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RESEARCH ARTICLE

Reinstatement of *Kayea assamica* Prain: evidences from morphological and molecular phylogenetic analysis

Prantik Sharma Baruah, Bhaben Tanti, Sachin Kumar Borthakur*

Department of Botany, Gauhati University, Jalukbari, Guwahati -781014, Assam, India.

*Corresponding author: skbgu1@gmail.com (S.K. Borthakur)
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Abstract

Mesua assamica, an evergreen tree previously included under Guttiferae [Clusiaceae] is now treated as a member of the family Calophyllaceae (APG III system of classification). The plant was originally described as *Kayea assamica* but later transferred to the genus *Mesua*. To overcome the ambiguity, we investigated the correct taxonomic status of the taxa using both morphological taxonomic tools and molecular analysis.

Keywords: *Mesua assamica*; *Kayea assamica*; micro morphology; *trn*L-*trn*F intergenic spacer sequence; phylogeny; reinstatement

1. Introduction

Mesua assamica (King & Prain) Kosterm., an evergreen tree, has been earlier treated under the family Guttiferae Juss. [Clusiaceae Lindl.]. However, in the APG III system of classification (APG 2009), the genus *Mesua* has been placed in a separate family Calophyllaceae along with 11 other genera. The species was first described by King and Prain (1901) as *Kayea assamica* on the basis of a specimen, (amongst several other collections) of H.G. Young on 29th June, 1900 from Dibrugarh, Assam, India. The plant has been found restricted only to a few localities in India, Myanmar and Malay Peninsula. In India, the plant is confined only to the sub-montane forests of Lakhimpur and Dhemaji districts of Assam (Kanjilal et al., 1934). The plant has been reported as rare and endangered species with a limited range of distribution (Choudhuri, 2007; Baruah et al., 2016, 2017, 2020).

In the family Guttiferae, one of the major taxonomic controversies is the status of two closely related genera, *i.e., Kayea* and *Mesua*. Linnaeus (1753) first introduced the genus *Mesua* with the type species *Mesua ferrea* in his "*Species Plantarum*" while, Wallich (1831) first introduced the genus *Kayea* in his "*Plantae Asiaticae Rariories*" with the type species *Kayea floribunda*. Since then, *Kayea* Wall. and *Mesua* L. were treated as two distinct genera under Guttiferae. Based on the nature of ovary and stigma structures *Kayea* has been distinguished from *Mesua*. Members of *Kayea* are characterised by the presence of one-celled ovaries with one seed and four-fid stigma, whereas members of *Mesua* have a two-celled ovary and peltate stigma (Bentham, 1862). Subsequently, Kostermans (1969) observed that one and two-celled fruits may be found with one or two seeds on the same individual tree of *M. ferrea*. Although he admitted he did not study adequate materials of different species of *Kayea* but he observed two-seeded fruits in several species of *Kayea*. As such Kosterman (1969) was sceptical about the congeneric status of *Kayea* and *Mesua*. Consequently, *Kayea assamica* Prain has been treated as basionym of *Mesua assamica* (King & Prain) Kosterm. in subsequent publications.

The decision of Kosterman (1969) of merging Kayea with *Mesua* has since been followed by subsequent workers (Whitmore, 1973; Keng, 1978; Corner, 1988; Chua, 1995; Turner, 1995; Kochummen, 1997). Subsequently, Stevens (1993) made the observations that the above two genera may also readily be distinguished by their usual growth pattern, morphological attributes, and anatomical features as well as xanthone chemistry. He also pointed out that there is no evidence that *Kayea* and *Mesua* form a monophyletic group and accordingly he treated both the genera separately, which was followed by Turner (2000). In the APG III system of classification (APG, 2009), the genus *Kayea* has been placed in a separate family Calophyllaceae along with 11 other genera including *Mesua*. The molecular phylogenetic analysis based on the trnL-trnF intergenic spacer sequence of *Mesua lepidota, M. kunstleri, M. racemosa* and *M. Corneri* (all formerly placed in *Kayea*) indicated that all these species are distinct from *M. ferrea*, and hence all these species of *Mesua* have been reverted to the genus *Kayea* (Zakaria, 2007). As such molecular phylogeny supports the classification of Stevens (1993) and Turner (2000) to separate *Kayea* from *Mesua*. Zakaria (2007) also recommended the reinstallation of the genus *Kayea* and transfer of *Mesua* species, except for *M. ferrea,* back to the genus *Kayea*. In the work of Zakaria (2007) *Mesua assamica* was not included probably because of its limited distribution and information for which it might have been overlooked. In this context the taxonomic study on *Mesua assamica* using both taxonomic tools and molecular analysis become imperative to determine its correct taxonomic status.

