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A taxonomic study of *Actinodaphne* (Lauraceae) in Southeast Asia based on multiplexed inter-simple sequence repeats genotyping by sequencing (MIG-seq) and Classic DNA barcodes

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Abstract

The genera *Actinodaphne* Nees and *Neolitsea* Merr. (Lauraceae) include ~100 each spp. of evergreen trees that mainly occur in Asia, and both morphological analysis and molecular phylogenetic analysis have supported that *Actinodaphne* and *Neolitsea* are closely related to *Litsea* Lam. Both *Actinodaphne* and *Neolitsea* can be distinguished from *Litsea* by leaves that are whorled or clustered in the nodes of branches, and *Actinodaphne* and *Neolitsea* can be distinguished on the basis of flower morphology. Recent molecular phylogenetic studies have suggested that *Neolitsea* is monophyletic but *Actinodaphne* is not. However, the resolution of the phylogenetic trees generated by these studies has been relatively low, owing to limited numbers of phylogenetically informative characters. In this study, we employed multiplexed inter-simple sequence repeats genotyping by sequencing (MIG-seq) to obtain finely resolved phylogenetic trees, in addition to phylogenetic analyses using internal transcribed spacer (ITS) sequences of ribosomal DNA. Here, we describe the results from phylogenetic analyses combined with morphological studies.

In Chapter I, a new species of *Actinodaphne* (Lauraceae), *Actinodaphne lambirensis* Tagane, Yahara & Okabe is described from Lambir Hills National Park, Miri District, Sarawak, Malaysia based on a MIG-seq tree, ITS tree, and morphological observation. Because only fruiting specimens were available for *A. lambirensis,* we confirmed its position in the phylogenetic trees obtained from 22 *Actinodaphne* spp. including the type species of the genus, *A. pruinosa* Nees, and 11 *Neolitsea* spp. from Southeast Asia, MIG-seq. In addition, we reconstructed a phylogenetic tree using ITS sequences for 36 *Actinodaphne* spp. and 40 *Neolitsea* spp. that included the 22 MIG-seq samples and additional species of *Actinodaphne* for which ITS sequences were

determined in previous studies. Both MIG-seq tree and ITS tree supported that *A. lambirensis* belongs to *Actinodaphne*.

In chapter II, we examined effectiveness of MIG-seq for phylogenetic reconstruction and species discovery of *Actinodaphne* and *Neolitsea* in Southeast Asia. We compared a MIG-seq tree reconstructed for 25 and 45 species of *Actinodaphne* and *Neolitsea*, respectively, with an ITS tree for 18 and 33 species of two genera. As a result, 119 of 162 (72 %) branches and 26 of 88 (30 %) branches were supported by bootstrap values of 85 % or larger in MIG-seq and ITS trees, respectively. In the 20 nodes supported by both ITS and MIG-seq trees, a bootstrap support to each node was always higher on the MIG-seq tree. In one of two inconsistent cases between the MIG-seq tree and the ITS tree, topologies of the MIG-seq tree agreed with morphological resemblance. In the MIG-seq tree, *Actinodaphne* was separated into two clades: *Actinodaphne* 1 including *A.* aff. *tsaii* 1 and *A.* aff. *tsaii* 2, and *Actinodaphne* 2 including the other 23 spp. *Actinodaphne* 1, *Actinodaphne* 2, and *Neolitsea* were almost equally differentiated. The MIG-seq tree supported sister relationship for 18 pairs of species, and sister species of each pair are distinguished by diagnostic traits. In both genera, morphologically similar species were often not sister to each other, suggesting repeated parallel evolution of leaf traits. On the MIG-seq tree, 6 *Actinodaphne* spp. and 30 *Neolitsea* spp. did not match any described species and are likely to be undescribed species. These results showed that a highly resolved phylogenetic tree by MIG-seq is effective to discover and delimitate new species.

In chapter III, a new genus *Neoactinodaphne* Okabe, Tagane & Yahara, including two new species and a variety were described from Vietnam and Thailand. This new genus is characterized by well-developed intervening veins perpendicularly extending between secondary veins. Phylogenetic analyses based on MIG-seq showed

that this new genus, having 3-merous flowers with 9 stamens, was sister to but distinct from *Neolitsea*, having 2-merous flowers with 6 stamens. Principal component analysis and a cluster analysis by Unweighted Pair Group Method using arithmetic Average were performed for a total of 67 species of *Actinodaphne* and *Neoactinodaphne* using six leaf traits: maximal number of leaves clustered on the branch top (MLC), midpoint petiole length (PL), midpoint leaf length (LL), midpoint leaf width (LW), midpoint lateral veins (LV), midpoint aspect ratio (AR). *Neoactinodaphne* is placed among species of *Actinodaphne,* showing that *Neoactinodaphne* is difficult to be distinguished from *Actinodaphne* spp by leaf shape. The MIG-seq tree showed that *A. acuminata* was placed not in *Actinodaphne* but in *Litsea*. The MIG-seq tree and morphological observations supported that eight species of *Actinodaphne* (24 %) are considered to be undescribed. Our results showed that phylogenetic analyses using MIG-seq are effective to discover and describe new species if it is combined with morphometric analyses.

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Chapter III

A phylogenetic analysis of *Actinodaphne* (Lauraceae) using multiplexed inter-simple sequence repeats genotyping by sequencing (MIG-seq) and a multivariate analysis of leaf morphological traits revealed 10 undescribed species including two species of *Neoactinodaphne*, a new genus from Vietnam and Thailand

Chapter I

A New Species of *Actinodaphne* (Lauraceae), *A. lambirensis* from Sarawak, Malaysia, and an Analysis of its Phylogenetic Position using MIG-seq and ITS sequences

Abstract

A new species of *Actinodaphne* (Lauraceae), *Actinodaphne lambirensis* Tagane, Yahara & Okabe from Lambir Hills National Park, Miri District, Sarawak, Malaysia is described and illustrated. This species is characterized by glabrous twigs and leaves, small lamina (4.3–9.2 × 1.7–2.8 cm), and long fruiting peduncles. Because only fruiting specimens were available for *A. lambirensis,* we confirmed its position in the phylogenetic trees obtained from 22 *Actinodaphne* spp. including the type species of the genus, *A. pruinosa* Nees, and 11 *Neolitsea* spp. from Southeast Asia, using multiplexed inter-simple sequence repeats genotyping by sequencing (MIG-seq). In addition, we reconstructed a phylogenetic tree using internal transcribed spacer (ITS) sequences for 36 *Actinodaphne* spp. and 40 *Neolitsea* spp. that include our MIG-seq samples and additional species for which ITS sequences were determined in our previous studies. Both MIG-seq tree and ITS tree supported that *A. lambirensis* belongs to *Actinodaphne*.

