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PHYLOGENETIC RELATIONSHIP BETWEEN THE BAMBOO SPECIES OF TRIBE BAMBUSEAE AND ARUNDINARIEAE BASED ON *mat*K GENE SEQUENCES

Kamlakar C. More and Prashant A. Gawande*

Department of Botany, Sant Gadge Baba Amravati University, Amravati – 444 602, Maharashtra (India)
**e-mail: prashantgawande@sgbau.ac.in*

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ABSTRACT

The identification and classification of bamboos based on morphological characters is a quite difficult task due to long and irregular flowering, lack of sufficient distinguished morphological characters and environmental influence. The present study was aimed to elucidate the phylogenetic relationship between the species of tribe Bambuseae and Arundinarieae based on *maturase* K (*mat*K) as a candidate gene marker. Fifteen bamboo species *mat*K genes were sequenced and their accession numbers (OM57488 to OM417520) generated from NCBI along with *mat*K gene sequences of additional bamboo species including the outgroup species retrieved from Gene Bank to infer the phylogenetic relationship. The phylogenetic tree of Maximum Liklihood (ML) and Maximum Parsimony (MP) revealed that the bamboo species of tribe Bambuseae and Arundinarieae were clustered in separate clades, with some exceptions. The ML tree showed a close relationship between *Ochlandrae bracteata* and *Schizostachyum polymorphum* with 96% bootstrapping support. Similarly, *Bambusa assamica*, *Gigantochloa atroviolacea* and *Bambusa polymorpha;* and *B. oliveriana*, *B. wamin* and *B. nutans* showed sister relationships. Clade-II showed the group of species of tribe Arundinarieae, except *Schizostachyum brachycladum* and *Gignatochloa rostrata*, the *Phyllostachys nidularia* and *Phyllostachys aureosulcata* seem to be recently evolved. The MP tree showed association between *Schizostachym polymorphum* and *Ochlandrae bracteata* with 91% bootstrapping support, while, *Gigantochloa atroviolacea* and *B. assamica* exhibited close association with 97% bootstrapping support. The inferences from the phylogenetic trees and DNA polymorphism analysis revealed that *mat*K gene possessed moderate revolutionary potential for species discrimination, for better phylogenetic resolution. The multigene barcode approach along with phenotypic observations need to be considered.

Keywords: Arundinarieae, bamboo, Bambuseae, DNA polymorphism, maturase K, phylogeny

INTRODUCTION

Bamboo is an important commercial plant belonging to the subfamily Bambusoidae of family Poaceae. Bambusoidae is further classified into three tribes i.e. Bambuseae (tropical) and Arundinarieae (temperate) and Olyreae (Wysocki *et al*., 2015). India is the 3rd largest genetic reservoir of bamboos having 136 species belongs to tribe Bambuseae and Arundinarieae (Meena *et al*., 2020). Worldwide, the bamboos are found in different regions of tropics and subtropics. Bamboo has gained huge commercial significance due to its diverse use in daily life activities such as house construction, agricultural and nutraceutical sectors (Zhu *et al*., 2014). Bamboo is a versatile source

to resolve some of the challenges in food, energy and climate change (Ramakrishnan *et al*., 2020). The traditional classification of bamboos is somehow controversial in some species, and it is also not proper to totally rely on the reproductive and vegetative characters (Zheng *et al*., 2020). Because of long and irregular flowering as well as limited distinguishable morphological characters, distinguishing the species is a quite challenging task, therefore it is essential to adopt molecular markers-based species discrimination methods (Amom *et al*., 2018). Further, the proper identification and classification of bamboos is essential to explore their sustainable utilization and conservation.

Till date very few bamboo species have been screened based on their plastid genome for species discrimination (Huang *et al*., 2020). The tribes of Bambusoideae, i.e. Bambuseae, Arundinarieae and Olyreae, were supported with high confidence as a monophyletic group, but the relationship between these tribes is still unclear (Triplett and Clark, 2010; Kelchner *et al*., 2013). Due to the lack of synapomorphy to differentiate tropical bamboos, phylogenetic classification within the tribe Bambuseae has not yet been established (BPG, 2012; Kelchner *et al*., 2013). Zhang and Clark (2000) reported that the tropical and temperate woody bamboos are related and based on *ndh*F sequences and anatomical characteristics formed a clade with woody bamboos. Bamboo classification issue can be resolved by using both morphological and molecular systematics. DNA sequencing is one of the popular molecular systematic techniques used for classifying the bamboos (Triplett and Clark, 2010). Therefore, the present investigation was aimed to study the phylogenetic relationship between various species of Bambuseae and Arundinarieae.