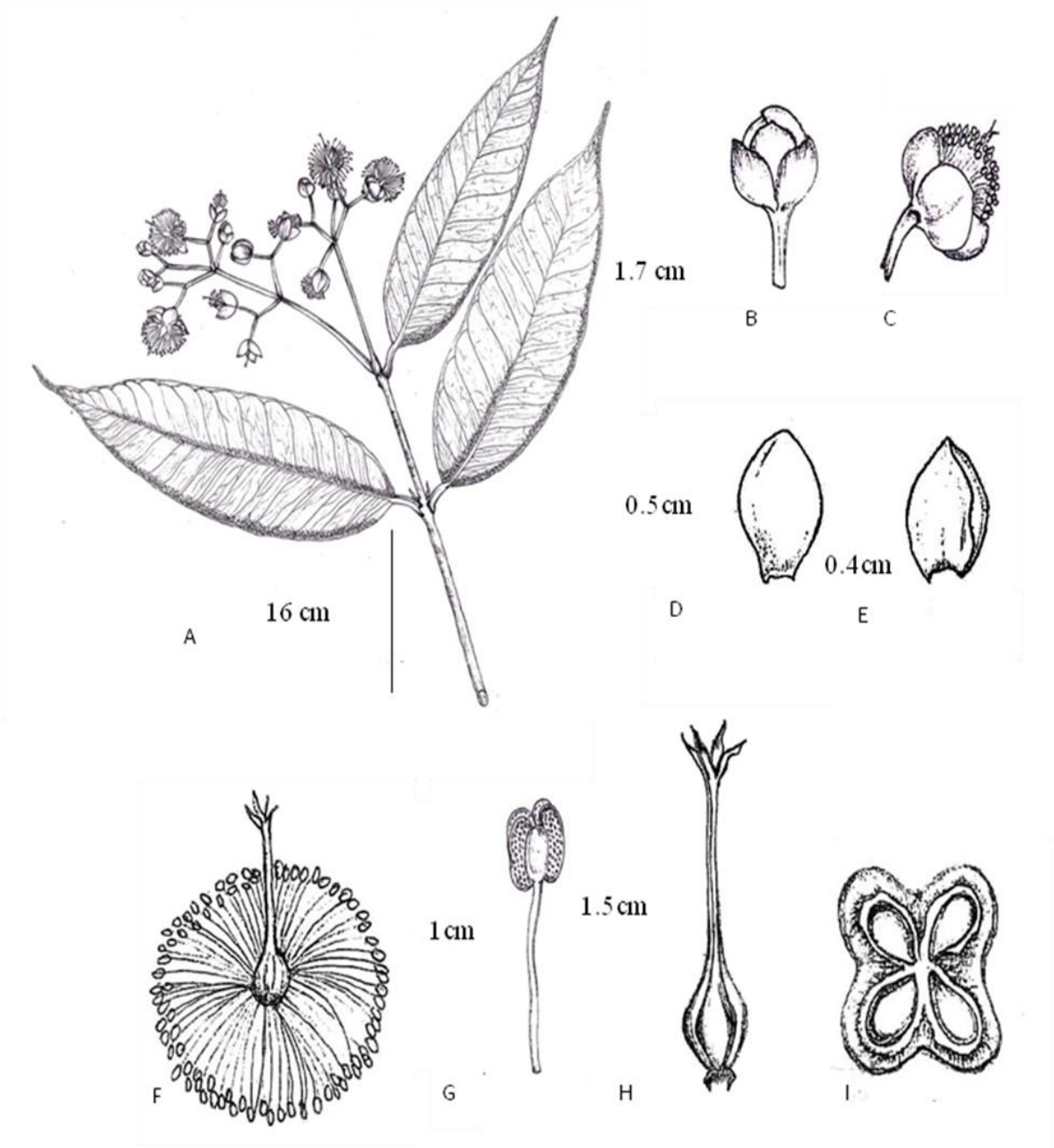

Figure 1. Illustration of *Mesua assamica*; **A.** habit (a flowering twig), **B.** flower bud, **C.** flower, **D.** sepal, **E.** petal, **F.** reproductive whorls bearing gynoecium and androecium, **G.** stamen, **H.** carpel, **I.** transverse section of ovary.

