Buitenzorg Journal of Tropical Science

Research Article

A Comparative Study of DNA Barcoding Markers for Bamboo

Seli Rizqi Awaliah[1]* & Aqwin Polosoro[2]

[1] Innovation Centre for Tropical Science (ICTS), Bogor 16113, West Java, Indonesia
[2] Research Center for Genetic Engineering, National Research and Innovation Agency, Cibinong, Bogor 16911, West Java, Indonesia

*Corresponding author: selirizqia30@gmail.com

ABSTRACT

The Bambusoideae subfamily, a significant group within the Poaceae family, contains diverse genera with complex taxonomic relationships. This study aimed to elucidate the phylogenetic relationships and genetic diversity within the bamboo, focusing on the utility of DNA barcoding markers, i.e., ITS2, *mat*K, and *rbc*L, in bamboo species identification. By analyzing sequence data from these markers, phylogenetic trees were constructed using the maximum likelihood method to infer evolutionary relationships among species. The results showed that ITS provides the highest resolution for species-level identification, distinguishing closely related species more effectively than *mat*K and *rbc*L. While *mat*K demonstrated robust genus-level classification, *rbc*L was limited by its high conservation, making it more suitable for broader taxonomic groupings. These findings contribute to a better understanding of bamboo taxonomy and highlight the importance of marker selection based on the taxonomic resolution required. The study also emphasizes the complementary use of these markers to provide a comprehensive view of bamboo phylogenetics.

Keywords: bamboo taxonomy, DNA barcoding, ITS2, *mat*K, *rbc*L

Introduction

The Bambusoideae subfamily, a diverse group within the Poaceae family, has been the subject of extensive taxonomic and phylogenetic research due to its ecological and economic importance. Bamboo provides crucial ecosystem services, including soil stabilization, carbon sequestration, and habitat provision, while also serves as a sustainable resource for construction and various industries. Despite its significance, bamboo taxonomy remains complex and contentious, largely due to the high morphological similarity between species and the challenges posed by traditional identification methods. This has driven the need for more precise molecular tools to resolve relationships within the subfamily.

Bamboo taxonomy is particularly challenging due to the morphological overlap between species and the complex evolutionary history of the group. The Bambusoideae subfamily is divided into two main tribes: Bambuseae (woody bamboos) and Olyreae (herbaceous bamboos) (Clark et al., 2007). Bambuseae genera such as *Phyllostachys* and *Chimonobambusa* exhibit close phylogenetic relationships, as demonstrated in molecular studies using markers like ITS rDNA and AFLP, which offer more robust species classification compared to traditional methods (Friar & Kochert, 1994;

Hodkinson et al., 2000). However, despite these advancements, issues such as the monophyly of certain genera and the precise delineation between species remain unresolved, necessitating further molecular analysis (Clark et al., 2007).

Bamboo species are widely distributed across tropical and subtropical regions, with the greatest diversity occurring in Asia, particularly in countries such as China, India, and Indonesia (Benton, 2015). Neotropical bamboo species also inhabit the Americas, from Mexico to the Andean regions of South America (Ruiz-Sanchez, 2011). These distributions are shaped by a combination of ecological factors such as altitude, climate, and soil type. However, ongoing environmental changes and human interventions may alter these patterns, making accurate species identification and classification critical for conservation efforts.

In recent years, DNA barcoding has emerged as a powerful molecular tool to address taxonomic challenges, providing a rapid and accurate means of identifying species based on short, standardized genetic sequences. For plants, markers such as the internal transcribed spacer 2 (ITS2), *mat*K, and *rbc*L are widely used for this purpose (Whitley et al., 2024). These markers, selected for their variability and ability to discriminate between species, have revolutionized plant taxonomy by complementing or even replacing traditional morphological approaches. DNA barcoding has been particularly effective in resolving complex species relationships and identifying cryptic species that are otherwise indistinguishable through morphological traits (Letsiou et al., 2024). Furthermore, the use of "super barcodes," such as those derived from chloroplast genomes, enhances species resolution, offering more precise tools for taxonomic classification and biodiversity conservation (Letsiou et al., 2024).