MATERIALS AND METHODS

Collection of plant material

Fifteen bamboo species belongs to tribe Bambuseae and Arundinarieae*viz*, *Ochlandrae bracteata, Gigantichola rostrata, G. atroviolacea, Bambus aassamica, B. nutan, B. oliveriana, B. cacharensis, B. wamin, B. polymorpha, Phyllostachys nigra, P. mannii, P. aurea, Schizostac hyumpolymorphum, S. dullooa,* and *S. brachycladum*, were collected from different geographical regions of India and maintained at the Central Forest Nursery, Wadali, Amravati (India) located between 20.922409" N, 77.793096" E and 20.922409" N, 77.793096" E by the Department of Forest, Government of Maharashtra (India). These bamboo species were selected to resolve the taxonomic ambiguities in species identification.

Preliminary identification of plant species

The plant specimens were identified through a key based on morphological descriptors elaborated in floras, monographs and books such as "Bamboos of India" (Seethalakshmi and Kumar, 1998), "Bamboos of Meghalaya" (Kumari and Singh, 2014) and the identity of plant species were confirmed by International Plant Names Index (IPNI) (*https://www.ipni.org/*), The Harvard University Herbaria and the Australian National Herbarium, hosted by the Royal Botanic Garden, Kew, England.

DNA extraction and PCR amplification

The plant genomic DNA was isolated from the leaves of plants under study by using Gene Elute TM Plant Genomic DNA Miniprep Kit (Sigma Aldrich) as per the manufacturer's instructions. The genomic DNA were quantified and analysed using UV-visible spectrophotometer (Simadzu) and agarose gel electrophoresis. A primer pair (forward *mat*K: 5′ACCCAGTCCATCTGGAAATCT TGGTCC 3′, reverse *mat*K: 5′ CGTACAGTACTTTTGTGTTTACGAG 3′) was used for PCR amplification on an S1000 thermal cycler (Bio-Rad, USA). The PCR cycle consisted of initial denaturation at 95°C for 1 min, then 35 cycles at 94°C for 30 sec, 52°C for 30 sec, 68°C for 1 min and extension step at 68°C for 10 min.

DNA sequencing, editing, consensus formation and NCBI blast

The PCR products were purified and bidirectionally sequenced using the same primer pair in 3730XL DNA Analyzer 96 capillary from Applied Biosystem, Eurofins Genomics, Bangalore, India. All these sequences were analysed, and streamed for low quality sequences, edited and refined manually using the software BioEdit version 7.0 (Hall, 2007). The consensus sequence files (FASTA files) were prepared using BioEdit version 7.0. The consensus sequences were used for NCBI blast to analyse the quality, identity percentage, quarry cover and frame shift mutations. The refined consensus sequences were submitted to NCBI gene bank using Bankit submission tool (*https://www.ncbi.nlm.nih. gov /WebSub/*) and the accession numbers for all the submitted sequences were generated from NCBI.

Pair-wise alignment and refinement of sequences

The refined consensus DNA sequences of *mat*K generated from the species under study along with the sequences retrieved from Gene Bank were assembled and aligned using the server *https://mafft.cbrc.jp/alignment/server/* with default parameters, alignment was further refined and trimmed using Gblocks web server *http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type= gblocks*. The refined files were saved in Phylip and Nexus formats for further analysis.

Nucleotide composition, substitution matrix and distance analysis

The nucleotide composition, maximum likelihood (ML) substitution pattern and distance matrix of gene sequences were calculated by using MEGA version 11.0 with Tajima-Nei model and Kimura 2 parameter model (Tamura and Nei, 1993; Tamura *et al*., 2021).

DNA polymorphism analysis

The aligned gene sequences of *mat*K in MEGA file were used for the analysis of DNA polymorphism by using various parameters like number of monomorphic sites, polymorphic sites, parsimony informative sites, singleton variable sites, number of mutations, number of indel sites, mean nucleotide differences (*k*), nucleotide diversity (π), haplotype diversity (Hd), conservation threshold (CT), sequence conservation (C) and GC content etc., in DnaSP6 software [*http://www.ub.edu/dnasp*] (Rozas *et al*., 2017).