Keywords:

Actinodaphne, Borneo, flora, Lambir Hills National Park, molecular phylogeny, *Neolitsea*, next-generation sequencing, taxonomy

Introduction

The tropical region of Southeast Asia harbors remarkable plant diversity as high as in tropical America (Kreft & Jetz 2007, Yahara *et al*. 2012, Middleton *et al*. 2019). However, taxonomic studies on vascular plants of this region remain incomplete, and about 3000 new species were described from Southeast Asia during 2011 to 2017 (Middleton *et al*. 2019). Considering the rapid loss of tropical forests in Southeast Asia, it is necessary to efficiently discover and describe new species (Yahara *et al*. 2012, Mase *et al*. 2020). Here, we describe a new species of *Actinodaphne* Nees (Lauraceae) based on fruiting specimens with its DNA sequences used to determine the genus.

The genus *Actinodaphne* includes ~100 spp. of evergreen trees that occur mainly in Asia (Rohwer 1993, van der Werff 2001), and both morphological analysis (Liou 1934) and molecular phylogenetic analysis (Rohwer 2000, Chanderbali *et al*. 2001) have supported that *Actinodaphne* is sister to *Neolitsea* (Benth. & Hook.f.) Merr. and these two genera are closely related to *Litsea* Lam. *Actinodaphne* and *Neolitsea* can be distinguished from *Litsea* by leaves that are whorled or clustered in the nodes of branches. *Actinodaphne* can be distinguished from *Neolitsea* on the basis of flower morphology (3-merous flowers with 9 stamens in *Actinodaphne vs.* 2-merous flowers with 6 stamens in *Neolitsea,* Li *et al*., 2008), but it is often difficult to distinguish two genera for sterile or fruiting specimens. Since only fruiting specimens are available for the new species, phylogenetic analysis is required to identify its genus.

Recent molecular phylogenetic studies have suggested that *Actinodaphne* is polyphyletic. By analyzing the *matK* and internal transcribed spacer (ITS) sequences of *Actinodaphne, Neolitsea,* and *Litsea*, Li *et al*. (2004) found that *Actinodaphne forrestii* (C.K.Allen) Kosterm. was sister to *Lindera megaphylla* Hemsl. and *A. obovata* (Nees) Blume was placed in the *Litsea* Clade. Subsequently, Li *et al*. (2006) analyzed the phylogenetic relationships among 13 spp. of *Actinodaphne* (11 from China and two

from Malaysia and Singapore) using ITS and external transcribed spacer (ETS) sequences and found that *Neolitsea levinei* Merr. and 11 *Actinodaphne* spp. (10 from China and *A. sesquipedalis* Hook.f. & Thomson ex Meisn. from Malaysia) were monophyletic, whereas the remaining two *Actinodaphne* spp. (*A. forrestii* from China and *A.* sp. from Singapore) were separated from this clade. Li *et al*. (2007) also analyzed the phylogenetic relationship of six *Actinodaphne* spp. from China, 29 *Neolitsea* spp., and four *Litsea* spp. using ITS and ETS sequences and reported that *A. forrestii* did not cluster with the other five *Actinodaphne* spp. Similarly, Mitsuyuki *et al*. (2018) analyzed the phylogenetic relationships among 46 *Neolitsea* spp., eight *Actinodaphne* spp., and one *Alseodaphne* spp. using ITS sequences and demonstrated that *Actinodaphne* was polyphyletic, with three *Actinodaphne* spp. (from China) being sister to *Neolitsea* and the other five, including *A. obovata* (from China) and *A. sesquipedalis* (from Cambodia), belonging to another clade. Meanwhile, Fijridiyanto & Murakami (2009) analyzed the phylogenetic relationships among 19 *Litsea* spp., six *Actinodaphne* spp., four *Neolitsea* spp., and seven *Lindera* spp. using *rpb2*, *matK*, *ndhF*, and nrITS sequences and found that all six *Actinodaphne* spp. (two from Indonesia and four from Malaysia) were monophyletic.

Considering the above molecular phylogenetic studies, we need to examine the phylogenetic position of the new species and justify that it is to be described as a species of *Actinodaphne.* The genus *Actinodaphne* is diversified in Southeast Asia where a total of 66 species is accepted in the Plant List (Anonymous 2019): four species from Vietnam, two from Thailand, four from Myanmar, 11 from the Phillipines, 23 from Indonesia, and 22 from Malaysia. However, previous phylogenetic studies of *Actinodaphne* examined limited number of species from Southeast Asia; two species in Li *et al*. (2006), three species in Mitsuyuki *et al*. (2018), and six species in Fijridiyanto

& Murakami (2009), and they did not include the type species of the genus, *A. pruinosa* Nees described from Peninsular Malaysia and Singapore (Nees 1831). To delimitate *Actinodaphne* and determine the phylogenetic postion of the new species, here we obtained a highly resolved phylogenetic tree for 22 *Actinodaphne* species gathered from Southeast Asia including the type species *A. pruinosa* using multiplexed ISSR genotyping by sequencing (MIG-seq; Suyama & Matsuki 2015). We also determined ITS sequences but less informative (details are in discussion). Using MIG-seq, Binh *et al*. (2018) successfully obtained a highly resolved phylogenetic tree of *Quercus langbianensis* Hickes & A.Camus and its relatives (Fagaceae), and described three new species. This study provides the second case where MIG-seq is effectively used for phylogenetic reconstruction and species discovery of vascular plants in Southeast Asia. The reconstructed molecular phylogenetic trees supported that a new species belongs to a clade of *Actinodaphne* including the type species *A. pruinosa*. Below, we first describe the species as *A. lambirensis* Tagane, Yahara & Okabe, *sp. nov.*, by characterizing its morphological traits. Then, we provide molecular phylogenetic evidence and discuss the phylogenetic position of *A. lambirensis*.

Materials and Methods

Field surveys

An undescribed taxon of *Actinodphne* was discovered during our field survey in Lambir Hills National Park in 2016. It grows near the summit of Mt. Lambir, at an elevation of 412 m.

