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# MOLECULAR PHYLOGENETICS AND DIVERGENCE DATING OF BYTTNERIOIDEAE IN BANGLADESH USING *MATK* DNA BARCODE

SHEIKH SUNZID AHMED AND M. OLIUR RAHMAN[1]

*Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh*

**Abstract**

Byttnerioideae Burnett is an economically and medicinally important subfamily belonging to Malvaceae. A molecular phylogenetic and dating approach was undertaken to elucidate the evolutionary relationships and divergence history of the Byttnerioideae species available in Bangladesh based on the *matK* gene. A multifaceted, algorithm-driven strategy was applied with Maximum-Likelihood (ML), Maximum-Parsimony (MP), and Bayesian Inference (BI) algorithms that resulted in a consistent tree topology for the four tribes, namely as Byttnerieae, Hermannieae, Theobromateae and Lasiopetaleae. All the tribes exhibited monophyletic origin with strong bootstrap and posterior probability support. Byttnerieae, Hermannieae and Theobromateae were found to be more closely related to each other than to Lasiopetaleae. Molecular dating analysis revealed Lasiopetaleae as the oldest tribe, diverging approximately 39.51 million years ago (MYA) in the Bartonian age of the Cenozoic era, while Byttnerieae was identified as the most recent tribe, diverging around 33.24 MYA in the Rupelian age of the same era. These findings would update the phylogenetic relationship of Byttnerioideae and provide valuable insights for the development of the historical biogeography of Byttnerioideae in future endeavors.

*Keywords:* Byttnerioideae, Molecular phylogenetics, Molecular dating, *matK*, Bayesian inference.

## Introduction

Byttnerioideae, one of the nine subfamilies of the angiosperm family Malvaceae, comprises approximately 650 species across 26 genera worldwide. These genera are categorized into four tribes, namely Byttnerieae, Hermannieae, Lasiopetaleae, and Theobromateae. Predominantly distributed in tropical and subtropical regions (Lima *et al*., 2019), Byttnerioideae is represented in Bangladesh by seven taxa belonging to seven genera under three tribes (Ahmed and Rahman, 2024). The characteristic features of the subfamily Byttnerioideae include a 5-lobed calyx, a 5-lobed corolla, numerous stamens united at the base to form a tube-like structure, schizocarpic or capsular fruits and seeds covered with hairs (Ahmed and Rahman, 2024). This subfamily includes numerous economically and medicinally significant species in Bangladesh including *Theobroma*

[1]Corresponding author. Email: oliur.bot@du.ac.bd

*cacao* L., *Melochia corchorifolia* L., *Abroma augustum* (L.) L. f. and *Kleinhovia hospita* L.

Molecular phylogenetic studies using the chloroplast (cp) genome are crucial for understanding evolutionary relationships, offering high-resolution insights into genetic divergence and species differentiation. The cp genome is particularly valuable due to its conserved structure and maternal inheritance, providing a stable genetic framework for phylogenetic analysis (Guan *et al*., 2024). Among chloroplast genes, *matK* (maturase K) is notable for its exceptional ability to differentiate closely related taxa, owing to its high evolutionary rate and substantial sequence variation, surpassing commonly used genes such as *rbcL* and *ndhF* (Kathriarachchi *et al*., 2005; Müller *et al*., 2006; Li *et al*., 2011; Ahmed *et al*., 2023). The *matK* gene ensures the correct maturation of RNA transcripts, supporting chloroplast functionality and efficiency, which are vital for plant growth and survival (Barthet and Hilu, 2007; Hertel *et al*., 2013). This dual role makes *matK* a powerful tool in evolutionary studies as well as a key component of the chloroplast's genetic machinery (Poovitha *et al*., 2016). While the *ndhF* gene has been used to study floral evolution and molecular phylogenetics within Byttnerioideae, research using the *matK* barcode for phylogenetic reconstruction in this subfamily is lacking (Whitlock *et al*., 2001). Molecular dating using *matK* data facilitates current understanding of evolutionary events within Byttnerioideae. The substantial sequence variation in *matK* enables more accurate estimation of divergence times, allowing reconstruction of detailed timelines of species differentiation and revealing historical biogeographical patterns as well as evolutionary dynamics (Konhar *et al*., 2016; Ahmed *et al*., 2023).