Phylogenetic analysis

Phylogenetic analyses of refined FASTA *mat*K sequences of plants under investigation were performed using maximum likelihood (ML) and maximum parsimony (MP) methods to elucidate the evolutionary relationship between species. The ML analysis was performed using RaxML GUI 2.0 (Daniel *et al*., 2021), with GTR+ GAMMA model for nucleotide evolution and bootstrapping of 1000 replicates were set. The MP analysis was performed with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch swapping algorithm in MEGA version 11.0. All the characters were unordered and of equal weight and the gaps were treated as missing data. Amongst the 100 trees generated the significant trees were selected for the analysis of evolutionary relationship between the species.

RESULT AND DISCUSSION

DNA polymorphism analysis

DNA polymorphism analysis of 24 nucleotide sequences of *mat*K were performed by using DnaSP6 software **[***http://www.ub.edudnasp/*] (Rozas *et al*., 2017). Various parameters were considered to study the pattern of DNA polymorphism. The aligned sequence length was found to be 705 bp. The mono-morphic sites, polymorphic sites, singleton variable sites, parsimony informative sites, number of mutations, number of indel sites, mean nucleotide differences (*k*), nucleotide diversity (π), haplotype

Table 1: DNA polymorphism analysis of 24 nucleotide sequences evaluated

S. No.	DNA polymorphism parameters	*mat*K
1.	Number of sequences (N)	24
2.	Aligned sequence length (bp)	705
3.	Monomorphic sites	654
4.	Polymorphic sites	51
5.	Singleton variable sites	36
6.	Parsimony informative sites (3 variants)	15
7.	No. of mutations	53
8.	No. of indel sites	197
9.	Mean nucleotide differences (*k*)	8.60870
10.	Nucleotide diversity (π)	0.01221
11.	Haplotype diversity (Hd)	0.851
12.	Sequence conservation (C)	0.933
13.	Conservation threshold (CT)	1
14.	G + C content	0.347

Table 2: Maximum likelihood estimates for substitution matrix of *mat*K gene sequences

From\To	A	T	C	G
A	-	8.1437	4.1653	7.8969
T	6.5401	-	11.1423	3.6352
C	6.5401	21.7848	-	3.6352
G	14.2076	8.1437	4.1653	-

diversity, sequence conservation, conservation threshold (CT) and GC content are given in Table 1. The polymorphism analysis revealed that the *mat*K was a potential source to discriminate species as it possessed adequate values of polymorphism analysis. Wang *et al*. (2022) have reported that *mat*K gene sequences display high nucleotide diversity and haplotype diversity, which are essential to differentiate the species. In the view of diversity information available, *mat*K was suitable barcoding candidates for identifying *Bambusa* species.

The maximum likelihood substitution matrix of *mat*K gene (Table 2) showed transitional and transversion substitutions The nucleotide frequencies were A = 29.09%, T/U = 36.22%, C = 18.53%, and G = 16.17%. For estimating ML values, a tree topology was computed. The maximum log likelihood for this computation was -1289.213. The highest transition substitution was between nucleotides CT, whereas least substitution was observed in AG. The highest transversion were found in AT and GT, whereas least transversions were observed in TG and CG.

Nucleotide composition

The average AT and GC content were found to be 65.29 and 34.68%, respectively. The maximum AT content (65.62) was observed in both *O. ebracteata* and *S. polymorphum*; whereas maximum GC content was found in *S. dullooa* (Table 3).

Phylogenetic analysis

The phylogenetic trees were constructed based on ML and MP methods, the *mat*K gene sequences of the species under study along with gene sequences of similar species (identified through blast analysis) were retrieved from NCBI for the generation of robust phylogenetic relationship between the species of tribe Bambuseae and Arundinarieae. The ML method-derived dendrogram exhibited two separate clades. The species belonged to tribe Bambuseae and Arundinarieae except *P. aurea* which was found to be associated with the members of Bambuseae. The clade-I showed the group of species belonging to Bambuseae, except *P. aurea*. The clade-I was further divided into 8 clusters where *P. aurea* and *S. dullooa* were found to be independent separate branches. The remaining species assembled in two groups. *Ochlandra ebracteata* and *S. polymorphum* exhibited a close relationship with 96% bootstrapping support. Similarly, *B. assamica* and *G. atroviolacea*; *B. polymorpha* and *B. oliveriana* and *B. wamin* and *B. nutans* showed sister relationships. The clade-II represents the species of tribe Arundinarieae, except *S. brachycladum* and *G. rostrata*. All the species of clade-II diverged as a separate branch, *P. nidularia* and *P. aureosulcata* seemed to be the recently evolved. The clusters showing > 50% bootstrap support are shown in Fig. 1, whereas as the clusters which exhibited < 50% bootstrap value were collapsed.