This study aimed to elucidate phylogenetic relationships within the *Bambuseae* tribe using molecular data from the ITS2, *mat*K, and *rbc*L markers. By constructing phylogenetic trees, we seek to address unresolved taxonomic questions and contribute to the broader understanding of bamboo evolution and classification. The results would have implications for taxonomy, conservation, and sustainable use of bamboo species worldwide.

Materials and Methods

Sequence Data Collection from NCBI

This study focused on DNA sequence data obtained from the NCBI database for three genetic markers commonly used in plant DNA barcoding, i.e., ITS2, *matK*, and *rbcL*. These markers were selected for their proven efficacy in resolving phylogenetic relationships in plants, particularly within the Bambusoideae subfamily. DNA sequences were retrieved using the Basic Local Alignment Search Tool for nucleotides (BLASTn) feature on NCBI, targeting sequences specific to the *Bambuseae* genus. For ITS sequences, the search was restricted to the rRNA/ITS database to ensure specificity, while *matK* and *rbcL* sequences were obtained using the highly similar sequences (MegaBLAST) option. All sequences matching the search criteria were downloaded in FASTA format for further analysis.

Data Preparation for Analysis

After downloading the sequence data, each FASTA file was manually inspected to ensure data quality. This step involved verifying sequence length, removing incomplete sequences, and checking for ambiguous nucleotides or other errors that could affect the accuracy of the analysis. Only sequences that met these quality control standards were retained for alignment and phylogenetic analysis. The selected sequences were then imported into the Molecular Evolutionary Genetics Analysis (MEGA) software for subsequent steps.

Sequence Alignment Using the MUSCLE Algorithm

Multiple sequence alignment was performed using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm within the MEGA software. MUSCLE was chosen due to its high accuracy and computational efficiency, particularly when handling large datasets. The aligned sequences were manually inspected for misalignments or incorrectly placed gaps to ensure accurate nucleotide placement. Additionally, we compared alternative alignments to verify the consistency of the results.

Sequence Trimming for Length Consistency

Once aligned, the sequences were trimmed to ensure uniform length across all samples. This trimming step focused on removing non-conserved regions, excessive gaps, and ambiguously aligned sections, leaving only the most informative and conserved regions for phylogenetic analysis. Regions with gaps exceeding 50% of the sequence length or exhibiting poor alignment quality were excluded from further analysis to maximize the reliability of the resulting phylogenetic tree.

Phylogenetic Tree Construction Using the Maximum Likelihood Model

To infer the phylogenetic relationships among species in the Bambusoideae subfamily, we used the ML method in MEGA. The Tamura-Nei substitution model was selected as best fit the evolutionary patterns observed in the data. The analysis included 1,000 bootstrap replicates to provide a measure of confidence in the tree branching structure, with bootstrap values above 70% considered statistically significant. All ambiguous positions were treated using partial deletion with a cutoff threshold of 95%, ensuring that only well-supported positions contributed to the tree topology.

Phylogenetic Tree Visualization and Interpretation

The resulting phylogenetic tree was exported in a publication-ready format for visualization and further interpretation. Bootstrap values were used to assess the reliability of each node, and the tree topology was examined to deduce evolutionary relationships between species within the Bambusoideae subfamily. Special attention was paid to the relative positions of taxa within *Phyllostachys* and related genera, providing insights into their genetic divergence and phylogenetic patterns.

Results and Discussion

This study used three molecular markers, i.e., ITS, *mat*K, and *rbc*L, contributing distinct insights into species identification and evolutionary relationships within bamboo. While these markers varied in terms of sequence length, variability, and utility across taxonomic levels, their combined analysis offered a robust framework for bamboo phylogenetic studies.

The ITS region, with a sequence length of 565 bp and identity values ranging from 93.39–100%, proved to be a valuable marker for distinguishing closely related bamboo species. The relatively high variability of ITS made it an ideal marker for exploring intraspecific variation or identifying cryptic species within bamboo populations. However, the limited number of sequences available in this study (13 sequences) restricted its broader applicability for large-scale phylogenetic analyses. Despite the high query coverage (98–100%), the limited representation of ITS sequences in public databases could hamper its use in comprehensive bamboo phylogenetic studies. Nevertheless, ITS remains essential for studies focused on fine-scale differentiation between bamboo species, where greater genetic variation is needed to resolve relationships.