In this study, 22 species of *Actinodaphne*, 11 species of *Neolitsea* and three species of *Litsea* (corresponding to 58 DNA samples used for MIG-seq analysis; 30

samples among them were used for sequencing the ITS region) were used, which gathered from a series of transect surveys in various locations of Southeast Asia (Tagane 2019). During these surveys, all the tree species within each 100 × 5 m plot were collected, even if plants were in sterile condition (Zhang *et al*. 2016, Mase *et al*. 2020). Among the species of *Actinodaphne* used in this study, we could collect flowering specimens only for *A. concinna* Ridl., *A. sesquipedalis*, and *A.* sp. 2, and fruiting specimens only for *A. lambirensis* and *A. perlucida* C.K. Allen.

Morphological observation

To evaluate the validity of the new species, we observed type specimens of the species of *Actinodaphne* accepted in the Plant List (Anonymous 2020) using the JSTOR Global Plants (http://plants.jstor.org/) and additional specimens deposited in the herbaria: ANDA, BK, BKF, BO, BRUN, FOF, KAG, NHL, RAF, SAR, TNS and VNM, and examined the taxonomic literature (Backer & van den Blink 1963, Wallich 1831, Kochummen 1989, van der Werff 2001, Julia 2005, Huang & van der Werff 2008, Tanaros et al. 2010, Pesler *et al.* 2011, de Kok 2019). If no type specimen image of a species is available on the web, we examined an original description for the species.

DNA extraction

Approximately 0.8 mm x 0.8 mm piece of silica gel-dried leaf samples were crushed using a QIAGEN TissueLyser and washed three times using 1-mL aliquots of buffer solution (0.1M HEPES, pH8.0; 2% mercaptoethanol; 1% PVP; 0.05M ascorbic acid), after which DNA was extracted from the leaf samples using the CTAB method of Doyle & Doyle (1987).

ITS sequencing and analysis

Ribosomal ITS sequences were amplified for 30 of the tree samples (23 spp.; Apendix 1, GenBank accession no: LC260478, LC504502–LC504529, LC502532) using Tks Gflex DNA Polymerase (Takara Bio, Kusatsu, Japan), previously described primers (ITS-18F: GTCCACTGAACCTTATCATTTAGAGG, ITS-26R: GCCGTTACTAAGGGAATCCTTGTTAG; Rohwer et al. 2009), and the following reaction conditions: 95°C for 4 min; 25 cycles of 94°C for 30 sec., 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. PCR products were subsequently purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). Purified amplification products were sequenced with Applied Biosystems 3730 DNA Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

In addition, ITS sequences were also obtained from the NCBI database (https://ncbi.nlm.nih.gov) for the 61 species of *Actinodaphne* and *Neolitsea* (84 sequences) studied by Li *et al*. (2006), Li *et al*. (2007), Mitsuyuki *et al*. (2018), and Fijridiyanto & Murakami (2009). Therefore, the final ITS dataset included 46 sequences from 36 *Actinodaphne* spp., 62 sequences from 40 *Neolitsea* spp., five sequences from three *Litsea* spp., and one sequence from *Machilus* sp. as an outgroup (Appendix 1).

For phylogenetic analysis, the DNA sequences were aligned using MEGA7 (Kumar *et al*. 2016), and after converting the alignment from fasta format to phylip format using kakusan4 (Tanabe 2011), a maximum-likelihood (ML) phylogenetic tree was constructed using RAxML (Stamatakis 2006) with 1000 bootstrap replicates.

MIG-seq

For 58 samples (37 species), we amplified 61,036–227,160 of short sequence from each genome using primers designed for MIG-seq following Suyama & Matsuki

(2015). The 1st PCR step was conducted to amplify inter-simple sequence repeats regions from genomic DNA using the MIG-seq primer set-1 (Suyama & Matsuki 2015). Those 1st PCR products were diluted 10 times for each 1st PCR product using deionized water, and purified, normalized, and size-selection was performed to remove ca. <250bp fragments using AMPure XP (Beckman Coulter, Brea, CA). The 2nd PCR step was performed independently to add individual indices to each sample with indexed primers. Then, 1 μL of each 2nd PCR product was pooled as a single mixture library. The mixture was purified and fragments in the size range ca. 400–800 bp were selected by AMPure XP. The concentration of size-selected library was measured by a SYBR green quantitative PCR assay (Library Quantification Kit; Clontech Laboratories, Mountain View, CA, USA), using approximately 12 pM of libraries that were used for sequencing on an Illumina MiSeq Sequencer (Illumina, San Diego, CA, USA), with a MiSeq Reagent Kit v3 (150 cycle, Illumina).

MIG-seq phylogenetic analysis

Quality control of the raw MIG-seq data was performed as described by Suyama & Matsuki (2015). Briefly, 14 bp of SSR region and 3 bp of anchor sequences in the first primers were trimmed from the MiSeq reads using fastx_trimmer, which is part of the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and high-quality reads were filtered using FASTQ Quality Filter in the FASTX-Toolkit with the criterion of q=30 and p=40 (q: quality cut-off value, p: percent of bases in sequence that must have quality equal to or higher than q). Next, TagDust (Lassmann *et al*. 2009) was used to remove reads from extremely short library entries (cut off for the false discovery rate = 0.01), the sequence primer region in the sequences of read 1 (forward sequences of the second PCR) and read 2 (reverse sequences of the second PCR) were searched

respectively, and the reads that had these sequences were removed.

After the quality control was complete, the remaining reads were assembled using de novo map pipelines (ustacks, cstacks, sstacks) in Stacks ver. 1.48 (Catchen *et al*. 2011). Homologous sequences (loci) were assembled in each sample using ustacks, with the following settings: minimum depth of coverage (m) = 3, maximum distance allowed between stacks (M) = 2, maximum distance allowed to align secondary reads to primary stacks (N) = 1, and maximum gaps = 2. A catalogue of consensus loci was built for each sample by using ustacks to assemble the loci, allowing only two mismatches between sample loci (n). A list of loci was obtained with following settings: minimum number of populations in a locus (p) = 1, and minimum percentage of samples in a population (r) = 0.025. We tested robustness of the position of *A. lambirensis* by changing r to 0.1, 0.2, 0.3, 0.4 and 0.5 (see Appendix 2 for a MIG-seq tree obtained using r = 0.5). The genotypes of the samples at each locus were provided by the populations pipeline output file "haplotypes.tsv". The bach_1.vcf-format file that included the SNP sites of all the samples was converted to phylip format and used to reconstruct a maximum likelihood (ML) tree in RAxML with 500 times bootstrap replicates. A total of 47,419 SNPs loci were used to construct the phylogenetic tree.