Molecular dating further provides the baseline to enhance our understanding of the mechanisms driving speciation and adaptation in Byttnerioideae species. Knowledge of evolutionary relationships and divergence times can inform breeding programs aimed at enhancing desirable traits, such as disease resistance, yield, and adaptability in Byttnerioideae taxa (Pessoa-Filho *et al*., 2017), potentially leading to the development of more resilient and productive crops. Therefore, molecular dating analysis holds a significant promise in refining classification, guiding conservation efforts, and linking evolutionary developments with geological and climatic shifts (Sauquet, 2013). However, similar to molecular phylogenetic studies, no efforts have yet been made to unveil molecular dating events in Byttnerioideae.

A comprehensive understanding of Byttnerioideae evolution requires an integrated approach that combines molecular phylogenetics and molecular dating. Molecular phylogenetics, particularly when using high-resolution markers such as the *matK* gene,

reconstructs evolutionary relationships, whereas molecular dating places these events within a temporal framework. By employing *matK*-based molecular phylogenetics and molecular dating, we aim to elucidate the evolutionary relationships and estimate divergence times of species in accordance with the geological time scale. This in-depth analysis provides a more detailed understanding of the evolutionary dynamics of Byttnerioideae in Bangladesh.

**Materials and methods**

*Molecular phylogenetic analysis*: Gene sequences from various taxa within Byttnerioideae, including outgroups, were retrieved from the NCBI Nucleotide database. Eleven taxa were selected based on the availability of *matK* gene sequences (Table 1). The *matK* sequence for *Ayenia elegans* Ridl. was unavailable in GenBank and could not be retrieved. The obtained sequences were downloaded in FASTA format and aligned using Multiple Sequence Alignment (MSA) on the MAFFT server, specifically employing the E-INS-i method and the BLOSUM62 scoring matrix for amino acid sequences (Katoh *et al*., 2019). Default settings were used for the alignment, and the resulting sequences were saved in FASTA format for further analysis (Tamura *et al.,* 2021). The Models module in MEGA was then used to calculate the transition-transversion bias. Phylogenetic trees were constructed using the Phylogeny module, employing both Maximum Likelihood (ML) and Maximum Parsimony (MP) methods with 1000 bootstrap replicates. The Kimura-2 parameter model was applied, which accounts for both transition and transversion substitutions with uniform rates across sites. Gaps or missing data were handled using partial deletion, with a site coverage cutoff of 95%. Bayesian evolutionary analyses were performed using Mr. Bayes v.3.2.6 (Ronquist *et al*., 2012), employing the General Time Reversible model with 10,000 generations in the Markov Chain Monte Carlo process. Trees were sampled every 10 generations after discarding the first 250 trees as burn-in. Posterior trees were then summarized to infer phylogenetic relationships and estimate associated parameters.

*Molecular dating analysis*: Molecular dating was conducted using the Clocks module in MEGA v.11, employing the RelTime-ML method (Mello, 2018), starting with matK alignment file and the Maximum Likelihood Tree file in NEWICK format. Outgroup species were then chosen, and the calibration was refined using the TimeTree server to select two calibration nodes based on available taxa and applying uniform distribution constraints (Kumar *et al.,* 2017). The Kimura-2 parameter model was employed for nucleotide substitution, assuming uniform substitution rates throughout the analysis. The

resulting time tree was then visualized using the default Tree Explorer (Srivathsan and Meier, 2012).

**Table 1. Taxa investigated in the present study for phylogenetic relationships among Byttnerioideae in Bangladesh.**

| No. | Taxa | Species codes | Tribe/Family | GenBank accession |
|---|---|---|---|---|
| Ingroup | | | | |
| 1 | *Abroma augustum* (L.) L. f. | Aba | Byttnerieae | HM488448.1 |
| 2 | *Ayenia grandifolia* (DC.) Christenh. & Byng | Ayg | Byttnerieae | KP093635.1 |
| 3 | *Commersonia fraseri* J. Gay | Cof | Lasiopetaleae | KM894706.1 |
| 4 | *Guazuma ulmifolia* Lam. | Guu | Theobromateae | GQ982003.1 |
| 5 | *Kleinhovia hospita* L. | Klh | Byttnerieae | HM488449.1 |
| 6 | *Melochia corchorifolia* L. | Mec | Hermannieae | KY607288.1 |
| 7 | *Rulingia magniflora* F. Muell. | Rum | Lasiopetaleae | HM488451.1 |
| 8 | *Theobroma cacao* L. | Thc | Theobromateae | GQ982111.1 |
| 9 | *Waltheria indica* L. | Wai | Hermannieae | OL538011.1 |
| Outgroup | | | | |
| 1 | *Daphne mucronata* Royle | Dam | Thymelaeaceae | MZ851783.1 |
| 2 | *Thymelaea hirsuta* (L.) Endl. | Thh | Thymelaeaceae | OK040774.1 |

