# Publications

**Publication in peer-reviewed journals.**

Das, M. M., P. Mahadani, R. Singh, K. Karmakar and S. K. Ghosh (2013). MatK sequence based plant DNA barcoding failed to identify Bambusa (Family: Poaceae) species from Northeast India. Journal of Environmental and Sociobiology **10**(1): 49-54.

Mahadani, P., M. M. Das, B. Dhar, P. R. Ghosh and S. K. Ghosh (2013). DNA passport of Indian Catmint (Anisomeles indica) from Northeast India. Journal of Environmental and Sociobiology **10**(1): 33-36.

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Das, M. M. and S. K. Ghosh (2014). Evaluation of psbA-trnH spacer as a molecular sequence based marker for determination of Bamboo phylogenetic relationship. (**Accepted in** BIOTECH journal).

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# *MATK* SEQUENCE BASED PLANT DNA BARCODING FAILED TO IDENTIFY *BAMBUSA* (FAMILY: POACEAE) SPECIES FROM NORTHEAST INDIA

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## ABSTRACT

Bamboos, popularly known as poor man's timber for their multipurpose use in the rural life are widely found in tropical and subtropical countries. India, the second largest producer of bamboo in the world has rich diversities of bamboos with about 130 species spreading over 18 genera. We tested the performance of *matK* as a species identifier of selected *Bambusa* species through Basic Local Alignment Search Tool. Genomic DNA from young leaves of selected species were isolated and *matK* gene (~800bp) of chloroplast DNA was amplified and sequenced for species level identification. Among the six determined *matK* sequences, *Bambusa nutan* (JX966234), *Bambusa arundinacea* (JX966235), *Bambusa balcooa* (JX966236) and *Bambusa cacharensis* (JX966237) are novel sequences. The *matK* sequence showed easy amplification and alignment but it showed very low variation and even in some cases no variable and parsimony site were found. These *matK* sequences were shown high similarity (99%-100%) with both inter and intra species. The core DNA barcode loci *matK* failed to provide species specific marker in *Bambusa* due to interspecies hybridization, introgression and polyploidization.

**Key words :** *Bambusa, matK, DNA barcoding, Northeast India, Bambusa arundinacea, Bambusa cacharensis*

## INTRODUCTION

Bamboos, popularly known as poor man's timber for their multipurpose use in the rural life are widely found in tropical and subtropical countries. A total of ~1400 species of bamboos are grouped under the subfamily Bambusoideae within the family Poaceae (Das *et al*., 2008). According to The Plant List (http://www.theplantlist.org/), 509 species names were recorded globally under the genus *Bambusa*, among which 130 are well described. Due to unusually long sexual cycle and late flowering, conventional taxonomic

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characterization of *Bambusa* are severely restricted and confusing (Bhattacharya *et al*., 2006). Other taxonomic parameters of *Bambusa* are also not considered reliable for high variation of environmental factors and need further support for accurate delineation at lower levels with sufficient resolution. Lack of proper molecular tools to resolve species level discrimination of the woody group incites to develop rapid and accurate DNA tags having commercial and conservation importance.

India, the second largest producer of bamboo in the world has rich diversities of bamboos with almost 130 species spreading over 18 genera, of which 78 species are distributed over Northeast India (Hore, 1998; NMBA, 2004). Six species of recognized *Bambusa* from NE India, *viz., Bambusa cacharensis*, *Bambusa balcooa, Bambusa bambos*, *Bambusa arundinacea*, *Bambusa nutans* and *Bambusa tulda* based on their economic and social importance were included in this study to test the effectiveness of *matK* region of chloroplast DNA, widely used for DNA barcoding of plants for accurate delineation of species (Hollingsworth *et al*., 2009).

## MATERIALS AND METHODS

***Sample collection and DNA isolation* :**

Young leaves of selected *Bambusa* species were collected aseptically from different sources in Northeast India (Table 1). About 40 mg wet fresh leaves were homogenized in DNA extraction buffer (50 mM Tris HCl pH 8.0, 25 mM EDTA pH 8.0, and 150 mM NaCl, 2 µL/mL â- mercaptoethanol). Genomic DNA was extracted using Potassium acetate (5M, pH 9.0) followed by phenol: chloroform extraction method (Mahadani *et al*., *2013*).