Taxonomy

Actinodaphne lambirensis Tagane, Yahara & Okabe, **sp. nov.**

Figure 1

Diagnosis. *Actinodaphne lambirensis* is distinct from all other *Actinodaphne* species in Borneo by a combination of glabrous twigs and leaves, small leaves (blade 4.3–9.2 ×

1.7–2.8 cm) and long fruiting peduncles (1.6–2.7 cm long in *A. lambirensis* vs. mostly sessile in the other species). The leaves are most similar to *Actinodaphne oleifolia* Gamble of the Malay Peninsular and Borneo, but easily distinguished by its midrib flat or shallowly sunken abaxially (vs. prominent in *A. oleifolia*) and long peduncles when fruiting.

Type. MALAYSIA, Sarawak, Miri District, Lambir Hills National Park, around the summit of Mt. Lambir, 04°11'56.3"N, 113°59'50.3"E, alt. 412 m, 23 July 2016, with fruits, *Yahara et al. SWK2556* (holotype SAR!, isotype K, KYO!).

Description. Small tree, 3 m tall. Bud scale ovate-triangular, ca. 1 mm long, apex acute, margin ciliate. Twigs terete, drying reddish brown to pale brown when young, grayish brown when old, glabrous. Leaves alternate, crowded; blade elliptic or ovate-elliptic, 4.3–9.2 × 1.7–3.8 cm, thinly coriaceous, apex shortly acuminate, base cuneate, margin entire, pale green adaxially, light pale yellow to light pale brown, glaucous abaxially, glabrous on both surfaces, midrib prominent adaxially, flat of shallowly sunken abaxially, secondary veins (6–)7–10 pairs, faintly visible adaxially, visible abaxially, tertiary veins reticulate, indistinct; petiole 1.2–2.2 cm long, glabrous. Flowers not seen. Infructescence solitary to shortened raceme appearing as pseudo-umbel consisting of 2–4 fruits, peduncle 1.6–3.5 cm long, glabrous. Fruits globose, ca. 6 mm in diam., with stigma-remnant at apex, blackish when dry, glabrous; perianth tube funnel-shaped, glabrous, decurrent to glabrous pedicel, the length of cupule and pedicel 7–8 mm long.

Additional specimens examined. SARAWAK. Miri District. Lambir Hills National Park: Mt. Lambir, 12 Jan. 1993, *Momose N8* (KYO); in Kerangas Forest, alt. 150–220

m, 20 Aug. 1994, with fruits, *Momose 1200* (Herbarium of the Japanese Laboratory in Lambir Hills National Park); ibid., 30 Aug. 1994, with fruits, *Nagamitsu 657* (Herbarium of the Japanese Laboratory in Lambir Hills National Park, KYO); ibid., Aug. 1995, *Momose 2456* (Herbarium of the Japanese Laboratory in Lambir Hills National Park); the summit of Mt. Lambir, in Kerangas, 8 Aug. 1992, *Nagamasu 4733* (KYO); ibid., 04°11'56.3"N, 113°59'50.3"E, alt. 412 m, 23 July 2016, *Yahara et al. SWK2554* (FU, SAR).

Phenology. Fruiting specimens were collected in July and August.

Distribution and habitat. This species is currently known only from Lambir Hills National Park, Miri District, Sarawak; 150–412 m elev. In our field observation, it grows along the edge of humid broad-leaved evergreen kerangas forest, at an elevation of 412 m just below the sumiit of Mt. Lambir. where we found a small population of less than 50 individuals.

Etymology. The specific epithet *lambirensis* reflects the type locality of this species.

GenBank accession No. *Yahara et al. SWK2556*: LC260477 (*rbcL*), LC260478 (ITS), LC260479 (*matK*).

Conservation status. The species is known only from the type locality and restricted to the peak area of Mt. Lambir. From our field observation, *A. lambirensis* is qualified for Critically Endangered (CR) according to the IUCN category (IUCN 2012) in that its limited distribution with an area of occupancy estimated to be less than 10 km^2

(criterion B2a) and a small population size estimated to be less than 50 (criterion D).

Results

Morphological observation

Among 19 species recorded in Sarawak (Julia 2005, Jawa & Chai 2007), six species are similar to *A. lambirensis* in having leaves shorter than 10 cm. Those species were carefully compared with *A. lambirensis* for nine morphological traits (Table 1). Among the six species, *A. oleifolia* is most similar to *A. lambirensis* in glabrous leaf surfaces, leaf shape and size with acuminate leaf apex, petiole length, the number of lateral veins, and reticulate tertiary veins. However, *A. lambirensis* is distinguished from *A. oleifolia* by obscure tertiary veins (vs. prominent on both surfaces), thinner leaf texture, midrib flat or shallowly sunken abaxially (vs. prominent in *A. oleifolia*), and much longer fruiting peduncle (1.6–2.7 cm long vs subsessile).

MIG-seq phylogenetic tree

The ML tree based on MIG-seq data showed high resolution, with 76 % (42/55) of the branches supported by bootstrap values of >90 % (Fig. 2). *Litsea* was placed outside of *Actinodaphne* and *Neolitsea* and was separated into two clusters (Fig. 2). One cluster (*Litsea* 1), which was supported by a bootstrap value of 100 %, included *L. johorensis* Gamble (T2421, T3066, SWK1917, SWK2629), and a second cluster (*Litsea* 2), which was supported by a bootstrap value of 100 %, included *L. accedens* Boerl. (SWK1827, SWK1896) and *L. verticillata* Hance (V3539). The monophyly of the clade that included both *Actinodaphne* and *Neolitsea* was supported by a bootstrap value of 100 % and was separated into three lower clades: (1) *Neolitsea* (bootstrap value

100 %), (2) *A.* aff. *tsaii*, and (3) the third clade that includes *A. pruinosa*, the type species of *Actinodaphne,* and the other *Actinodaphne* spp. examined (bootstrap value 100 %, Fig. 2). We hereafter refer to the third clade as *Actinodaphne s.str.* since the clade includes the type species of the genus, *A. pruinosa.* The second clade composed of *A.* aff. *tsaii* was not sisiter to *Actinodaphne s.str.* but to *Neolitsea*, and characterized by lanceolate to oblanceolate leaves usually with more than 12 lateral veins and prominent veinlets on both surfaces.