## Results and discussion

Molecular phylogenetic analysis of *matK* sequence data revealed distinct patterns among the selected Byttnerioideae taxa, which were retrieved from the NCBI Nucleotide database along with the outgroups using their GenBank accession numbers (Table 1). Following multiple sequence alignment (MSA), transition and transversion sites were analyzed, revealing sequence variability across the *matK* alignment (Table 2).

The transitional substitution rate was 54.75% and the transversional rate was 45.25%, indicating that transitional substitution was predominant over transversional substitution. This finding is consistent with a recent molecular phylogenetics study of the palm family Arecaceae (Ahmed *et al*., 2023). Predominant transitional substitutions can lead to better resolution and more reliable phylogenetic relationships, as transitions are generally less disruptive to protein structure and function (Drummond and Wilke, 2008). Similarly, a DNA barcoding study on *Mentha longifolia*, *Mentha* × *piperita*, and *Lavandula dentata* employing the *ITS*, *ITS2*, *rbcL*, and *trnH* genes found that transition mutations occurred more frequently than transversions (Ahmed, 2018). In addition, *matK* sequences were

used to study the phylogenetic relationships of six *Saraca* L. species (Fabaceae), revealing higher transitional than transversional substitutions (Sil *et al*., 2021). Together, these findings support the use of *matK* gene, with its high rate of transitional substitutions, as a reliable marker for resolving phylogenetic relationships among closely related species.

**Table 2. Substitution matrix analysis showing transition/transversion rates of the *matK* sequences after multiple sequence alignment.**

| DNA bases | A | T | C | G |
|---|---|---|---|---|
| A | - | *5.02* | *4.92* | **8.15** |
| T | *4.20* | - | **10.22** | *5.02* |
| C | *8.62* | **21.44** | - | *3.15* |
| G | **14.94** | *11.02* | *3.30* | - |

Each entry indicates probability of substitution from one base (row) to another base (column). Substitution pattern and rates were estimated under the General Time Reversible model. Rates of different transitional substitutions are shown in **bold** face, while those of transversional substitutions are shown in *italics*.

Of the four tribes of Byttnerioideae, Byttnerieae, Hermannieae, and Theobromateae are represented in Bangladesh, whereas Lasiopetaleae does not occur; however, all four tribes, including Lasiopetaleae, were included in the present study to reconstruct phylogenetic relationships within the subfamily Byttnerioideae. Taxa from the family Thymelaeaceae were chosen as outgroup due to their close resemblance to Malvaceae, as both families belong to the order Malvales (Bredenkamp and Van Wyk, 2001). The Maximum Likelihood (ML) tree showed the monophyletic origin for all four tribes of Byttnerioideae. All nine taxa representing the four tribes exhibited a clear segregation pattern in the phylogenetic tree (Fig. 1). Bootstrap support was higher than 80% for most terminal nodes, confirming the accuracy of the constructed tree.

Monophyletic segregation is crucial for understanding relationships among closely related Byttnerioideae taxa, as it ensures that all members of this subfamily share a common ancestor and include all its descendants. This clear delineation helps accurately reconstruct evolutionary histories and understand the genetic and phenotypic similarities and differences among the studied taxa. Focusing on the monophyletic groups helps avoid misleading conclusions that might arise from convergent evolution or horizontal gene transfer (Chaw *et al*., 2000). The Maximum Parsimony (MP) tree corroborated the topology revealed by the ML tree, exhibiting a similar clustering pattern and a clear segregation of taxa (Fig. 2). Cross-validation using these two approaches is significant, as

the convergence of ML and MP trees indicates that the inferred evolutionary relationships are not artifacts of a specific analytical method but instead reflect true biological patterns.

