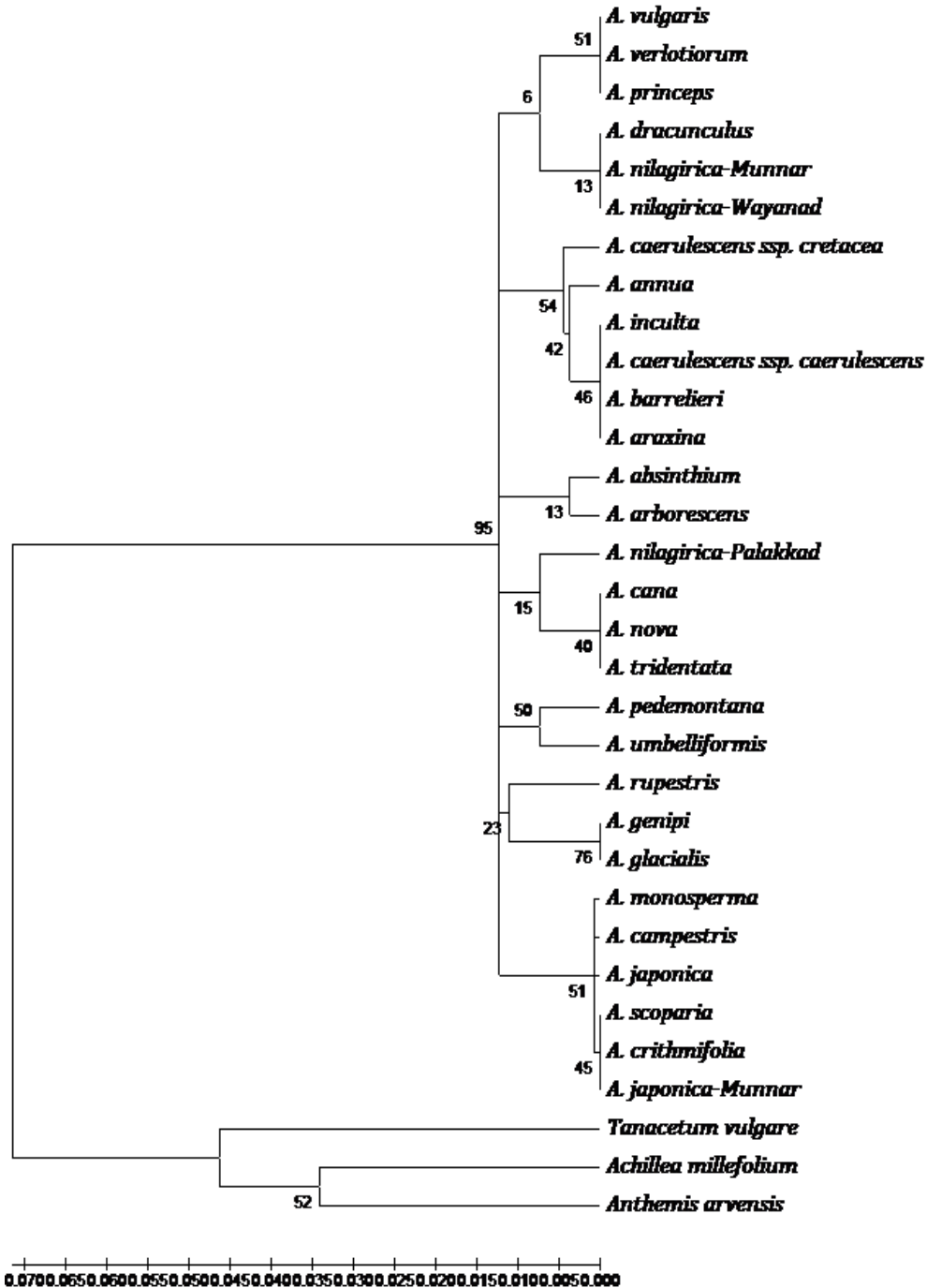
The third clade groups the section *Absinthium* which includes *A. absinthium* and *A. arborescens*.

The fourth clade includes the members of the section *Tridentatae* which include *A. cana*, *A. nova* and *A. tridentata*.

The fifth clade comprised of the section *Absinthium* which include *A. pedemontana* and *A. umbelliformis*.

The next clade included the section *Absinthium* which have *A. rupestris* and *A. glacialis*. *A. genipi*, a member of the section *Artemisia* is seen as a clade next to *A. glacialis*, included in the section *Absinthium* supported by a bootsrap value of 76. Both of the species are European in distribution. The works<sup>(23)</sup> confirms *A. genipi* (which is given to the section *Abrotanum* = *Artemisia* by many authors) to this section.

The seventh clade includes the section *Dracunculus* which includes all the members of this section. It also includes *A. japonica* collected from Munnar which is seen next to the *A. japonica* deposited in the Gen Bank.



**Figure 2: Phylogenetic tree constructed by neighbour joining method using ITS2 sequences**

All the examined species of the section *Seriphidium* are grouped in one clade with comparatively good bootstrap value. The outgroups taken from the tribe Anthemideae, which is most related to the analysed taxa is represented with a bootstrap value of 52.

The present analysis corroborates with the systematic studies on the genus *Artemisia* [12]. The molecular data, the morphological characters and the biogeographical studies are supporting each other and all are necessary for drawing conclusions about the relationships between the taxa. But, some studies [15] reported the phylogenetic relationships among *Artemisia* species based on nuclear ITS and chloroplast psbA-trnH DNA markers and showed that the ITS and trnH-psbA sequence data is capable for systematic revision of problematic taxa at intra-genus level in plants. They confined the genus *Artemisia* into three sections namely, *Artemisia*, *Dracunculus* and *Seriphidium* by their studies.

## Conclusion

The ITS2 sequence analysis of the genus *Artemisia* gives a clear demarcation in five groups according to the classification based on morphological features. The genus *Artemisia* which is represented by around 500 species which span in five different sections *Artemisia*, *Dracunculus*, *Absinthium*, *Seriphidium* and *Tridentata* may represent a taxon with high evolutionary rate.

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