2. Material and methods

2.1. *Collection of plant materials*

Mesua ferrea (type of *Mesua*) and *Mesua assamica* (plant of interest) were studied from Lakhimpur and Dhemaji districts of Assam, India, and *Kayea floribunda* (type of *Kayea*) was studied from Garo Hills of Meghalaya, India by undertaking field work during 2015-2017 in different seasons of the year. Fresh material was collected for corroborating the morphological features as well as for molecular analysis. The specimens collected have been preserved as herbarium specimens following the standard herbarium techniques (Jain and Rao, 1977) and deposited in the Gauhati University Herbarium (GUBH). Three replicates of each were used throughout the study.

2.2. *Foliar and floral morphology*

Foliar micro-morphological characters such as foliar epidermal characters, nature of stomata, venation pattern along with floral morphological characters such as inflorescence architecture, nature of androecium, gynoecium, and ovary structures were taken into consideration.

2.3. *Epidermal treatments*

Fresh leaves have been treated with 8.0 N nitric acid and 10% sodium hydroxide solution to remove the epidermal peels, followed by staining with safranin (1%) and mounted in glycerine. Camera lucida sketches were made and photographed (Nikon Eclipse E200). Stomatal frequency and stomatal index were calculated out of ten readings. The terms used for describing stomata were that of Hickey (1973) and Metcalfe and Chalk (1950). The classification

and terminology of epidermal morphology was elaborated following Ramayya and Rajagopal (1980).

2.4. *Scanning electron microscopic analysis*

Leaf samples were fixed in 3% glutaraldehyde followed by washing in 0.1 M sodium cacodylate buffer thrice at 15 min interval at 4 °C. This was followed by dehydration of the leaf samples twice in each of 30%, 50%, 70%, 80%, 90%, 95% and 100% acetone at 15 min interval at 4°C. Dehydrated samples were then immersed in tetra methyl silane for 5-10 min twice at 4°C, allowed to dry at room temperature, mounted on brass stubs gold coated with sputter (cc. 35 nm thick) and finally observed under scanning electron microscope (Model-JEOL JSM-6390LV).