***PCR amplification and DNA sequencing* :**

PCR amplification of matK region was performed using primers pairs, *matK* X F 5´-TAATTTACGATCAATTCATT-3´, *matK* 5R 5´-GTTCTAGCACAAGAAAGTCG-3´. The PCR reaction of 30 µl mixture contained 20 ng genomic DNA, 20 pmole each primer, 0.2 mM of each dNTPs, 0.5 units of high fidelity *Taq polymerase* enzyme (Applied Biosystem), 1X buffer, and 1.5 mM $MgCl_2$. PCR thermal conditions were 94º C for 3 minutes, 30 cycles at 94º C for 1 minute, 48º C for 45 seconds and 72º C for 45 seconds. The PCR products of the expected size were extracted using QIA quick PCR purification kit (QIAGEN, Cat. No.28704) and sequenced using automated DNA sequencer (ABI 3700).

***Sequence analysis***

Raw traces were manually edited, and both forward and reverse sequences were subsequently aligned to generate targeted sequences. The 3´ and 5´ terminals were clipped to generate consensus sequences for each taxon. The Open Reading Frame (ORF) for *matK* was checked and correct amino acid sequences were determined by online software ORF prediction (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The aligned sequences were corrected manually using BioEdit program (Hall, 1999).

GenBank database was searched using megablast during November-December 2012 with default parameter adjusted to retrieve 5000 sequences. In most of the cases, this corresponded to the sequence with the high BLAST score. In other cases, the closest match was a shorter target with a higher percent identity. Ambiguous bases in target sequence were considered as matching. A similar procedure was not performed using BOLD searches which is less populated with plant DNA barcode sequences.

## RESULTS AND DISCUSSION

Using a single set of primer, readable *matK* barcode sequences (681 bp-843 bp) were recovered from selected *Bambusa* species. In this study, we uncovered six sequences of the *matK* region from the studied specimens. Among them *Bambusa nutan* (JX966234), *Bambusa arundinacea* (JX966235), *Bambusa balcooa* (JX966236) and *Bambusa cacharensis* (JX966237) were determined for the first time and submitted to GenBanK (Table 1). Phylogenetic analysis based on *matK* sequences cannot be performed due to very low and in some cases due to absence of variable and parsimony sites in *matK* of the group.

**Table 1.** List of studie *Bambusa* species and their sample ID, place of collection, latitude and longitude, major uses, Accession Number of sequences submitted to NCBI

| Species name | Sample ID | Place of collection | Latitude and Longitude | Accession No | Major use |
|---|---|---|---|---|---|
| *Bambusa nutans* | AUS-MB04 | Durgakona, Assam | 24.8617N 92.7991E | JX966234* | To supply paper mills |
| *Bambusa arundinacea* | AUS-MB09 | Durgakona, Assam | 24.8617N 92.7991E | JX966235* | Ethnomedicine, raw materials for handicrafts |
| *Bambusa balcooa* | AUS-MB12 | Tripura University, Tripura | 23.7607N 91.2651E | JX966236* | Construction/structural purpose |
| *Bambusa cacharensis* | AUS-MB03 | Tripura University, | 23.7607N 91.2651E | JX966237* | Young shoots are often used in Tripura for preparing delicious soups and pickles; to supply paper mills |
| *Bambusa bambos* | AUS-MB14 | Tripura University, Tripura | 23.7607N 91.2651E | JX966238 | Raw materials for handicrafts |
| *Bambusa tulda* | AUS-MB15 | Tripura University, Tripura | 23.7607N 91.2651E | JX966239 | Fencing materials; to supply paper mills |

* Indicate novel sequence

For each *matK* barcode, BLAST searches of GenBank were performed to match the closest within same species and nearest neighbour (NN) of same or different genus. *matK*