Actinodaphne s.str. was further separated into three clades, all with bootstrap values of 100 %. Clade 1 included *A. rehderiana* (C.K.Allen) Kosterm. ex Yahara (published in Nagahama et al. 2019; V4084), *A. leiophylla* (Kurz) Hook.f. (MY446, T4258), and *A. lambirensis* (SWK2556). Clade 2 supported by a bootstrap value 100 % included *A.* sp. 1 (S72), *A. diversifolia* Merr. (SW1727), *A.* aff. *diversifolia* (SWK620), *A.* sp. 2 (IK9), *A. glabra* Blume (SWK1028), *A. montana* Gamble (IS45, MY661), *A. heterophylla* Blume (IS854), *A. sesquipedalis* Hook.f. & Thoms. ex Hook.f. var. *cambodiana* Lecomte (1920, 708, 4722) and *A. sesquipedalis* var. *sesquipedalis* (MY366, V1594). Clade 3 supported by a bootstrap value 100 % included *A. pilosa* (Lour.) Merr. (V2960, V1363), *A.* sp. 5 (V2703), *A. henryi* Gamble (T3571), *A. perlucida* (V445, V508, V616), *A. amabilis* Kosterm. (T4910), *A. borneensis* Meisn. (SWK2517 and SWK2575), *A. sulcata* S.Julia (SW1107), *A. pruinosa* (SWK1199) and *A. concinna* (M178). Meanwhile, *Neolitsea* was separated into two clades: Clade 1 supported by a bootstrap value 100 % included *N. cassiifolia* Merr. (IJ598 and IJ740) and *N. latifolia* S.Moore (IS778) whereas Clade 2 supported by a bootstrap value 100 % included the remaining *Neolitsea* spp.

ITS-based phylogenetic tree

The ML tree based on ITS sequences showed much lower resolution than the MIG-seq tree, and only 18 of the 110 branches (16 %) had bootstrap values of > 90 % (Fig. 3). Among 23 species of *Actinodaphne* included in the MIG-seq tree, we could not determine ITS sequence for *A. pruinosa* and *A. perlucida*. Among the remaining 21 species, *A.* aff. *tsaii* was sister to *A. tsaii* Hu (AY817119), and the other 20 species were located in a clade with a bootstrap value of 55 %. This clade, corresponding to *Actinodaphne s.str.* in the MIG-seq tree, was separated into three clades supported by 52 %, 92 %, and 38 % bootstrap values, respectively. These three clades corresponded to Clade 1, Clade 2, and Clade 3 of the MIG-seq tree. As in the MIG-seq tree, Clade 1 of the ITS tree included *A. rehderiana*, *A. leiophylla*, and *A. lambirensis*. Clade 2 of the ITS tree included *A. glomerata* (Blume) Nees (AB260849), *A. procera* Nees (AB260854), *A. macrophylla* (Blume) Nees var. *angustifolia* (AB260850), *A. maingayi* Hook.f. (AB260851), and *A. myriantha* Merr. (AB260853), in addition to eight species of Clade 2 in the MIG-seq tree. Clade 3 of the ITS tree included *A. malaccensis* Hook.f. (AB260852), in addition to eight species of Clade 3 in the MIG-seq tree. The ITS tree included additional nine species that were placed outside of *Actinodaphne s.str*. First, *A. forestii* (AY265399) was basal to the clade including *Litsea*, *Actinodaphne* (except *A. forestii*) and *Neolitsea.* Second, seven *Actinodaphne* spp. from China including *A. lecomtei* C.K. Allen (AY817112) were clustered, and placed outside of a clade including *Actinodaphne s.str., Neolitsea,* and a clade including *A.* aff. *tsaii* and *A. tsaii*. Third, *A. paotingensis* Y.C.Yang & P.H.Huang (AY817118) was sister to *Neolitsea*.

Discussion

The resolution of the MIG-seq tree was clearly greater than that of the ITS tree: branches supported by bootstrap values of 90 % or higher amounted to 76 % in the

MIG-seq tree, but only 16 % in the ITS tree. In particular, the monophyly of *Actinodaphne s.str.* including *A. lambirensis* was supported by a bootstrap value of 100 % in the MIG-seq tree, but only by 55 % in the ITS tree. On the other hand, the topology of branches supported by bootstrap values of > 90 % was identical between the MIG-seq tree and the ITS tree. Based on these results, we consider the phylogenetic position of *A. lambirensis* mainly based on the MIG-seq tree.

The MIG-seq tree strongly supported that *A. lambirensis* belongs to *Actinodaphne s.str.* (a clade including the type species) and is closely related to *A. rehderiana* from southern Vietnam and *A. leiophylla* from Myanmar and Thailand. *Actinodaphne rehderiana* is endemic to Lamdong Province of sourthern Vietnam and distinct from *A. lambirensis* in having 1.6–2 cm long, thick peduncles of fruits (Allen 1938) and larger leaves originally described as 12–17 cm long (Allen 1938) but often attaining to 30 cm long (Nagahama *et al*. 2019). *Actinodaphne leiophylla* is a species described from Tenasserim Region of Myanmar (Hooker 1890), and distinct from *A. lambirensis* in having semi-triplinerved leaves 12–15 cm long; fruiting specimens of *A. leiophylla* have never been collected. Our collection MY446 collected from Tanintharyi, correspoinding to Tenasserim Region, and another collection T4258 from Peninsular Thailand neighboring to Tanintharyi, Myanmar, are sterile, but is identical with the type specimen of *A. leiophylla* in leaf morphology. These three species belonging to Clade 1 is sister to a clade (Clade 2 and Clade 3) including the other 19 species of *Actinodaphne s.str.* from Myanmar, Thailand, Cambodia, Vietnam, Malaysia and Indonesia. Among six species morphologically similar to *A. lambirensis* (Table 1), *A. pruinosa* and *A. borneensis* belonged to the latter clade. Further studies on the rest four species, *A. fuliginosa* Airy Shaw*, A. oleifolia, A. spathulifolia* S.Julia*,* and *A. semengohensis* S.Julia, are waited to deepen our understanding on the phylogenetic affinity of *A. lambirensis*

with those species. The ITS tree suggests that *A. glomerata, A. macrophylla* var. *angustifolia*, and *A. procera* of Indonesia and *A. maingayi, A. malaccensis,* and *A. myriantha* from Malaysia belong to the latter clade of *Actinodaphne s.str*. No Chinese species was placed in *Actinodaphne s.str.*

The ITS tree showed that *Actinodaphne* is unlikely to be monophyletic, as was suggested in previous studies (Li *et al*. 2004; Li *et al*. 2006; Li *et al*. 2007; Mitsuyuki *et al*. 2018). The following three groups are located outside of *Actinodaphne s.str.*: (1) *A. forestii*, (2) seven *Actinodaphne* spp. from China, and (3) *A.* aff. *tsaii* and *A. tsaii*. In addition, *A. paotingensis* was sister to *Neolitsea*. The resolution of the ITS tree is, however, too limited to determine the phylogenetic positions of these three groups. To determine phylogenetic positions of the above three groups, further phylogenetic studies are required and this study showed that MIG-seq provides a promising approach to obtaining more highly resolved phylogenetic trees.