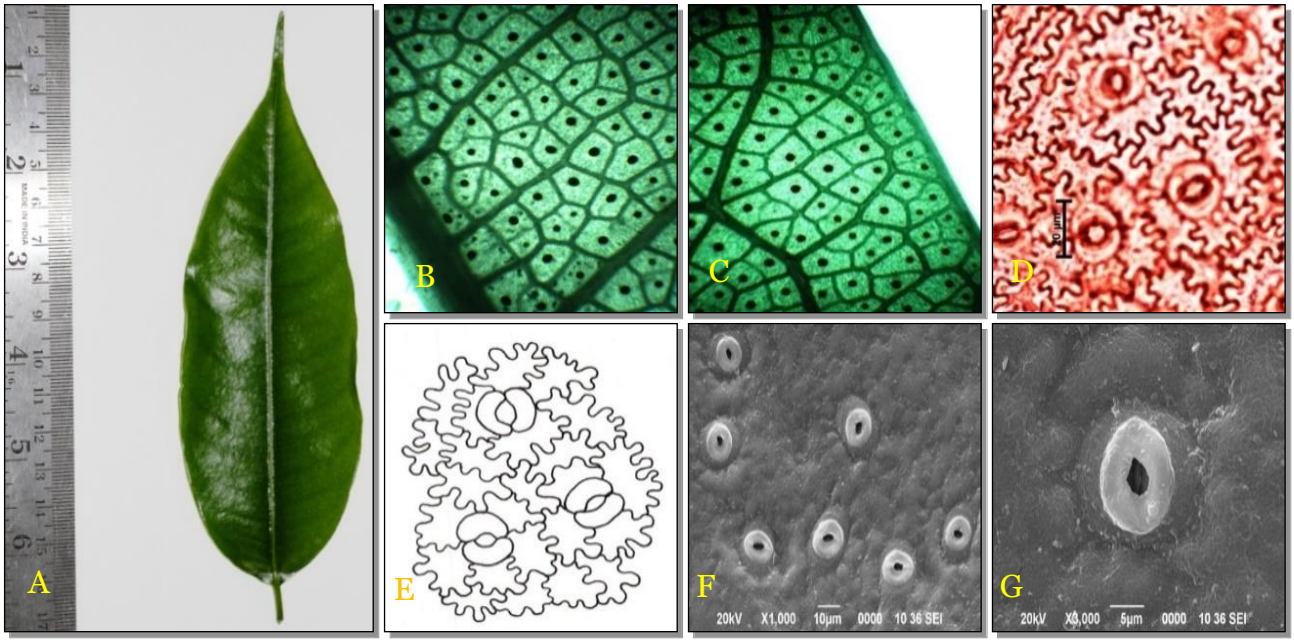

Figure 2. Foliar morphological features of *M. assamica*; **A.** ovate leaf with acuminate apex and rounded cuneate base, **B.** semi-craspedodromous venation (upto 5° veins), **C.** vein endings forming distal loops, **D.** anomocytic stomata (10x), **E.** camera lucida drawing of stomata, **F.** scanning electron microscopic image of stomata (1000x), **G.** scanning electron microscopic image of stomata (3000x).

2.5. *DNA extraction, amplification and sequencing*

DNA extraction of *Mesua assamica* was done from fresh leaf materials following the CTAB method (Doyle and Doyle, 1987). The *trn*L-*trn*F intergenic spacer of the chloroplast genome was amplified using universal primers (*trn*L-*e*:5′-GGTTCAAGTCCCTCTATCCC-3′and*trn*L-*f*:5′-ATTTGAACTGGTGACACGAG-3′) (Taberlet et al., 1991). PCR amplification was performed in a total volume of 10 µl with 50 ng DNA template, 1 µl 10x PCR buffer, 0.2 mM dNTPs, 5 pmol each primer (forward and reverse) and 1 unit *Taq* DNA polymerase following 5 min pre-heating at 95 °C, 1 min denaturation at 95 °C, 1 min annealing at 58 °C, and 1 min extension at 72 °C for 35 cycles with final extension at 72 °C for 5 min and the reaction was stopped at 4 °C (SimpliAmp-Applied Biosystems). The PCR product was checked by 1% agarose gel electrophoresis and stained with ethidium bromide; fragment sizes were estimated by comparison to molecular marker of 100 bp.

Amplified product of *trn*L-*trn*F intergenic spacer region of *Mesua assamica* was then sequenced using cycle sequencing Kit along withDS-35 dye setusing Automated DNA Sequencer (Applied Biosystems, ABI3730 xl). Sequencing was performed bidirectionally for *trn*L-*trn*F intergenic spacer with the same primers**.** Chromatograms were manually checked and visualized using ChromasPro. Chromatograms were further converted to FASTA format by codon code aligner software. The sequence was submitted to NCBI GenBank and accession number was obtained as MK513658.

2.6. *Phylogenetic analyses*

Sequences of *Mesua assamica* were compared with corresponding DNA sequences of other related species available in the NCBI by BLAST and few sequences with high similarity were downloaded for phylogenetic analysis (Altschul et al., 1997). BLASTn was performed to ascertain its homology to non-redundant nucleotide databases (nr). Significance of BLAST results were tested by expected values (e-value) generated by search algorithm (Table 1).

The *trn*L-*trn*F intergenic spacer sequence were aligned using CLUSTAL X2 (Thompson et al., 1994) as offline application and corrected visually using Bioedit. Ambiguous regions in the alignment were excluded from the phylogenetic analysis manually. Pairwise distances among the individuals were calculated using MEGA6.2 with default parameters. Maximum parsimony (MP) tree was generated using default parameters (Tanti et al., 2012; Sarma and Tanti, 2017). The phylogenetic trees were tested for authenticity using bootstrap method at 1000 replicates (Felsenstein, 1985).