The *mat*K gene, at 1,072 bp, offered a broader genetic window and served as a

robust marker for plant DNA barcoding. With identity values ranging from 99–100% and query cover of 99.72–100%, *mat*K provided strong support for species identification, particularly at higher taxonomic levels. The availability of 100 sequences in this study reflected the widespread use of *mat*K in plant phylogenetics. However, its relatively high conservation across species limited its resolution to lower taxonomic levels, such as between closely related species or within genera. This marker is especially effective when used in conjunction with more variable regions, such as ITS, to improve taxonomic resolution. While *mat*K excels in distinguishing between genera, its limited intragenus variability suggests that it should be complemented by more variable markers for detailed species-level investigations.

Similarly, the *rbc*L marker, spanning 514 bp, displayed high identity (100%) and query cover (99.81–100%), demonstrating its reliability in species identification. With 100 available sequences, *rbc*L is one of the most well-represented and commonly used markers in plant barcoding studies, including those on bamboo. The highly conserved nature of *rbc*L makes it particularly effective for identifying bamboo species at broader taxonomic scales, such as between genera or subtribes. However, like *mat*K, its limited variability reduced its ability to distinguish closely between related species or subspecies. This makes *rbc*L less suitable as a standalone marker for resolving complex phylogenetic relationships at the species level. Nonetheless, *rbc*L remains valuable for its reliability and high representation in bamboo studies, particularly when used in combination with more variable regions like ITS.

The ITS phylogenetic tree provided detailed insights into the genetic relationships among bamboo species from the Bambusoideae subfamily (Figure 1). Traditional bamboo taxonomy has largely relied on morphological traits such as culm structure, leaf shape, and flowering patterns. Interestingly, genetic groupings based on the ITS marker strongly correlated with these traditional classifications, particularly for species like *Bambusa tulda*, *Bambusa auriculata*, and *Bambusa burmanica*. These species aligned well with their morphological groupings, supporting the reliability of ITS data in capturing evolutionary divergence that reflects observable characteristics used in taxonomy.

Table 1. Comparison of Bambusoideae DNA barcoding markers from NCBI database.

Markers	Size (bp)	Query cover (%)	Identity (%)	Ns
ITS2	565	98–100	93.39–100	18
*mat*K	1,072	99–100	99.72–100	100
*rbc*l	514	100	99.81–100	100

Ns = number of sequences

However, Figure 1 also demonstrated discrepancies between molecular and morphological data. For example, *Bambusa bambos* groups with species like *Dendrocalamus strictus* and *Gigantochloa atroviolacea* in the phylogenetic tree, despite their distinct morphological traits, such as culm size and type, traditionally placing them in different subgenera (Yang et al. 2010). This incongruence demonstrated that *B. bambos* share closer genetic ties with species traditionally classified in other genera. The genetic component analysed in this study may not be related to the morphological traits in the classification. Such findings indicate the limitations of relying solely on physical traits for classification, hinting at the need for re-evaluating genus boundaries based on genetic data rather than morphological observations alone.

The ITS marker is highly variable, which enhances its ability to distinguish between closely related species. In the context of bamboo, ITS has demonstrated better resolution compared to chloroplast markers like *mat*K and *rbc*L, making it the