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Table 1. Morphological characteristics of *Actinodaphne lambirensis* and six similar species.

Characters	*A. fuliginosa*	*A. lambirensis*	*A. oleifolia*	*A. spathulifolia*	*A. pruinosa*	*A. semengohensis*	*A. borneensis*
Hairliness of leaf blade	glabrous	glabrous	glabrous	hairy	glabrescent	hairy	glabrous
Leaf apex	rounded	shortly acuminate	shortly acuminate	obtuse	long acuminate	acute or acuminate	cuspidate
Leaf shape	obovate	elliptic, ovate-elliptic	elliptic-oblong	oblanceolate	elliptic or obovate	oblanceolate or narrowly elliptic	obovate to elliptic
Lamina size (cm)	2.5–4.5 × 1.5–2.5	4.3–9.2 × 1.7–2.8	4.0–9.5 × 1.5–3.0	5.0–7.5 × 2.5–4.0	7.5-13.5 × 2.5-4.0	7.5–9.5 × 2.0–2.5	9.0–14.5 × 3.5–5.5
Petiole (cm)	0.5–1.0	1.2–2.2	0.5–2.0	1.2–1.5	1.0–1.5	1.0–2.0	0.8–2.0
Lateral veins	4–6 pairs	7–10 pairs	6–10 pairs	5–6 pairs	7–9 pairs	4–6 pairs	3–7 pairs
Venation	reticulate	reticulate	reticulate	scalariform	scalariform	scalariform	scalariform
Tertiary veins	obscure	obscure	prominent	prominent	obscure	obscure	obscure
Fruit peduncle (cm)	unknown	1.6–2.7	subsessile	unknown	<1.0	unknown	subsessile

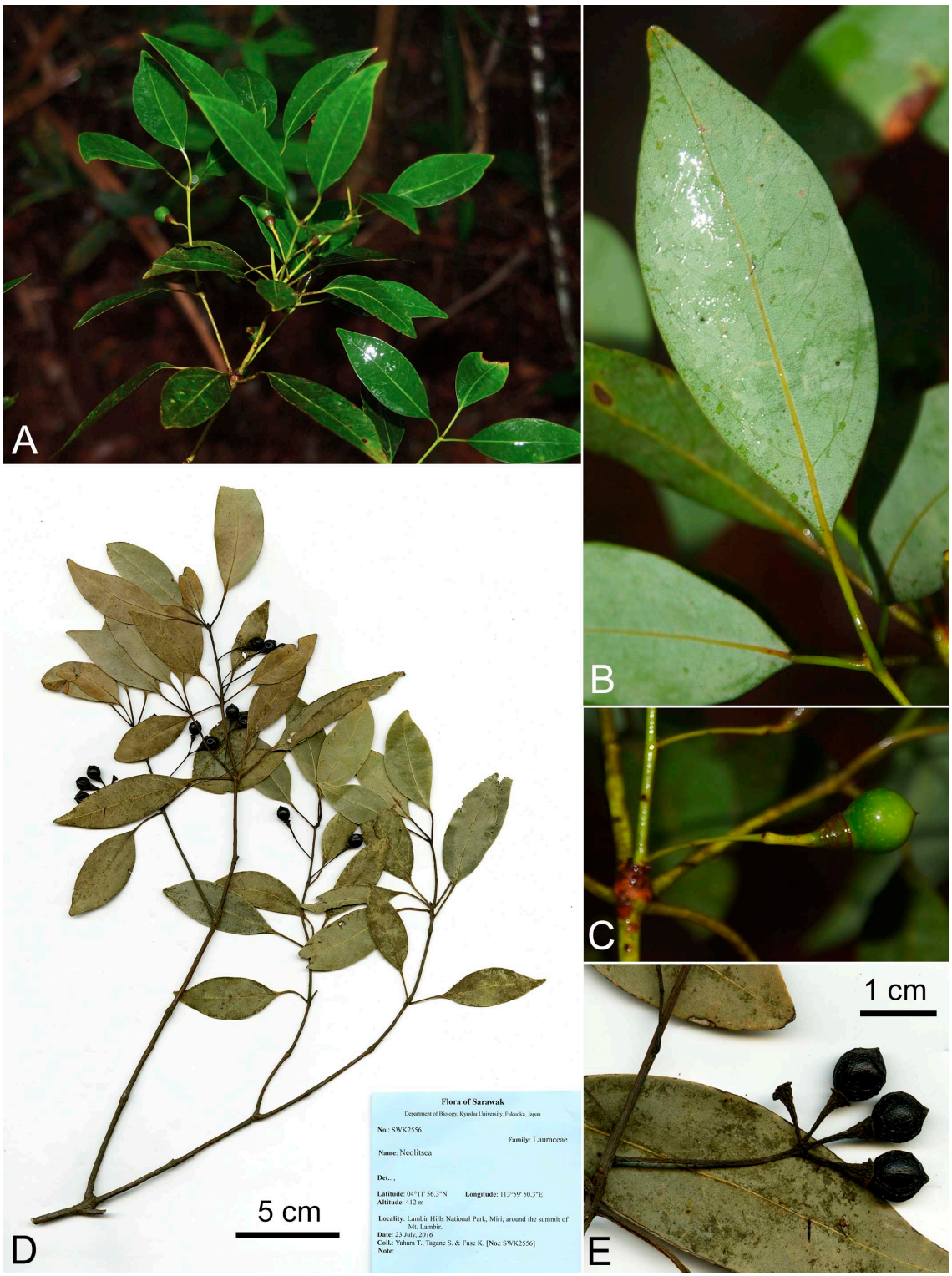


Figure 1. Photos of *Actinodaphne lambirensis* Tagane, Yahara & Okabe. **A** fruiting branch, **B** abaxial leaf surface, **C** fruit, **D** holotype, **E** infructescence. **A–C** photos taken on 23 July 2016. **D & E** material from *Tagane et al. SWK2556* (KYO).