Table 1. Downloaded sequences from GenBank showing high similarity with *Mesua assamica*

SN	Description of closest species match	Gene Bank accession number	References	Query cover	Identity
1	*Calophyllum inophyllum*	GQ456079	Zakaria et al., 2007	99%	93%
2	*Calophyllum rupicola*	AY389781	Zakaria et al., 2007	100%	100%
3	*Calophyllum soulattri*	GQ456080	Zakaria et al., 2007	96%	90%
4	*Clusia major*	AY144086	Zakaria et al., 2007	90%	86%
5	*Clusiarosea*	AY144095	Zakaria et al., 2007	90%	85%
6	*Kayea kunstleri*	AJ606678	Zakaria et al., 2007	100%	94%
7	*Kayea lepidota*	AJ606677	Zakaria et al., 2007	81%	96.31%
8	*Mammea brevipes*	AY389790	Zakaria et al., 2007	99%	93%
9	*Mammea siamensis*	AJ606679	Zakaria et al., 2007	100%	96%
10	*Mesua ferrea*	AY389792	Zakaria et al., 2007	81%	93.44%

3. Result

3.1. Taxonomic treatment

Mesua assamica (King & Prain) Kosterm., in *Reinwardtia*. 1969.7:426; *Assam's Flora* (*Present status of vascular plants* by Chowdhury (2005), pp. 83; *Indigenous Plants and Birds of Assam*, 2010. p. 48; *Plant Diversity of Assam-A Checklist of Angiosperms & Gymnosperms*, 2014. pp. 56; *Kayea assamica* Prain (basionym) in *Indian Forester* 27: 62. 1901 et in Notes and Papers 420.1901 (reprints); typus: Baker, Young (BM, G, K). [Some confusions with author-citation].

Evergreen slow growing trees, 25 m tall, trunk straight, bark glabrous. Leaves simple, opposite; lamina acuminate, symmetrical, light to dark green in colour, coriaceous, 9.0-17.5 × 2.8-6.5 cm, margin entire, apex and base angle acute, apex acuminate, base rounded, petiole 0.7-1.3 cm long, venation semicraspedodromous with 28–34 secondary veins, forming distinct distal loops, inter-secondary veins bold and several. Tertiary veins transversely orientated, alternate or opposite; higher order veins other than quaternary veins absent; areolae well developed, usually pentagonal, rarely hexagonal without free ending veins (FEVs). Epidermal cells elongated, irregular, mixed (invasive and symplastic) on both adaxial and abaxial surfaces. Leaves hypostomatic, stomata anomocytic, 11.50-12.50 µm, stomatal index 16.67. Cymes paniculate, Flowers 2-3 cm in diameter, ebracteate, bisexual, corolla white or creamy white fragrant, floral buds sub-globose; sepals 4, 0.6-0.8 cm, coriaceous, depressed at the base; petals 4, obovate, 0.8-1.1 cm, entire, white, deflexed over the calyx on opening; stamens numerous, 0.90-0.95 cm long; filament white, anthers bithecal, spherical, golden yellow; carpels two, syncarpous, c. 1.0 cm; ovary superior, four-chambered; style linear; stigma four-fid. Mature fruit upto 5 cm in diameter, globose, 1- seeded (Figure 1).

3.2. Comparative morphological analyses of *M. assamica* with the type of the genus *Mesua* and *Kayea*

Comparative analysis of morphological attributes of both foliar and floral features exhibits distinction of *Mesua assamica* from the type material of the genus *Mesua* with respect to its characteristic generic features; rather showed similarities with the characteristic features of the genus *Kayea*. The reddish green newly borne leaves form a characteristic foliage crown which is a distinguishing feature of members of the genus *Mesua,* but not in the members of the genus *Kayea*. Leaves in species of *Mesua* are intensely glaucous on the abaxial surface while shiny pale brown on the adaxial surface. Veins and veinlets are indistinct on both the sides while secondary veins are without forming distal loops. In contrast, leaves of *Kayea* spp. are found to be glossy green with prominent veins and veinlets on both the sides and secondary veins terminate forming distal loops (Figure 2). The comparative morphological analysis of floral characters of *Mesua assamica* with the type material of the genus *Mesua* and *Kayea* is presented in Table 2. The most remarkable points of similarities of *Mesua assamica* with the generic characters of *Kayea* are in leaf shape (elliptic-lanceolate), nature of lamina-tip (acuminate), venation pattern semi-craspedodromous, inflorescence type (cymose-panicle), nature of stigma (four fid), number of ovules present in ovary (four), number of seeds present in mature fruit (one); these are rather contradictory from the generic characters of the genus *Mesua*.