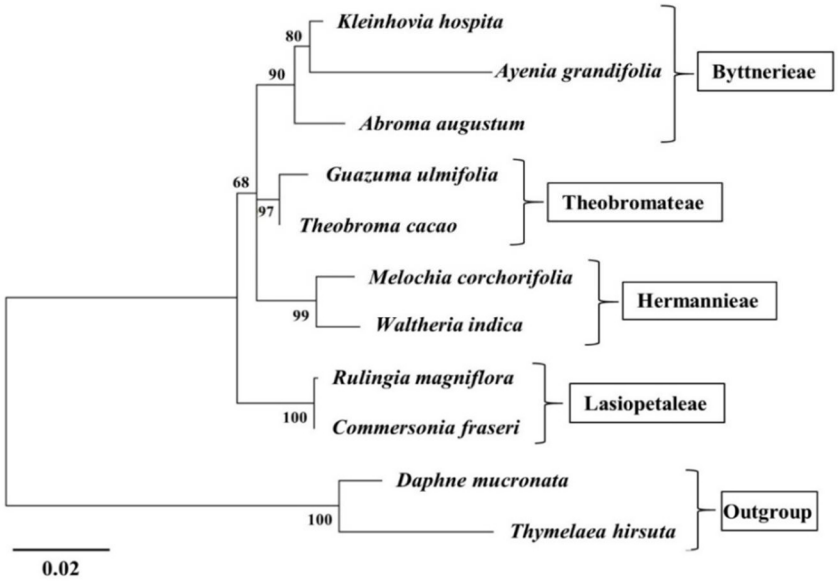


Fig. 1. Maximum-Likelihood tree with 1000 bootstrap replicates showing inter-relationships of Byttnerioideae taxa based on *matK* sequences.

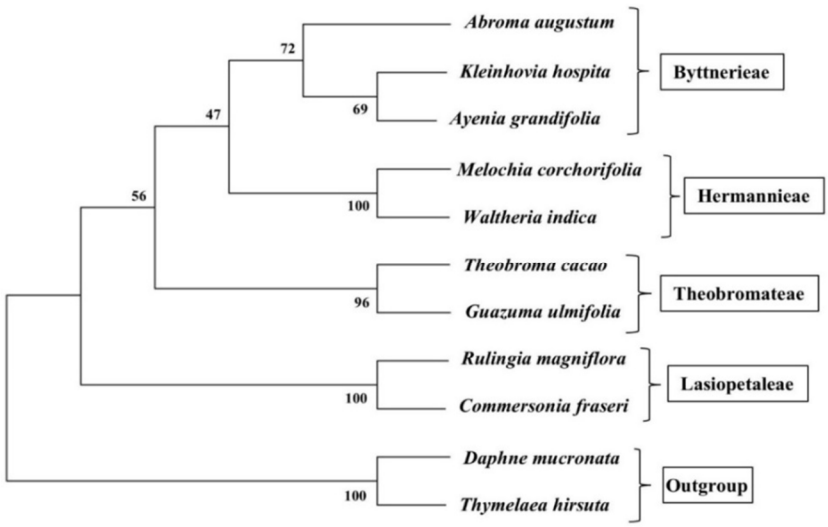


Fig. 2. Maximum-Parsimony tree with 1000 bootstrap replicates showing inter-relationships of Byttnerioideae taxa based on *matK* sequences.

In the MP tree, the tribe Lasiopetaleae, represented by *Rulingia magniflora* and *Commersonia fraseri,* demonstrated a distant relationship from the other three tribes, viz. Byttnerieae, Hermannieae and Theobromateae. Similar to the ML tree, the MP tree also unraveled the monophyletic nature of all four tribes. The genetic distance matrix identified the lowest distance (0.001), indicating maximum similarity between *Rulingia magniflora* and *Commersonia fraseri* (Table 3). In contrast, the highest genetic distance (0.183), indicating minimum similarity was observed between *Melochia corchorifolia* and *Thymelaea hirsuta*