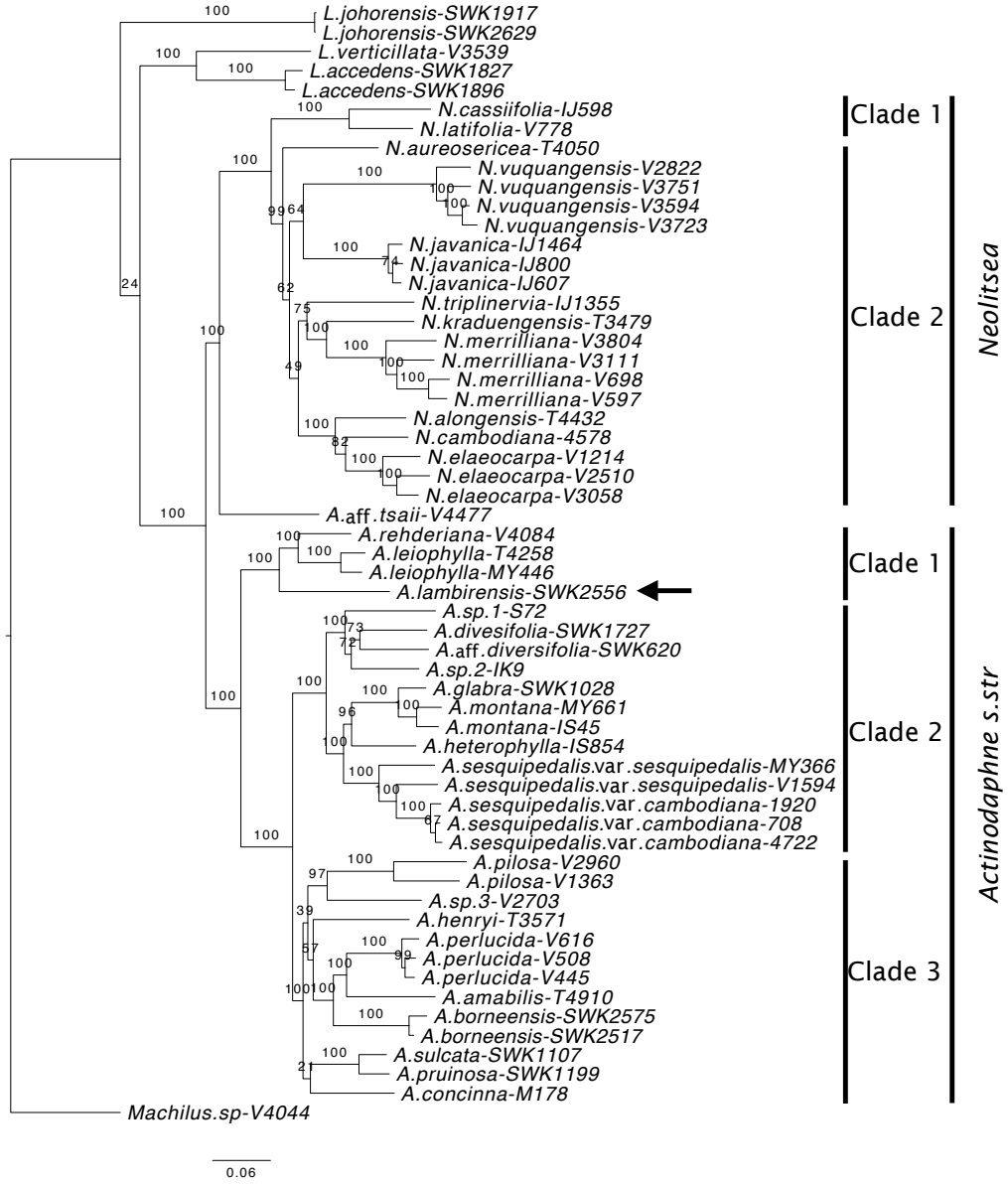


Figure 2. A MIG-seq ML tree for 31 samples (22 species) of *Actinodaphne*, 21 samples (11 species) of *Neolitsea*, five samples (three species) of *Litsea,* and one sample of *Machilus*. Branches are labeled with bootstrap values. Voucher specimen ID or GenBank accession number is added after each specimen name.