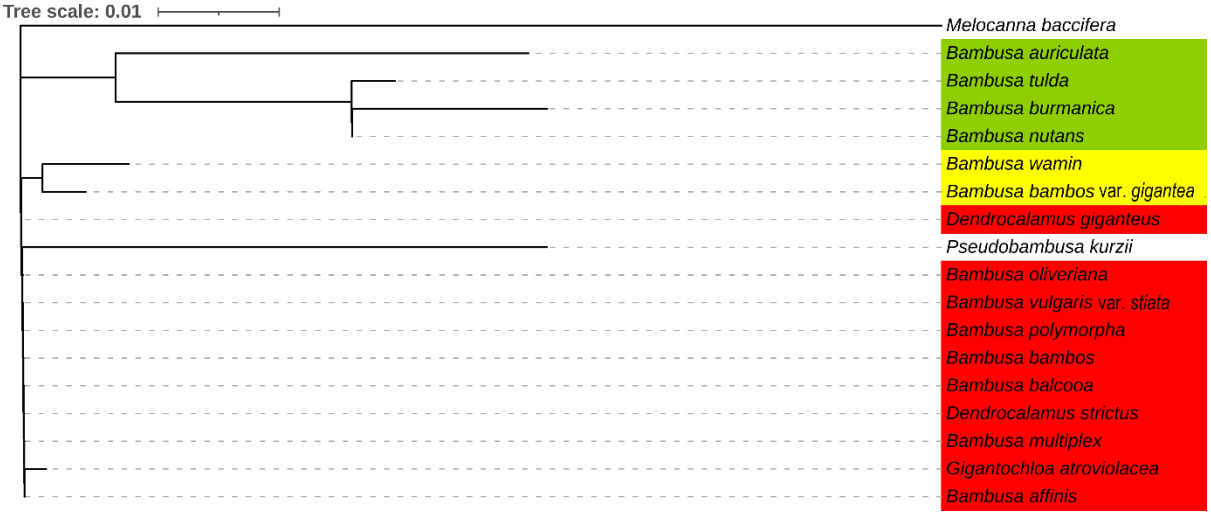

Fig. 1. Phylogenetic analysis of the Bambusoideae subfamily ITS2 DNA barcode.

preferred choice for resolving complex species relationships. This variability is particularly useful in identifying species like *D. strictus*, which might not be easily separated from species in the *Bambusa* genus based on morphology alone. Studies on other plant groups, such as *Bulbophyllum lobbii* and *Cassia* ssp., have also highlighted the superior phylogenetic resolution provided by ITS (Jeevitha & Anandan, 2024; Su'udi et al., 2024). This suggests the importance of marker selection based on the specific goals of the research—whether fine-scale species resolution or broader taxonomic classification is needed.

The ITS phylogenetic tree also revealed genetic relationships across different bamboo genera, such as *Dendrocalamus*, *Gigantochloa*, and *Melocanna*. Traditional taxonomy segregated these genera based on significant morphological differences, yet the ITS data suggest that some genera may be polyphyletic, that is, containing species that do not share a single common ancestor. For example, *D. strictus* clustered with species from the *Bambusa* genus, indicating deeper genetic relationships that are not reflected on morphological classifications. This finding emphasized the possibility that traditional morphology-based taxonomy may overlook these genetic relationships, prompting the need for reassessment of genus-level classifications.

Additionally, the inclusion of varieties and subspecies, such as *Bambusa vulgaris* var. *striata* and *B. bambos* var. *gigantea*, highlights their close genetic relationship to their parent species while maintaining distinct genetic identities. This supports their classification as valid taxonomic units, despite their physical similarities to the parent species. Genetic data reinforce the need to consider these varieties and subspecies in taxonomic classifications, ensuring that they are not overlooked in biodiversity and conservation studies.

The ITS phylogenetic tree confirmed many traditional taxonomic groupings based on morphology but also revealed previously hidden genetic relationships. This provided a more comprehensive understanding of bamboo classification, suggesting that a combined approach is crucial for accurately defining the evolutionary history of bamboo species. While traditional morphological characteristics remain valuable, molecular data like ITS can refine our understanding of genetic divergence, resolving ambiguities and uncovering cryptic species. This dual approach is essential for developing robust taxonomic frameworks that reflect both evolutionary and morphological relationships, ultimately

contributing to more effective conservation strategies for bamboo species.

The *mat*K phylogenetic tree for bamboo species provides a detailed molecular perspective, offering both confirmation of traditional taxonomy based on morphological traits and new insights that challenge established classifications. Traditional bamboo taxonomy has largely been based on physical characteristics such as culm structure, leaf shape, and reproductive features. In many cases, phylogenetic analysis aligns with these traditional classifications. For instance, species like *B. vulgaris*, *B. bambos*, and members of the *Dendrocalamus* genus (*D. strictus* and *D. minor*) clustered together in both the phylogenetic tree and traditional taxonomic systems due to shared morphological traits (Yang et al. 2010). This congruence indicates that certain morphological traits remain reliable indicators for taxonomic grouping probably due to their association, reaffirming the value of traditional methods in bamboo classification.