**Table 3. Genetic distance matrix showing pairwise distance generated after multiple sequence alignment.**

| Species | Aba | Ayg | Cof | Dam | Guu | Klh | Mec | Rum | Thc | Thh | Wai |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Aba | 0 | | | | | | | | | | |
| Ayg | 0.057 | 0 | | | | | | | | | |
| Cof | 0.043 | 0.073 | 0 | | | | | | | | |
| Dam | 0.155 | 0.179 | 0.153 | 0 | | | | | | | |
| Guu | 0.033 | 0.059 | 0.033 | 0.149 | 0 | | | | | | |
| Klh | 0.019 | 0.043 | 0.036 | 0.156 | 0.026 | 0 | | | | | |
| Mec | 0.045 | 0.075 | 0.043 | 0.159 | 0.034 | 0.038 | 0 | | | | |
| Rum | 0.045 | 0.075 | 0.001 | 0.153 | 0.034 | 0.038 | 0.045 | 0 | | | |
| Thc | 0.026 | 0.055 | 0.026 | 0.143 | 0.006 | 0.019 | 0.028 | 0.028 | 0 | | |
| Thh | 0.172 | 0.174 | 0.174 | 0.043 | 0.170 | 0.168 | 0.183 | 0.174 | 0.164 | 0 | |
| Wai | 0.045 | 0.073 | 0.038 | 0.155 | 0.034 | 0.038 | 0.019 | 0.039 | 0.028 | 0.176 | 0 |

Aba: *Abroma augustum*, Ayg: *Ayenia grandifolia*, Cof: *Commersonia fraseri,* Dam: *Daphne mucronata*, Guu: *Guazuma ulmifolia,* Klh: *Kleinhovia hospita,* Mec: *Melochia corchorifolia,* Rum: *Rulingia magniflora*, Thc: *Theobroma cacao*, Thh: *Thymelaea hirsuta*, Wai: *Waltheria indica.*

Bayesian inference analysis elucidated strong posterior probability support for nearly all nodes. Consistent with the ML and MP trees, the Bayesian tree also supported the overall tree topology and confirmed the monophyletic origin of Byttnerioideae (Fig. 3). Bayesian posterior probability estimation differs somewhat from bootstrap calculation. Bootstrap validation entails resampling the original dataset with replacement to generate multiple pseudo-replicates, reconstructing a phylogenetic tree for each, and determining how frequently each branch appears across these trees. This method provides support values as percentages, indicating the frequency of a branch's occurrence, which can imply confidence but not direct probabilities. In contrast, Bayesian posterior probability uses a model-based approach, employing Markov Chain Monte Carlo (MCMC) simulations to sample trees according to their likelihood and prior information (Chib, 2001). The

posterior probability for each branch is then calculated from the proportion of sampled trees containing that branch, providing a direct probability measure. While bootstrap validation is simpler and widely used, it may overestimate support and is computationally intensive. Bayesian methods, though also computationally demanding and sensitive to model and prior selection, offer a more accurate and probabilistic interpretation of branch support (Efron, 2012).

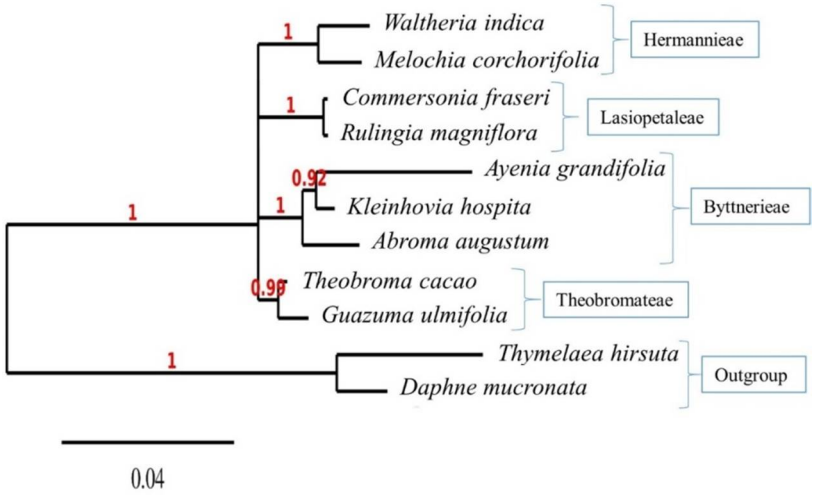


Fig. 3. Bayesian tree showing evolutionary relationships of Byttnerioideae taxa based on *matK* sequences. Posterior probability support has been denoted with red color.