sequence from *Bambusa nutan* (AUS-MB 04) showed 99% identical match with *Bambusa vulgaris, B. ventricosa, B. pachinensis, B. dolichomerithalla, B. oldhamii, B. bambos, B. beecheyana* and *B. tulda.* In case of *Bambusa balcooa* (AUS-MB 12) and *Bambusa arundinacea* (AUS-MB09), barcode sequence showed 100% identity with *B. vulgaris , B. ventricosa, B. pachinensis, B. dolichomerithalla, B. oldhamii, B. bambos, B. beecheyana, B. tulda* and *B. malingensis.* Barcode from *Bambusa cacharensis* (AUS-MB03) showed 100% similarity with *B. vulgaris, B. ventricosa, B. pachinensis, B. dolichomerithalla, B. oldhamii, B. malingensis, B. bambos, B. beecheyana, B. tulda, Dendrocalamus brandisii, D. dumosus, D. sinicus, D. latiflorus, D. asper, D. giganteus* and *D. barbatus*. 99% similarity with *Bambusa bambos* and also with *Bambusa vulgaris, Bambusa ventricosa, etc.* was found against the *matK* barcode sequence of *Bambusa bambos* (AUS-MB14). In case of *Bambusa tulda* (AUS-MP15), 99.9% closest matches were found in same species and 100% with *Dendrocalamus brandisii, D. dumosus, D. sinicus, D. latiflorus, D. asper, D. giganteus* and *D. barbatus* (Fig. 1).

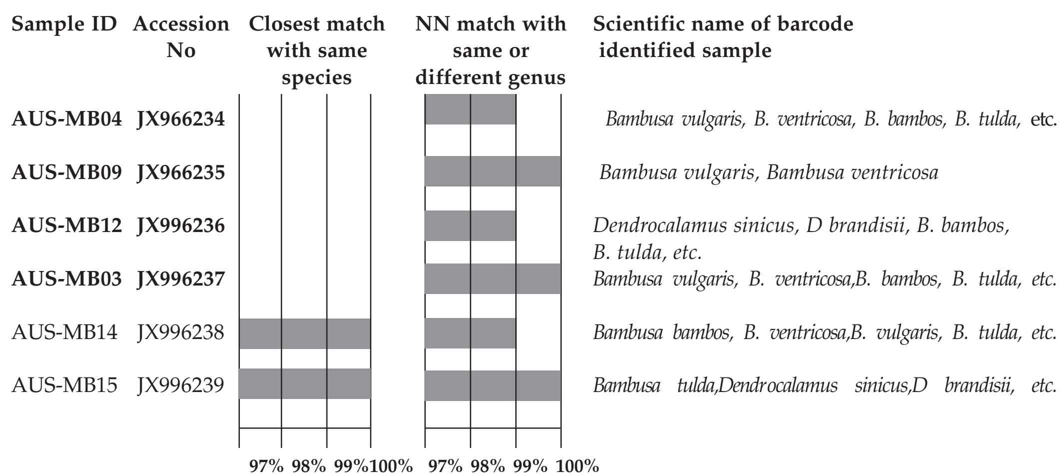


**Fig. 1.** *matK* barcode identification. For each sample, sample ID, graphical representation of match result and identification are shown. Bars depict percentage identity to close match with same species and Nearest Neighbor (NN) in the same or different genus, with scale at bottom

The core DNA barcode loci *matK* failed to provide species specific marker in *Bambusa* though it suitably discriminates species in land plants (Lahaye *et al.*, 2008; Mahadani *et al.*, 2013). The accuracy of DNA barcoding for plant species identification depended to sequence divergences of closely related species (Hollingsworth *et al.*, 2009). Barcode regions are often useful to detect new species, but quite challenging in search of universal loci due to biological complications by interspecies hybridization, introgression and allopolyploidy (Roy *et al.*, 2010). Lack of sufficient polymorphism due to longer generation

time of the woody bamboos probably resulted slower nucleotide substitution rate in *matK* in comparison to other grasses (Gaut *et al*., 1997). Since rates of molecular evolution are linked to the life history of flowering plants (Smith and Donoghue, 2008), this low rate of molecular evolution in *Bambusa* species failed to resolve DNA barcode identification employing *matK*.

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# DNA PASSPORT OF INDIAN CATMINT (*ANISOMELES INDICA)* FROM NORTHEAST INDIA

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## ABSTRACT

The plant *Anisomeles indica* (Lamiaceae) is commonly known as 'Indian Catmint' which is traditionally used as an anticancer and anti-inflammatory and as a source of new anti-metastatic agent for food and pharmaceutical industries. Here, we adopt the plant DNA barcoding technique to develop species level DNA passport for *Anisomeles indica* based on *matK* sequence (~800bp) of chloroplast DNA isolated from *Anisomeles indica* with subsequent amplification and sequencing for species level identification. Our result demonstrated 100% similarity with other *Anisomeles indica* sequences retrieved from global databases. The phylogeny analysis revealed that *Anisomeles indica* formed distinctive clusters with its different closely related species assigning correct taxonomic position. We propose that *matK* gene may be used as DNA passport for medicinal plants.