Table 2. Comparative morphological analyses of *Mesua assamica* with the type materials of the genus *Mesua* and *Kayea*

Features of comparison	Qualitative morphological distinction		
	Mesua assamica	*Mesua ferrea*- type material of *Mesua*	*Kayea floribunda*- type material of *Kayea*
Leaf shape and organisation	Ovate-lanceolate, Simple	Elliptic, Simple	Lanceolate, Simple
Leaf apex	Acuminate	Attenuate	Shortly acuminate
Leaf base	Rounded-Cuneate	Cuneate (Straight)	Cuneate
Symmetry	Symmetrical	Symmetrical	Symmetrical
Texture	Coriaceous	Coriaceous	Coriaceous
Tooth type	Absent	Absent	Absent
Hairs	Absent	Absent	Absent
Venation	Semi-Craspedodromous	Reticulodromous	Semi-Craspedodromous
Petiolar feature and attachment	Pulvinate, Marginal	Pulvinate, Marginal	Pulvinate, Marginal
Leaf margin type and Lobation	Entire, unlobed	Entire, unlobed	Entire, unlobed
Inflorescence type	Panicle-like Cyme	Single flowered Cyme	Panicle-like Cyme
Gynoecium type	Monocarpous (unicarpellate)	Monocarpous (unicarpellate)	Monocarpous (unicarpellate)
Nature of stigma	Four-fid	Peltate	Four-fid
Number of ovules present in ovary	Four	Two	Four
Number of seeds present in mature fruit	One	Two	One
Type of adhesion of anther to filament	Anther globose, Basifixed	Anther elongated, Basifixed	Anther globose, Basifixed

3.3. Molecular phylogenetic analysis

PCR amplified product of *trn*L-*trn*F intergenic spacer region of *Mesua assamica* yielded 462 bp sequences which was deposited in the NCBI and obtained the accession no (MK513658). Based on the blast search of *Mesua assamica* (MK513658), the closest sequences downloaded from NCBI i.e., GQ456079, AY389781, GQ456080, AY144086, AY144095, AJ606678, AJ606677, AY389790, AJ606679 and AY389792 representing *Calophyllum inophyllum* L., *Calophyllum rupicola* Ridl., *Calophyllum soulattri* Burm.f., *Clusia major* L., *Clusia rosea* Jacq., *Kayea kunstleri* King, *Kayea lepidota* Pierre, *Mammea brevipes* (Craib) Kosterm., *Mammea siamensis* T. Anders. and *Mesua ferrea* L. respectively were subjected for phylogenetic analysis. To evaluate the phylogenetic relationships among the selected taxa, phylogenetic tree was constructed using maximum parsimony (MP) method. In this investigation, all the individual samples were clustered into two major groups constituting four different clades with high bootstrap support. As shown in the results of the phylogenetic tree, all the four clades reflected the intergeneric variation. *Mesua ferrea* was clustered with the members of *Calophyllum* (clade I). However, in this investigation, our experimental plant *i.e., M. assamica* was found taking place in clade III with the members of *Kayea*. On the other hand, the members of *Mammea* and *Clusia* were differentiated into two separate clades (clade II and clade IV respectively) (Figure 3). In this investigation, our experimental plant (*Mesua assamica;* MK513658) along with the other members of *Kayea* revealed paraphyletic lineage with the other clades.

4. Discussion

Bentham (1862), Ridley (1910 and 1922) and Melchior (1964) used generative characters to distinguish *Kayea* and *Mesua*. Generative characters appear to be more consistent than the fruit characters in *Mesua* and *Kayea*. However, Kostermans (1969) merged *Kayea* under *Mesua* based on number of seeds per fruit cell. Consequently, all the taxa previously included under *Kayea* had been transferred to *Mesua,* which was indeed supported by several workers (Whitmore, 1973; Keng, 1978; Corner, 1988; Chua, 1995; Kochummen, 1997) for a long time. Subsequently, molecular phylogeny based on *trn*L-*trn*F intergenic spacer sequences established that *Mesua lepidota, M. kunstleri, M. racemosa* and *M. corneri*, formerly placed in *Kayea* were distinct from *Mesua ferrea* (Zakaria, 2007). Moreover, Ruhfel et al (2011) reported all genera of Calophyllaceae as monophyletic in their analyses with tribe

Calophylleae containing several well-supported subclades. The first subclade contains the strictly 'New World genera' *viz*., *Caraipa*, *Clusiella*, *Haploclathra*, *Kielmeyera*, *Mahurea* and *Marila*. However, the second subclade includes *Kayea*, *Mammea* and *Poeciloneuron*. On the other hand, the third subclade includes *Calophyllum* and *Mesua*.