In the present study, the similar clustering patterns observed in the Bayesian, ML, and MP trees reveal robustness and confidence in the inferred evolutionary relationships of Byttnerioideae. The agreement among multiple analytical methods reduces sensitivity to methodological variation and enhances the overall reliability of the findings. This concordance further helps detect and mitigate method-specific biases, ensuring a more accurate representation of the underlying evolutionary mechanisms of Byttnerioideae. A similar approach was undertaken to understand the phylogenetics of Amaranthaceae using *matK* and *trnK* sequences (Müller and Borsch, 2005), which further supports our protocol. A study using *ndhF* sequences reported that Byttnerieae, Hermannieae, and Lasiopetaleae are more closely related to each other than to Theobromateae in the maximum parsimony (MP) tree (Whitlock *et al*., 2001). Conversely, our investigation using *matK* sequences indicated that Byttnerieae, Hermannieae, and Theobromateae are more closely allied with each other than to Lasiopetaleae in the MP tree. The variation in tree topology between the *ndhF* and *matK* sequences may be attributed to several factors, including gene-specific evolutionary rates, horizontal gene transfer, incomplete lineage

sorting, sampling error, or differing functional constraints. The *ndhF* gene may be evolving under different selective pressures or experiencing more rapid changes compared to the *matK* gene, leading to discrepancies in inferred relationships. Alternatively, the *matK* gene may provide a more accurate reconstruction of overall evolutionary history due to its critical role in cellular functions. Further comprehensive genomic studies are required to resolve these discrepancies.

Molecular dating analysis was performed after estimating pairwise divergent times to establish the nodes (checkpoints) of calibration. Two nodes were selected based on their availability in the TimeTree server, as no additional calibration points were accessible. These nodes represent well-supported relationships within Malvaceae and provide reliable divergence estimates for calibration. The TimeTree server revealed pairwise divergence times for two calibration nodes. The first node, between *Theobroma* and *Guazuma* showed a median divergence time of approximately 18.4 million years ago (MYA). The second node, between *Melochia* and *Waltheria* exhibited a median divergence time of approximately 11.2 MYA (Fig. 4).

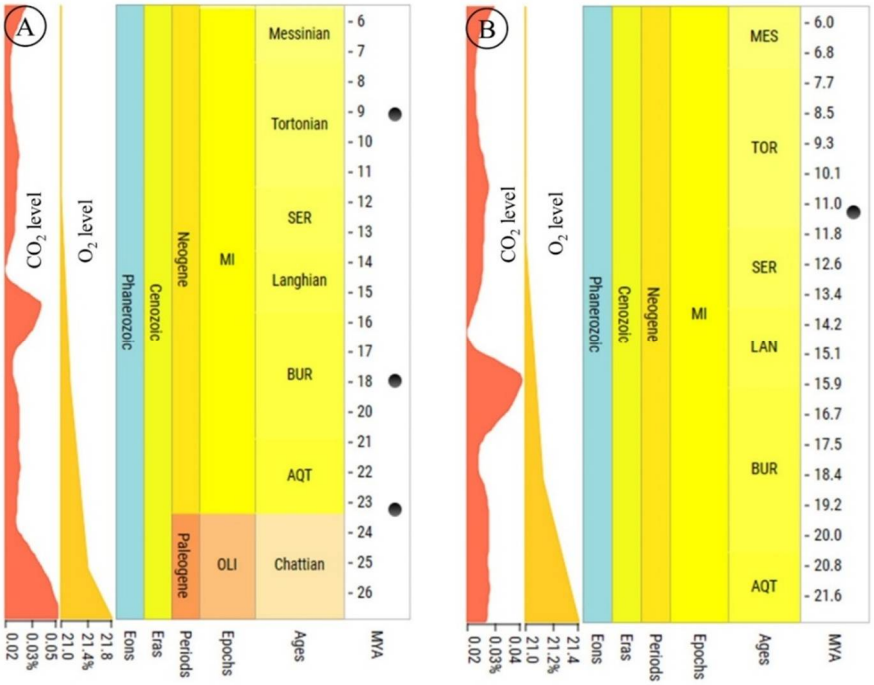


Fig. 4. Pairwise divergent times according to the geological time scales used to calibrate the time tree for molecular dating analysis. A. Divergence between *Theobroma* and *Guazuma*, B. Divergence between *Melochia* and *Waltheria.*

The credibility interval (CI) for the first node ranged from 9.0 to 22.9 MYA, and the adjusted divergence time was 18.0 MYA. The CI for the second node could not be retrieved from the server due to the paucity of available data; therefore, the adjusted divergence time remained 11.2 MYA.