**Key words :** *Anisomeles indica, DNA passport, matK, sequence homology, species level*

## INTRODUCTION

The plant, *Anisomeles indica* (Lamiaceae), commonly known as 'Indian Catmint' is native to southeast Asia and is distributed throughout India, China, Japan and southwards from Malaysia to Australia. It is traditionally used as an anticancer and anti-inflammatory agent and also considered as a source of new anti-metastatic agent for food and pharmaceutical industries (Liao *et al.*, 2012). Batish *et al.* (2007) reported that *A. indica* holds good promise for use as a natural herbicide for managing weeds. Under the WTO regime for export of medicinal plants, it is important that plants to be exported have precise "passport data" in the way of their authentic characterization using modern biotechnological tools.

A passport is a document issued by a national government, which certifies the identity and nationality for international travel (http://en.wikipedia.org/wiki/Passport). Similarly in biological science, important distinguishable gathered data of samples including their collection sites are called the passport data. The availability of such data could help in

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planning the exploration route in future collection/recollection programmes and deciding ecological regions and analogous agro-climatic conditions where such materials are to be evaluated. Further, passport data are an asset to understand nature, cause and even consequences of variation among plant populations. The passport data are very useful to curators/breeders and other users to draw valid conclusions about the utility of the material. With the implementation of Convention on Biological Diversity (CBD) and patenting of genetic resources, the passport data assume additional significance. To ensure fair benefit sharing and to prevent unwarranted bio piracy of indigenous plant genetic resource it is important to authentically characterize and precisely evaluate the medicinal plant diversity of our country using modern biotechnological tools that allow specific tag in species of the plants and other organisms.

A large number of molecular techniques have been used to authenticate medicinal plants based on species-specific variations in the sequences of various chloroplast and nuclear DNA regions. Using PCR-based methods, species identification has been achieved using DNA that was isolated from fresh and dried plant parts, plant extracts, processed herbal drugs, as well as finished products, such as, herbal teas, tablets and capsules (Hollingsworth *et al*., 2011; Stoeckle *et al*., 2011). In fact, molecular taxonomists now envision cataloging all living species on earth using DNA barcodes and species specific nucleotide sequence of a short DNA fragment. The generation of molecular "barcodes" of medicinal plants and deposition of sequence data in publicly accessible databases will strengthen the concerted effort of the medicinal plant research experts and contribute to the ongoing effort of defining barcodes for every species on earth (Hebert *et al*., 2003). DNA barcode based authentication of medicinal plants is a work in progress that offers powerful new tools and entry points for measures aimed at quality control and quality assurance in medical plant research as well as production, clinical use, and forensic examination of herbal medicine and as species level DNA passport for medicinal plants. Here, we developed species level DNA passport for *Anisomeles indica* based on matK sequence of chloroplast DNA.

## MATERIALS AND METHODS

Young leaves of *Anisomeles indica* (AUS-MP 73) were collected aseptically from southern Assam. For sequencing, total DNA was extracted and amplification of *matK* was obtained from young leaves according to the method described in Mahadani *et al*. (2013). The PCR products of the expected size were extracted using QIA quick PCR purification kit (QIAGEN, Cat. No.28704)(Mahadani *et al*., 2013). The purified PCR products were sequenced both bidirectionally using automated DNA sequencer (ABI 3700).

Raw traces were manually edited and both forward and reverse sequences were subsequently aligned to generate targeted sequences. The 3´ and 5´ terminals were clipped to generate consensus sequences. The aligned sequences were corrected manually and nucleotide compositions were calculated using BioEdit program (Hall 1999). In addition, 12 sequences of *mat*K for same or related taxa of the studied specimen were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The neighbour-joining (NJ) analysis was done in MEGA 4.2 (Tamura *et al*., 2007) to examine phylogenetic relationship. A total of 1000 bootstraps replicates were calculated for the NJ tree construction.

## RESULTS AND DISCUSSION

Successful amplifications of *matK* about 800 bp were obtained from the species. The chromatograms analysis shows no high ambiguities in the sequences. The obtained sequences was deposited in GenBank under Accession No. KC150885. These sequences are matched to 100% similarity when aligned and compared with GenBank *matK* sequences of *Anisomeles indica*. One obtained sequences and the additional 12 *matK* sequences of closely related species were downloaded from the GenBank (Table 1). Total 13 sequences were aligned to yield an equal length of 741bp with no gaps and no indels. The 741bp *matK* sequences had 85 variable sites and 72 parsimony informative sites.