Table 3: The summary of nucleotide composition and GC and AT contents of *Mat*K sequences

S. No.	Plant species	Accession numbers	T(U)	C	A	G	Total	A + T (%)	G + C (%)
1.	*Bambusa assamica*	OM574884	36.06	18.81	28.99	16.12	707	65.05	34.93
2.	*B. cacharensis*	OM574867	36.06	18.81	28.99	16.12	707	65.05	34.93
3.	*B. oliveriana*	OM574871	36.20	18.67	29.13	15.98	707	65.33	34.65
4.	*B. nutans*	OM574874	36.11	18.69	29.03	16.14	706	65.14	34.83
5.	*B. polymorpha*	OM574873	36.20	18.52	28.99	16.26	707	65.19	34.78
6.	*B. vulgaris wamin*	OM574882	36.20	18.67	28.99	16.12	707	65.19	34.79
7.	*Phyllostachys aurea*	OM574870	36.11	18.69	28.89	16.28	706	65	34.97
8.	*P. nigra*	OM574880	36.06	18.52	29.13	16.26	707	65.19	34.78
9.	*P. violascens*	KX146433.1	36.20	18.38	29.13	16.26	707	65.33	34.64
10.	*P. aureosulcata*	KX146430.1	36.20	18.38	29.13	16.26	707	65.33	34.64
11.	*P. sulphurea*	MH659938.1	36.20	18.38	29.13	16.26	707	65.33	34.64
12.	*P. mannii*	OM574881	36.20	18.52	28.99	16.26	707	65.19	34.78
13.	*P. nidularia*	JN247146.1	36.20	18.38	29.13	16.26	707	65.33	34.64
14.	*P. rubromarginata*	MN043649.1	36.20	18.38	29.13	16.26	707	65.33	34.64
15.	*P. makinoi*	MN043642.1	36.20	18.38	29.13	16.26	707	65.33	34.64
16.	*P. meyeri*	MN043644.1	36.20	18.38	29.13	16.26	707	65.33	34.64
17.	*P. flexuosa*	MN043640.1	36.20	18.38	29.13	16.26	707	65.33	34.64
18.	*Gigantochloa atroviolacea*	OM574866	36.06	18.81	28.99	16.12	707	65.05	34.93
19.	*G. rostrata*	OM574879	36.06	18.52	29.13	16.26	707	65.19	34.78
20.	*Ochlandrae bracteata*	OM574875	36.49	18.52	29.13	15.84	707	65.62	34.36
21.	*Schizostachyum dullooa*	OM574877	35.92	18.95	28.99	16.12	707	64.91	35.07
22.	*S. brachycladum*	OM574878	36.20	18.38	29.13	16.26	707	65.33	34.64
23.	*S. polymorphum*	OM574876	36.63	18.67	28.99	15.70	707	65.62	34.37
24.	*Triticum aestivum*	KM212081.1	36.91	17.68	29.42	15.98	707	66.33	33.66
	Average		36.21	18.52	29.08	16.16	706.91	65.29	34.68

The MP-derived phylogenetic tree showed the formation of two clades that separated the species of tribe Bambuseae and Arundinarieae with the exception of *P. aurea* which was associated with the members of Bambuseae. *S. brachycladum* and *G. rostrata* exhibited association with the species of Arundinarieae. The clade-I was divided into two clusters. The cluster -I split into two sub-clusters, the sub-cluster-I exhibited a relationship between *P. nigra* and *G. rostrata* and seemed to be recently diverged having >50% bootstrap support. The sub-cluster-II of cluster-I exhibited the group of species which were diverged as a separate branch. The evolutionary relationship between the species under clade-I were significantly supported by bootstrap value. The clade-II having 80% bootstrap support were further split into 2 clusters. The cluster-I showed 9 species. The *S. polymorphum* and *O. ebracteata* exhibited close relationship with 91% boot-strapping support. Similarly, *G. atroviolacea* and *B. assamica* showed closed association with 97% bootstrapping support. The *B. cacharensis* was exceptionally associated with *P. aurea*; whereas, the *B. polymorpha*, *B. nutans* and *S. dullooa* showed separate evolutionary branching in cluster-I.