In the present study, comparative morphological attributes of foliar as well as floral features show a clear distinction of *M. assamica* from the type specimen of *Mesua*, and rather showed similarities with the type specimen of *Kayea*. The occurrence of new drooping young reddish green leaves in members of *Mesua* is entirely absent in *Kayea* spp. The leaves of *Mesua* spp. are shiny green on adaxial surface while glaucous on abaxial surface with indistinct veins and veinlets on both the surfaces. Moreover, secondary veins do not form any distal loops along the margin of the leaf blade. On the other hand, leaves of *Kayea* spp. are glossy green with prominent veins and veinlets on both the surfaces. The secondary veins terminate along the leaf margin forming distal loops. Foliar morphological features *viz*., ovate with acuminate apex, rounded base, semi-craspedodromous venation pattern with anomocytic stomatal type in *Mesua assamica* differ from the characters of the type species of *Mesua* (*M. ferrea*) in having elliptic leaf with attenuate apex, cuneate-straight base, reticulodromous venation pattern with paracytic stomatal type. Moreover, occurrence of paniculate inflorescence, nature of anther, four-fid style and ovary containing four ovules in *Mesua assamica* marked its distinction from the type species of *Mesua* (*M. ferrea*) bearing single flowered cyme, nature of anther, peltate stigma and ovary bearing two ovules. Apart from distinctions in morphological and reproductive features, the phylogenetic tree (maximum parsimony) produced four separate clades with high bootstrap support. In the dendrogram, with the exception of *Mesua assamica*, the *Mesua ferrea* included with the *Calophyllum* clade with high bootstrap support. All the *Kayea* taxa formed a well-supported separate clade along with *Mesua assamica*. Thus, *Mesua assamica* was found to be closely related to *Kayea kunstleri* and *Kayea lepidota*. On the other hand, *Clusia* and *Mammea* formed individual clades with high bootstrap support. The results of phylogenetic analysis not only supported the findings of Stevens (1993) and Turner (2000) to separate the taxa *Kayea* from *Mesua* but also with the findings of Ruhfel et al (2011).

5. Conclusion

The present investigation revealed a positive correlation between morphological and molecular attributes which distinctly separated *Mesua assamica* from the *Mesua* taxa and nested with members of *Kayea*. Here, molecular phylogenetic analysis supported the findings of Stevens (1993) and Turner (2000) to separate *Kayea* from *Mesua*. Based on the overall experimental findings, it is therefore, strongly recommended to reinstate *Kayea assamica* from *Mesua assamica*.

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Author's contributions

BT and SKB have conceptualized the problem, designed the experiment. PSB has conducted the experiments and compiled the manuscript and finally SKB and BT have corrected and finalized the manuscript.

Conflict of interests

The authors declares that there is no conflict of interest

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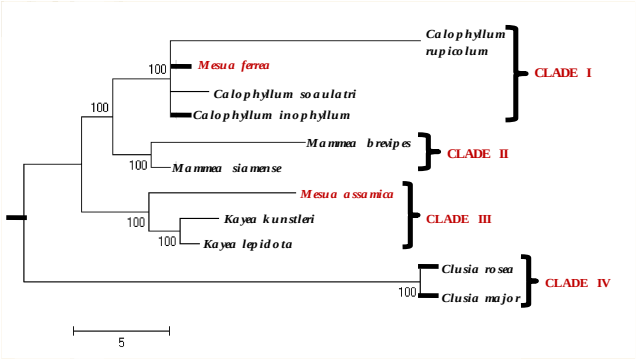

Figure 3. Dendrogram based on the *trn*L-*trn*F intergenic spacer sequences of the experimental plant (*Mesua assamica*) along with other related members obtained from NCBI.

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