However, the *mat*K phylogenetic tree also revealed genetic relationships that diverge from traditional morphological groupings. For example, *Bambusa polymorpha* and *Bambusa ventricosa* are grouped near species from the *Dendrocalamus* and *Gigantochloa* genera in the phylogenetic tree. This suggests that taxonomy based on physical characteristics alone may sometimes overlook deeper evolutionary connections, implying that genetic markers like *mat*K can uncover evolutionary relationships that are not readily apparent from morphological characteristics. These findings suport the importance of molecular data in providing crucial insights into bamboo evolution and taxonomy, prompting a reconsideration of certain taxonomic boundaries.

The *mat*K gene, located in the chloroplast genome, is particularly useful for resolving species-level relationships within certain genera due to its relatively high mutation rate compared to other chloroplast genes. In various plant phylogenetic studies, *mat*K has shown high effectiveness in species-level resolution. For instance, *mat*K successfully distinguished *Paeonia arietina* from closely related species, highlighting its utility in phylogenetic research (Cetiz et al., 2023). However, despite these advantages, *mat*K exhibited lower variability compared to nuclear markers such as ITS, limiting its effectiveness in resolving closely related species within the same genus (Table 1). This lower variability can result in the inability to fully differentiate species that are morphologically distinct, emphasizing the need for complementary markers in certain cases.

A key insight from the *mat*K phylogenetic tree was the closer genetic ties observed between species across traditionally separate genera, such as *Bambusa*, *Gigantochloa*, and *Dendrocalamus* (Figure 2). These findings suggest that genus-level classifications may require revision based on genetic evidence. The phylogenetic relationships inferred from *mat*K data indicate that species within these genera may share more recent common ancestors than previously thought. This challenges traditional morphological classifications and raises important questions about the monophyly of certain genera. The presence of polyphyletic groupings—where a genus contains species that do not share a single common ancestor—suggests that some genera may need redefinition based on genetics rather than morphological criteria.

Additionally, the *mat*K phylogenetic tree revealed the genetic proximity of subspecies and varieties, such as *B. vulgaris* clone Green and *B. bambos* var. *gigantea*, to their parent species. This close relationship reinforced their traditional status as varieties rather than distinct species. However, the genetic data also highlighted subtle differences that could warrant further investigation. The ability of *mat*K to differentiate between