**Table. 1.** List of species and their Accession Number of sequences analyzed in this study.

| Species name | Accesion No |
|---|---|
| *Anisomeles indica* | KC150885* FJ513162 |
| *Betonica officinales* | JN895102, JN895211, JN895213 |
| *Lagopsis supina* | HQ839710 |
| *Phlomis mongolia* | HQ839711 |
| *Pogostemen cablin* | EF529559, FJ513165, HQ839697 |
| *Holochelia longipedunculata* | AF315304 |
| *Leucosceptrum canum* | JF954333, JF954334 |

*indicate the generated sequence from this study

*Anisomeles indica* and its different closely related species formed distinctive clusters. Evidently, the database sequences (FJ513162) and the conspecific generated sequences of *Anisomeles indica* were clustered cohesively. Similarly, all the database sequences of *Betonica officinales, Pogostemen cablin* and *Leucosceptrum canum* were clustered cohesively (Fig. 1). Our study showed that species identification of *Anisomeles indica* was possible

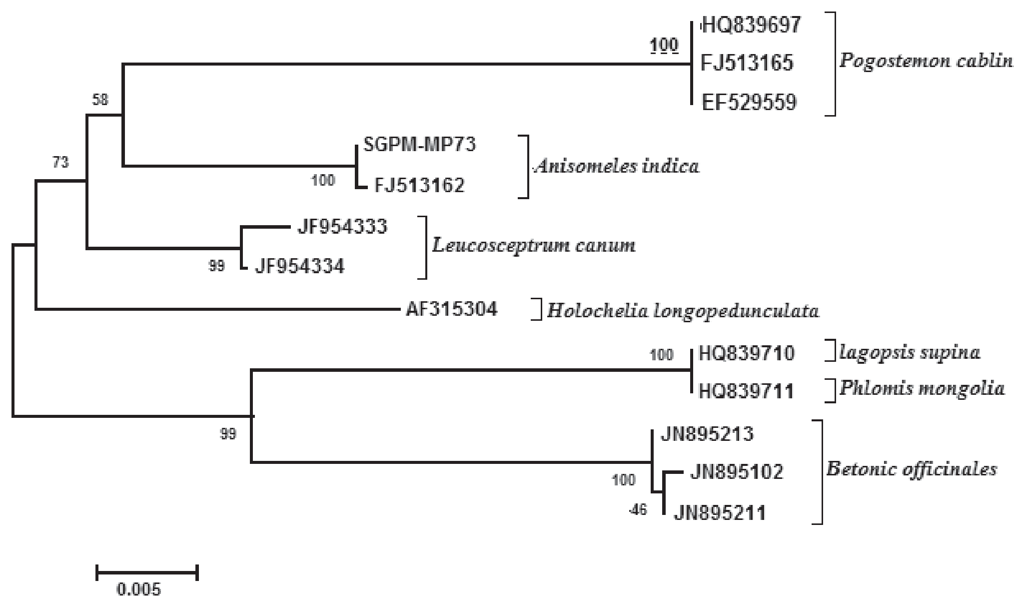


**Fig.1**. Neighbour-joining analysis of Kimura 2-parameter (K2P) distance of *matK* sequences of *Anisomeles indica*. A total of 1000 bootstrap replicates were calculated for the NJ tree construction. Sample ID Accession Nos of *Anisomeles indica* are SGMP-MP73 and KC150885.

using phylogenetic analyses constructed from *matK* sequences. Using of the partial *matK* sequences, which is comparable to that of the full-length sequences, also had species discrimination power. The *matK* gene showed easy amplification and alignment in the analyzed species and high level of discrimination values (Hollingsworth *et al*., 2009). This gene is identified as a universal DNA barcode for flower plants (Lahaye *et al*., 2008). Thus, *matK* gene would be a potential tool in ethnobotany research and the generated sequences may be used as DNA passport for medicinal plants.