The molecular dating analysis suggested that the divergence of Byttnerioideae began with Lasiopetaleae approximately 39.51 MYA, during the Bartonian age of the Eocene epoch within the Paleogene period of the Cenozoic era (Fig. 5). The Bartonian age (41.2 to 37.8 MYA) was a critical period for angiosperm evolution and diversification. This period witnessed an expansion of tropical and subtropical forests, facilitated by warm and humid climate conditions, which provided diverse habitats for angiosperms to thrive. Moreover, many advanced angiosperm families diversified during this time, resulting in increased richness in trees, shrubs, and herbaceous plants. Additionally, there was a significant advancement in plant-pollinator interactions, with angiosperms developing specialized floral structures and mechanisms to attract insects, birds, and mammals. These evolutionary developments likely played an important role in shaping the existing diversity and complexity of Byttnerioideae (Moraweck *et al.*, 2015).

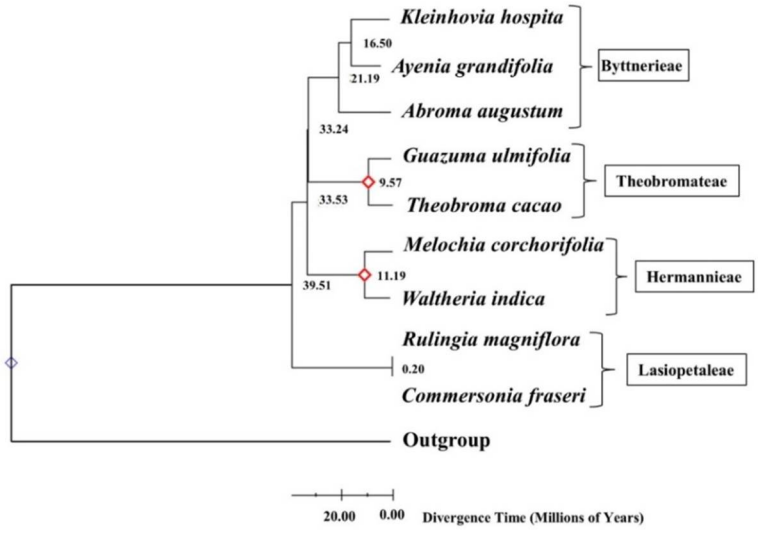


Fig. 5. Molecular dating tree showing divergence time of the four tribes of Byttnerioideae.

Molecular dating analysis further unveiled Lasiopetaleae as the oldest tribe (39.51 MYA), while Byttnerieae was identified as the most recent tribe (33.24 MYA). Except for Lasiopetaleae, all three tribes diverged during the Rupelian age of the Oligocene epoch

within the Paleogene period of the Cenozoic era. The Rupelian age, spanning approximately 33.9 to 28.1 MYA, was marked by significant global cooling and the expansion of Antarctic ice sheets, leading to substantial changes in both marine and terrestrial ecosystems. Marine organisms had to adapt to cooler water, resulting in shifts in species composition and distribution. On land, mammals continued to diversify, evolving new forms and adapting to the cooler climatic conditions. Overall, the Rupelian age represents a critical period of ecological transition, reflecting the broader climatic shifts of the Oligocene epoch (Fornelli *et al.*, 2020).

## Conclusion

Molecular phylogenetics and divergence-time analyses were carried out for the first time on Bangladeshi members of Byttnerioideae using the *matK* gene. The study unveiled a higher rate of transitional over transversional substitutions. The reconstructed phylogeny of the four tribes - Byttnerieae, Hermannieae, Theobromateae and Lasiopetaleae, confirmed the monophyletic origin of Byttnerioideae, with all nine taxa exhibiting distinct segregation patterns. Multi-model inference using ML, MP, and Bayesian approaches further corroborated the tree topology and inter-relationships of the species with high bootstrap and posterior probability support. Molecular dating analysis suggested that Lasiopetaleae is the earliest diverging tribe, originating around 39.51 MYA, whereas Byttnerieae represents the most recent lineage, diverging approximately 33.24 MYA. Overall, the findings enrich current understanding of the phylogenetic relationships within Byttnerioideae and provides valuable insights into the evolutionary history of this subfamily in Malvaceae.

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