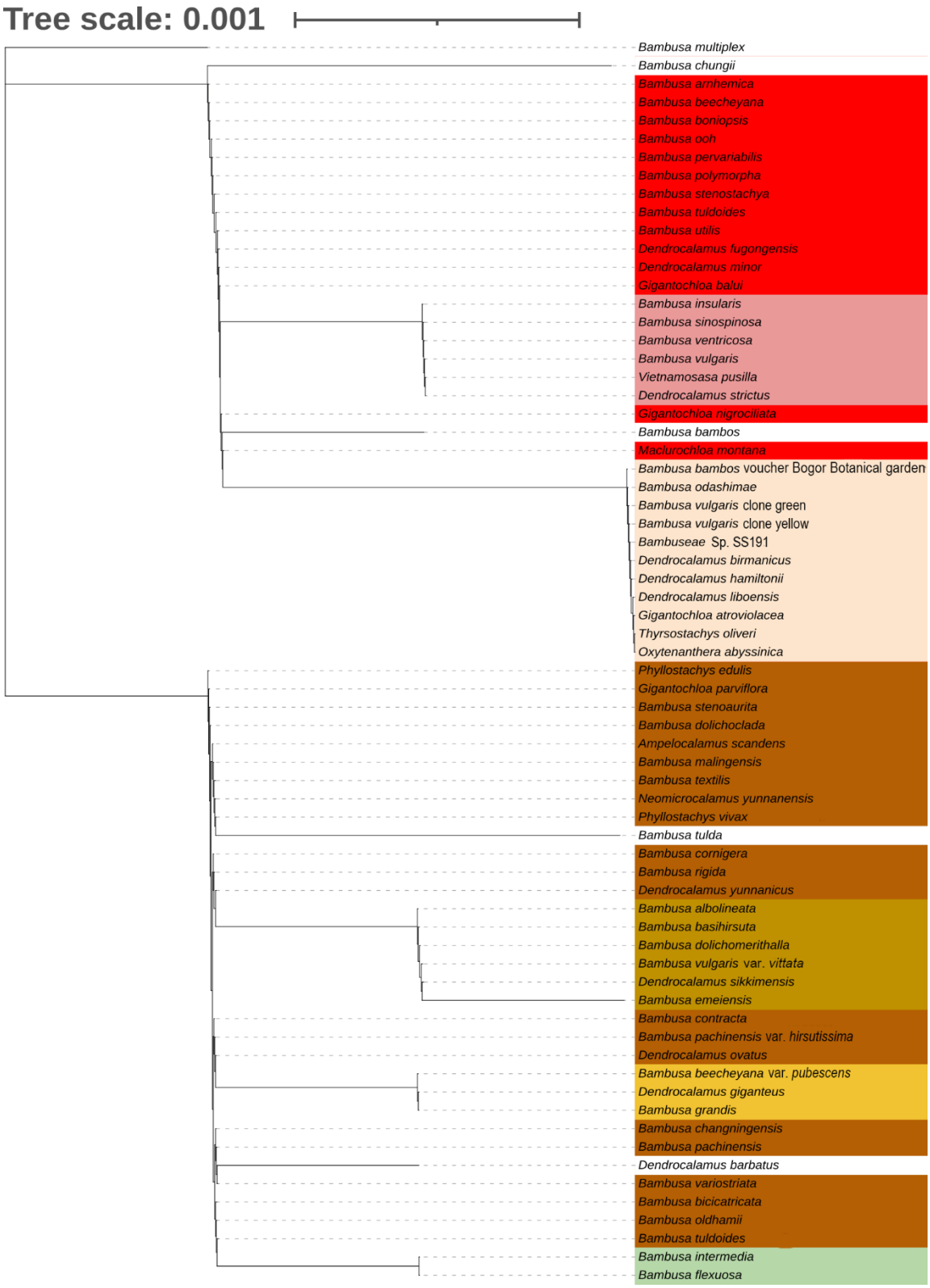

Fig. 2. Phylogenetic analysis of the Bambusoideae subfamily *matK* DNA barcode.

varieties and their parent species underlines its value for fine-scale taxonomic resolution, particularly when identifying genetic divergence within traditionally recognized taxa.

The *rbc*L phylogenetic tree offered a molecular perspective on the genetic diversity and evolutionary relationships within bamboo species, complementing and at times challenging traditional

taxonomy based on morphological traits. Molecular approaches using the *rbc*L gene, a core component of the photosynthetic apparatus, were expected to provide a deeper understanding of these relationships. Studies using *rbc*L sequences have been successful in differentiating plant taxa across a wide range of environments, including aquatic plants and seaweed, uncovering both known species and identifying previously unrecognized taxa (Lonthor et al., 2023; Susanti et al., 2023). These demonstrate gene utility not only in species identification but also in elucidating ecological and evolutionary contexts. For example, research on *Crassocephalum crepidioides* utilized *rbc*L data to assess genetic diversity, a crucial step for developing effective conservation strategies (Omonhinmin, 2023).

In bamboo species, the *rbc*L phylogenetic tree frequently aligned with traditional classifications. Species such as *B. vulgaris*, *B. bambos*, and various *Dendrocalamus* species clustered together in both the *rbc*L phylogenetic tree (Figure 3) and traditional taxonomy. This congruence underscores a strong correlation between certain morphological traits and genetic structure, indicating association between the morphological traits and the genetic structure, thus validating the use of these traits in traditional bamboo classification systems. For instance, the morphological similarities in culm and leaf characteristics among these species were reflected in their close genetic relationships, reinforcing the continued use of these traits in bamboo taxonomy. Strong phylogenetic groupings were observed for species like *B. tulda* and *D. strictus*, which are solidly positioned within their respective taxonomic frameworks. These examples highlight the complementary role of *rbc*L data in confirming well-established taxonomic groupings while also identifying areas where reclassification may be necessary. The close alignment between genetic data and traditional morphology in these cases reaffirms the reliability of certain morphological traits for species identification and classification.