The cluster-II of clade-II exhibited relationship between the *B. wamin* and *B. oliveriana* with 56% bootstrapping support (Fig. 2), with the exception of *G. rostrata* and *S. brachycladum*. The other members of Bambuseae and Arunidinarieae were grouped into separate clades, wherein most branches were supported with >50% bootstrap. Compared with ML method, the MP phylogenetic tree were found to be well-supported with bootstrap value, but overall, the results with respect to bootstrap support were insufficient for significant resolution of phylogenetic relationship. This is due to high sequence similarity between most of taxa belonging to tribe Bambuseae and Arundinarieae. The results of present study were moderately consistent with those of Meena *et al*. (2020) who observed *mat*K discriminatory potential in Himalayan temperate woody bamboos with >60% boot-strap support as reported by Bamboo Phylogeny Group (2012) and Kelchner *et al*. (2013). Phylogenetic

Table 2: Pair-wise distance matrix of bamboo species evaluated [calculated by Kimura-2 parameter model]

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A																								
B	0.000																							
C	0.000	0.000																						
D	0.001	0.001	0.001																					
E	0.002	0.002	0.002	0.004																				
F	0.001	0.001	0.001	0.002	0.001																			
G	0.001	0.001	0.001	0.002	0.004	0.002																		
H	0.002	0.002	0.002	0.004	0.002	0.001	0.004																	
I	0.005	0.005	0.005	0.007	0.005	0.004	0.007	0.005																
J	0.015	0.015	0.015	0.017	0.015	0.014	0.012	0.015	0.018															
K	0.015	0.015	0.015	0.017	0.015	0.014	0.012	0.015	0.018	0.000														
L	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001													
M	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000												
N	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000	0.000											
O	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000	0.000	0.000										
P	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.004	0.004	0.002	0.002	0.002	0.002									
Q	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000	0.000	0.000	0.000	0.002								
R	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000	0.000	0.000	0.000	0.002	0.000							
S	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000	0.000	0.000	0.000	0.002	0.000	0.000						
T	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000					
U	0.014	0.014	0.014	0.015	0.014	0.012	0.011	0.014	0.017	0.001	0.001	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000				
V	0.001	0.001	0.001	0.002	0.001	0.000	0.002	0.001	0.004	0.014	0.014	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012			
W	0.012	0.012	0.012	0.014	0.012	0.011	0.014	0.012	0.007	0.026	0.026	0.024	0.024	0.024	0.024	0.024	0.024	0.024	0.024	0.024	0.024	0.011		
X	0.054	0.054	0.054	0.056	0.054	0.053	0.051	0.054	0.056	0.059	0.059	0.057	0.057	0.057	0.057	0.054	0.057	0.057	0.057	0.057	0.057	0.053	0.059	

The bamboo species evaluated: *A: Bambusa assamica, B: B. cacharensis, C: Gigantochloa atroviolacea, D: Schizostachyum dullooa, E: B. oliveriana, F: B. nutans, G: Phyllostachys Aurea, H: B. polymorpha, I: Ochlandra ebracteata, J: G. rostrata, K: P. nigra, L: S. brachycladum, M: P. violascens, N: P. aureosulcata, O: P. sulphurea, P: P. mannii, Q: P. nidularia, R: P. rubromarginata, S: P. makinoi, T: P. meyeri, U: P. flexuosa, V: B. vulgaris wamin, W: S. polymorphum, X: Triticum aestivum*