## ACKNOWLEDGEMENTS

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# GURUCHARAN COLLEGE

## DEPARTMENT OF BIOTECHNOLOGY

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Date: 21/09/2014

To,

**Mridul Mohan Das,**
Asst. Professor, Dept. of Botany,
Gurucharan College, Silchar.

Sub: **Acceptance Letter**

**Dear Mr. Das,**

It is to inform you that your paper entitled "**Evaluation of *psbA-trnH* spacer as a molecular sequence based marker for determination of Bamboo phylogenetic relationship**" has been accepted for publication in the next issue our journal "BIOTECH" (bearing ISSN: 2230-9098; Vol: 5, Issue No. 1).

Yours sincerely,

**Dr. Bibhas Deb,**
Associate Prof. and Head, Dept. of Botany,
Coordinator, Dept. of Biotechnology,
Editor, BIOTECH
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# Evaluation of *psbA-trnH* spacer as a molecular sequence based marker for determination of Bamboo phylogenetic relationship.

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**Abstract:** The identification of Bamboo on the basis of morphological studies is a very tenuous and rigorous process requiring high level of skill in determining all the characters. An error in 1 or 2 characters may lead to wrong identification. Molecular based markers holds great promise as a tool for correctly determining the phylogenetic relationship in Bamboo. *psbA-trnH* spacer region has proved its capability in determining the phylogenetic relationship in many plant groups. In this study, we evaluate the efficiency *psbA-trnH* region to do the same in Bamboo.

Keywords: *Bamboo, psbA-trnH, MEGA6*

**Introduction:**

Bamboos are members of the sub-family Bambusoideae within the grass family Poaceae. The bambusoid grasses do not comprise only grasses whose culms are lignified, called "woody bamboos" or simply "bamboos", but also of several of grasses with herbaceous culms, called "herbaceous bamboos" or "bamboo allies". The identification of Bamboo on the basis of morphological studies is a very tenuous and rigorous process requiring high level of skill in determining all the characters. An error in 1 or 2 characters may lead to wrong identification (Stapleton 1997). Traditional plant taxonomy is highly skewed towards the floral characters as deterministic of plant identification. But in case of Bamboo, flowering is a rare occurrence and some species have never been reported to have flowered. Many important characters in Bamboo are influenced by environmental factors and thus provide erroneous identification.

Different molecular marker based techniques have been used to identify the different members of the bamboo group. Friar and Kochert (1991, 1994) used restriction fragment length polymorphism (RFLP) technique for phylogeny assessment of 61 accessions and 20 species of Phyllostachys but it proved to be inconclusive. Amplified fragment length polymorphism (AFLP) has also been used to understand the Bamboo phylogenetic relationship by Loh, Kiew et al. (2000). Unique banding patterns were obtained in 13 out of 15 species and the cluster pattern helped reveal the polyphyletic nature of the genus

Bambusa. Sequence characterized amplified regions (SCARs) is an extension of the RAPD procedure and this technique was in an attempt to make species specific markers in bamboos by Das, Bhattacharya et al. (2005).

Apart from the molecular markers, DNA sequence based techniques has been used in determining the phylogenetic relationship and species identification. Different plant chloroplast regions have been studied in different plant groups with varied success. Newmaster, Fazekas et al. (2006) used *rbcL* for land plants. Lahaye, van der Bank et al. (2008) proposed the use of *matK* as a DNA Barcode for plants.

**Methodology:**

In the present study, *psbA-trnH* intergenic spacer region is being evaluated for its efficiency to determine the phylogenetic relationship in Bamboo. 56 *psbA-trnH* intergenic spacer region sequences were downloaded from the NCBI database for the study. The Accession Numbers of the sequences are clearly written at the beginning of each entry of the Phylogenetic tree.

The sequences were saved together in a fasta file. The phylogenetic studies were performed using the molecular evolutionary genetic analysis (MEGA6) software in accordance with the Kimura 2-Parameter (K2P) model. DNA Barcoding sequences were analyzed by using the phylogenetic tree reconstruction methods such as Neighbor-joining (NJ) which is a heuristic method for estimating the minimum evolution tree originally developed by Saitou and Nei (1987) and modified by Studier and Keppler (1988).

**Result and Discussion:**

The phylogenetic tree given below clearly shows that DNA sequence of *psbA-trnH* intergenic spacer region has partial capability in correctly determining the phylogenetic relationship and species identification in Bamboo. In the area marked in rectangle, it is seen that the Genus *Bambusa* and *Gigantochloa* has been put together in the same clade, whereas it should have been in different clades. Again in the same rectangle, it is seen that Genus *Bambusa* and *Dendrocalamopsis* has been put together in the same clade which is wrong. Looking at the rounded rectangle below, it again shows genus *Melocanna* and *Schizostachyum* in the same clade which is incorrect. A clade indicates that the entities share a common ancestor and hierarchy. But looking at the other sequences which has been correctly placed (marked in oval); we can easily see that in some cases *psbA-trnH* intergenic spacer region is incapable of phylogenetic elucidation.