As with the ITS2 and matK phylogenetic trees, the *rbc*L one also revealed discrepancies that challenge traditional classifications. For example, species like *Gigantochloa levis* and *Dendrocalamus bambusoides* were placed differently than expected based on morphological traits. These inconsistencies suggest that morphological characteristics may have evolved convergently in different bamboo lineages, or that traditional classifications based solely on physical features may require revision considering genetic data. This reinforces the notion that relying exclusively on morphology can sometimes mask deeper genetic relationships, especially in groups like bamboo, where phenotypic plasticity or environmental adaptation may influence physical traits.

The *rbc*L data further complicated traditional taxonomic boundaries by revealing close genetic relationships between species across different genera. For instance, species within the *Gigantochloa* and *Dendrocalamus* genera sometimes appeared more closely related genetically than their morphological traits would suggest. This blurring of generic boundaries called into question the rigidity of genus-level classifications and suggests the need for taxonomic revision to better reflect underlying genetic relationships. Revising genus boundaries based on genetic evidence could provide a more accurate picture of bamboo evolution and improve the classification's utility in evolutionary and ecological research.

The integration of *rbc*L data into bamboo taxonomy has broader implications beyond species classification. Understanding the genetic diversity and evolutionary relationships within bamboo species is essential for developing conservation strategies, especially for endangered species and populations with restricted habitats. The *rbc*L gene provides

Fig. 3. Phylogenetic analysis of the Bambusoideae subfamily *rbc*L DNA barcode.

a molecular tool for assessing genetic diversity between species, which is critical for the sustainable management of bamboo resources. Conservation efforts can be informed by genetic insights into population structure, helping to prioritize species and populations that are most at risk. Additionally, the genetic relationships

uncovered by *rbc*L data can guide breeding programs aimed at improving bamboo species for agricultural, ecological, and economic purposes.

Compared to *rbc*L, *mat*K gene, a chloroplast marker, is favored for its higher variability, making it more efficient in identifying closely related species. In bamboo studies, *mat*K has demonstrated excellent genus-level resolution, achieving 100% accuracy in multiple studies (Utama et al., 2024; Yong et al., 2024). Similar findings have been observed in other plant groups, such as pitcher plants, where *mat*K effectively distinguished between species and genera (Gogoi et al., 2018). Its broader taxonomic utility makes *mat*K a valuable marker for genus-level classification in bamboo. However, despite its higher variability compared to *rbc*L, *mat*K struggles to resolve species-level relationships with the same precision as nuclear markers like ITS. For closely related bamboo species, the resolution provided by *mat*K is often insufficient, highlighting the need for complementary markers in species-level studies.

In contrast, *rbc*L utility lies primarily in broader taxonomic studies where species are more distantly related. For bamboo taxonomy, where species differentiation is challenging due to overlapping morphological traits, *rbc*L provides valuable insights into higher-level evolutionary relationships but falls short for species-level resolution.

The ITS marker excels in species-level studies across a variety of plant and fungal taxa and has proven to be the most effective marker for species identification in bamboo. Its capacity to resolve fine-scale genetic differences gives it a distinct advantage over *mat*K and *rbc*L, making it the preferred choice when detailed species-level differentiation is required. While each marker has its specific strengths, their combined use offers a more comprehensive understanding of bamboo taxonomy. *mat*K generally provides superior genus-level resolution compared to *rbc*L, but both markers serve distinct roles in bamboo phylogenetics.

Conclusion

The comparison of the *mat*K, *rbc*L, and ITS markers in bamboo taxonomy demonstrates that each offers distinct advantages at different taxonomic levels. *mat*K provides strong resolution at the genus level, making it useful for broad taxonomic classification, though it struggles with distinguishing closely related species. *rbc*L, being highly conserved, excels in broad evolutionary analyses, but its low variability limits its effectiveness for species-level identification. In contrast, ITS offers the best species-level resolution, thanks to its higher variability, making it ideal for distinguishing closely related bamboo species where morphological traits and chloroplast markers fall short. An integrated approach that combines these three markers is essential for a comprehensive understanding of bamboo taxonomy, allowing for more accurate species identification and revealing deeper evolutionary relationships that traditional methods may miss. This multi-marker strategy complements morphological classification, helping to refine and resolve taxonomic ambiguities in bamboo.

Conflict of Interest

We declare that we have no conflicts of interest, whether financial, personal, or otherwise, with any individuals or organizations connected to the subject matter presented in this manuscript.

Acknowledgement

N/A.

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