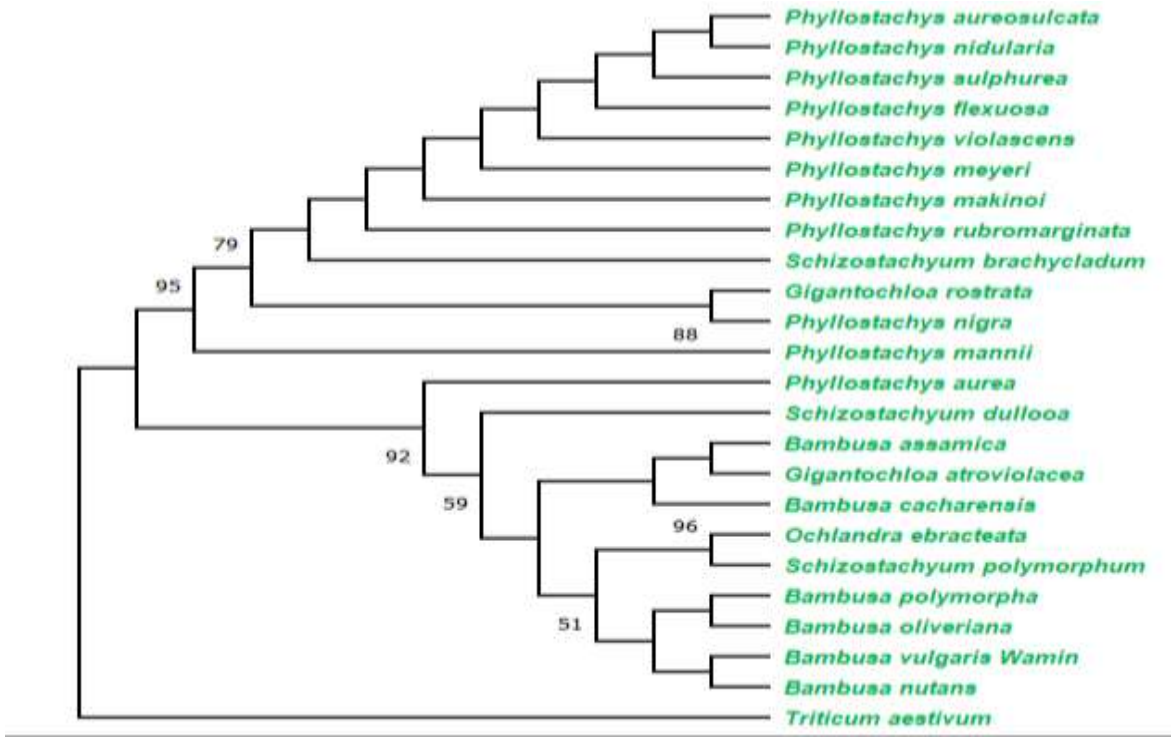

Fig. 1: Maximum Likelihood (ML) phylogenetic tree constructed in RaxML GUI 2.0, with GTR+ GAMMA model for nucleotide evolution and bootstrapping of 1000 replicates

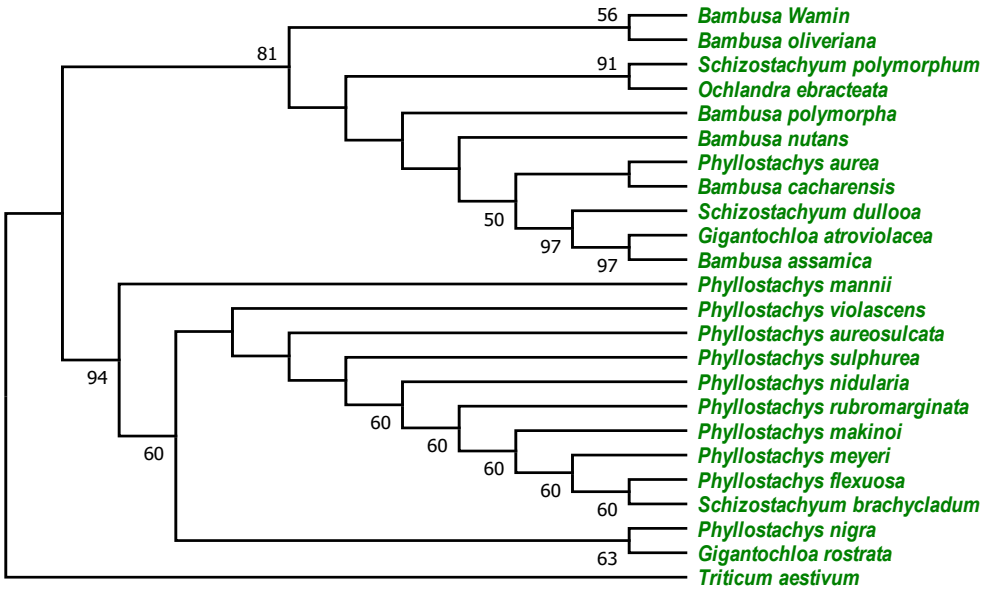

Fig. 2: The Maximum parsimony (MP) tree constructed in MEGA version 11.0 with 1000 random taxa addition and tree bisection and reconnection (TBR) as the branch swapping algorithm

classification within tribe Bambuseae has not yet been defined due to the lack of morphological synapomorphy to differentiate tropical bamboos. Therefore, this study elucidated the evolutionary relationship between the tribes of Bambuseae and Arundinarieae.

Conclusion: The present study revealed that *mat*K gene sequences can be the suitable barcodes to identify the species to some extent. The evolutionary relationship inferred from this study provide a supportive information to identify the species. Moreover, the screening of multiple gene loci along with assessment of morphological descriptors need to be evaluated for better resolution of phylogenetic relationship of Bamboo species.

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