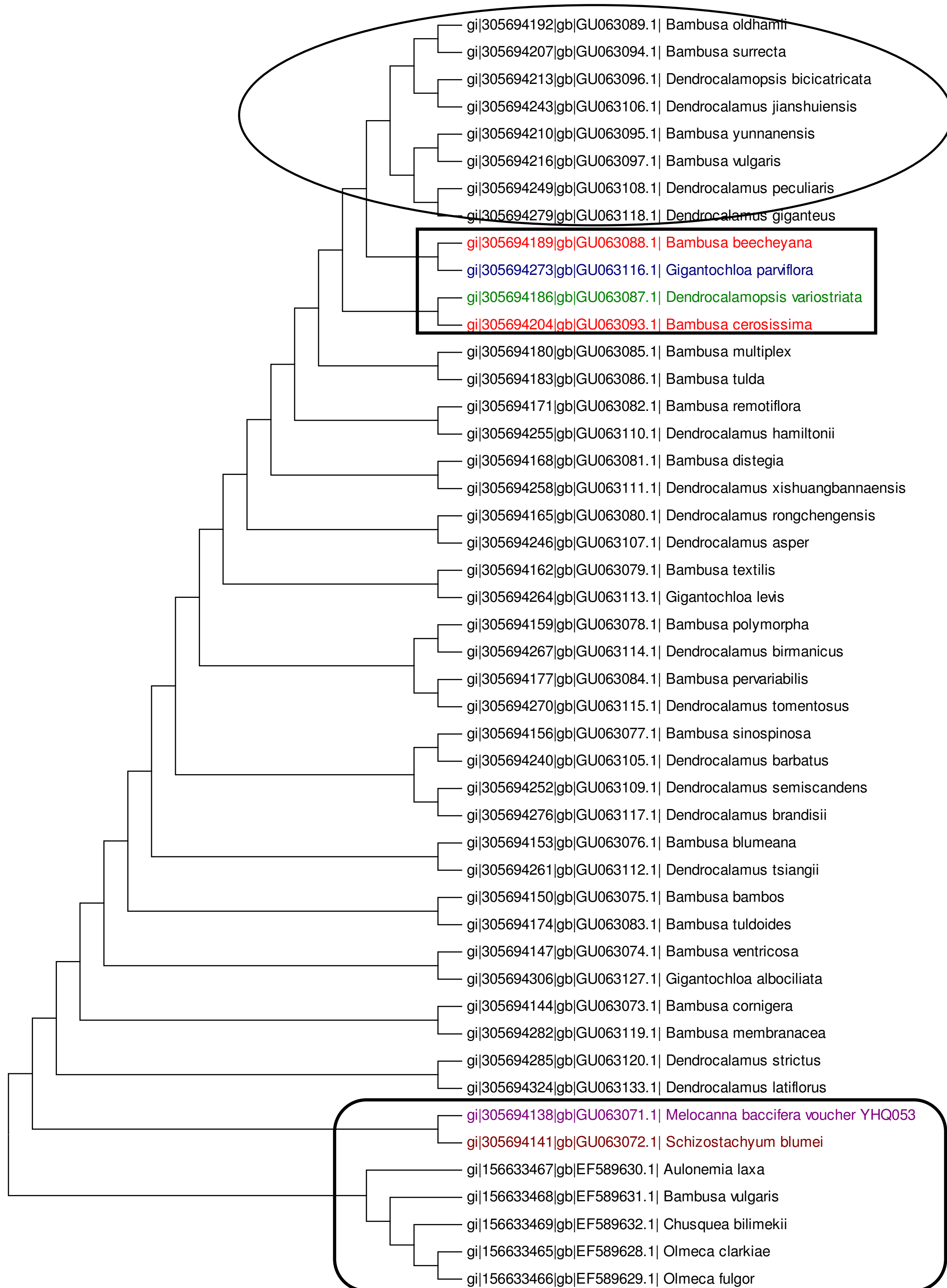


Fig: Phylogenetic tree by NJ method for psbA-trnH sequences

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