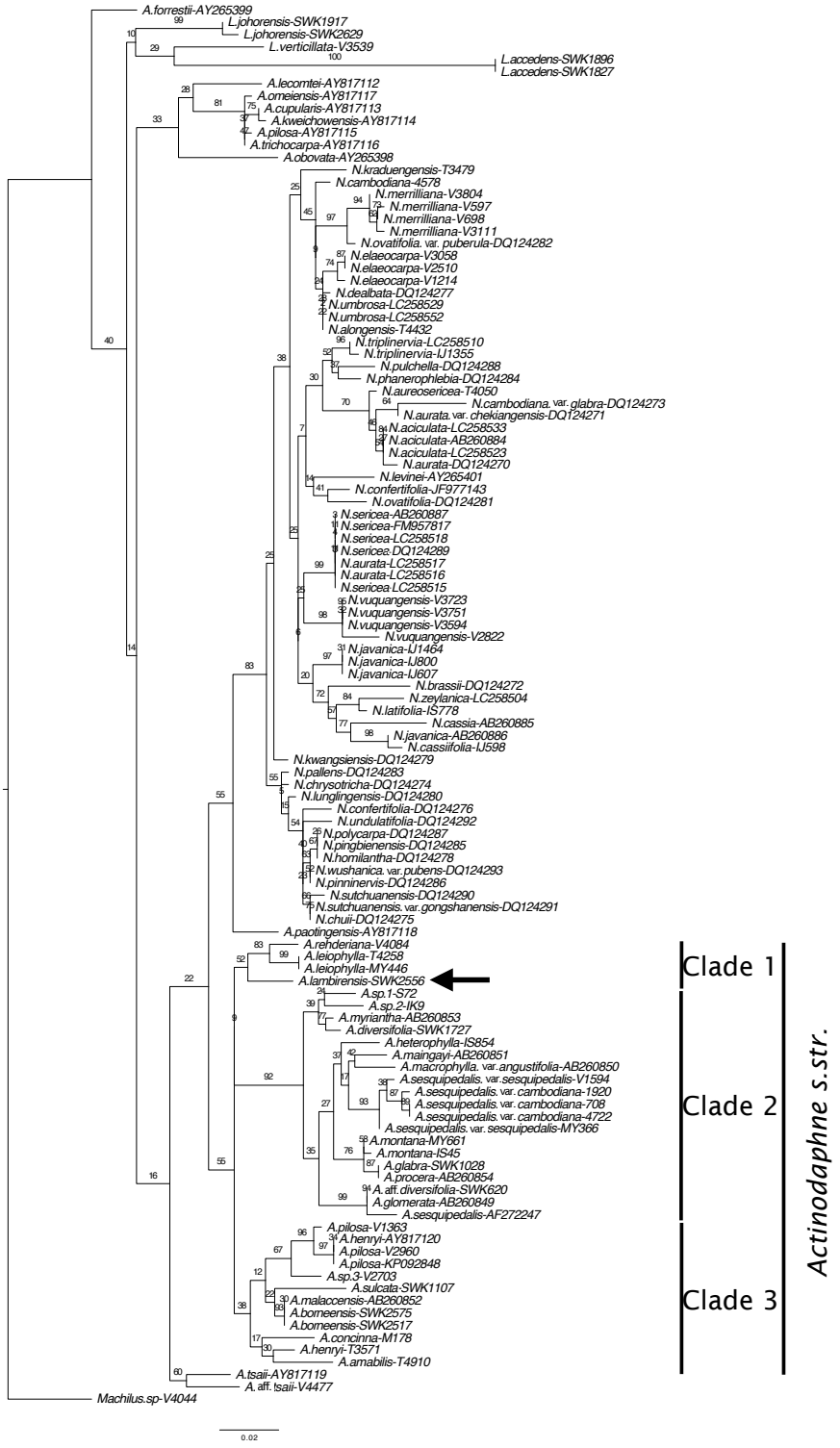


Figure 3. An ITS ML tree for 46 samples (36 species) of *Actinodaphne*, 62 samples (40 species) of *Neolitsea*, five samples (three species) of *Litsea,* and one sample of *Machilus*. Branches are labeled with bootstrap values. Voucher specimen ID or GenBank accession number is added after each specimen name.

Appendix 1. A list of samples used for sequencing ITS regions and genotyping genome-wide SNPs with MIG-seq.

Species	**Countries / Regions**	**Areas**	**Voucher specimens / References**	**GenBank accession no.**	**MIG-seq**
Actinodaphne amabilis Kosterm.	Thailand	Khao Luang National Park, Nakhon Ratchasima	T4910 (FU) / –	LC504502	+
A. borneensis Meisn.	Malaysia	Lambir Hills National Park, Sarawak	SWK2517 (FU) / –	LC504520	+
A. borneensis Meisn.	Malaysia	Lambir Hills National Park, Sarawak	SWK2575 (FU) / –	LC504521	+
A. concinna Ridl.	Malaysia	Fraser's Hill, Pahang	M178 (FU) / Mitsuyuki *et al*. (2018)	LC258564	+
A. cupularis (Hemsl.) Gamble	China	Shidian, Guizhou	– / Li *et al*. (2006)	AY817113	–
A. diversifolia Merr.	Malaysia	Tatau, Bintulu, Sarawak	SWK1727 (FU) / –	LC504503	+
A. aff. *divesifolia*	Malaysia	Watercatchment Camp Ayam, Bintulu,	SWK620 (FU) / –	LC504517	+

		Sarawak			
A. forrestii (C. K. Allen) Kosterm.	China	Mengla, Yunnan	– / Li *et al*. (2006): Li *et al*. (2007)	AY265399	–
A. glabra Blume	Malaysia	Water Catchment Sekawei, Sarawak	SWK1028 (FU) / –	LC504504	+
A. glomerata (Blume) Nees	Indonesia	Bogor Botanical Garden, Java	– / Fijridiyanto & Murakami (2009)	AB260849	–
A. henryi Gamble	China	Mengla, Yunnan	– / Li *et al*. (2006)	AY817120	–
A. henryi Gamble	Thailand	Phu Kradueng National Park, Loei	T3571 (FU) / –	LC504507	+
A. heterophylla Blume	Indonesia	Airsirah, Padang, Sumatra	IS854 (FU) / –	LC504524	+
A. kweichowensis Y.C.Yang & P.H.Huang	China	Dongshan, Guangxi	– / Li *et al*. (2006); Mitsuyuki *et al*. (2018)	AY817114	–
A. lambirensis Tagane, Yahara & Okabe	Malaysia	Lambir Hills National Park, Sarawak	SWK2556 (FU) / –	LC260478	+
A. lecomtei C.K.Allen	China	Without precise	– / Li et al. (2006)	AY817112	–

		locality, Guangxi			
A. leiophylla (Kurz) Hook.f.	Myanmar	Taninthayri Nature Reserve, Tanintharyi	MY446 (FU) / –	LC504509	+
A. leiophylla (Kurz) Hook.f.	Thailand	Karome Waterfall, Khao Laung National Park, Nakhon Ratchasima	T4258 (FU) / –	LC504510	+
A. macrophylla (Blume) Nees *var. angustifolia*	Indonesia	Bogor Botanical Garden, Java	– / Fijridiyanto & Murakami (2009)	AB260850	–
A. maingayi Hook.f.	Malaysia	Lambir Hills National Park, Sarawak	– / Fijridiyanto & Murakami (2009)	AB260851	–
A. malaccensis Hook.f.	Malaysia	Lambir Hills National Park, Sarawak	– / Fijridiyanto & Murakami (2009)	AB260852	–
A. montana Gamble	Indonesia	Pinang Pinang, Padang, Sumatra	IS45 (FU) / –	LC504505	+
A. montana Gamble	Myanmar	Taninthayri Nature Reserve, Tanintharyi	MY661 (FU) / –	LC504506	+
A. myriantha Merr.	Malaysia	Lambir Hills National	– / Fijridiyanto &	AB260853	–

		Park, Sarawak	Murakami (2009)		
A. obovata (Nees) Blume	China	Mengla, Yunnan	– / Li *et al*. (2006); Mitsuyuki *et al*. (2018)	AY265398	–
A. omeiensis (Liou) C.K.Allen	China	Mt. Emeishan, Sichuan	– / Li *et al*. (2006)	AY817117	–
A. paotingensis Y.C.Yang & P.H.Huang	China	Baoting, Hannan	– / Li *et al*. (2006); Mitsuyuki *et al* (2018)	AY817118	–
A. perlucida C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V445 (FU) / –	–	+
A. perlucida C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V508 (FU) / –	–	+
A. perlucida C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V616 (FU) / –	–	+
A. pilosa (Lour.) Merr.	China	Yongning, Guangxi	– / Li *et al*. (2006); Mitsuyuki *et al* (2018)	AY817115	–
A. pilosa (Lour.) Merr.	China	–	– / Mitsuyuki *et al*. (2018)	KP092848	–

A. pilosa (Lour.) Merr.	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1363 (FU) / –	LC504519	+
A. pilosa (Lour.) Merr.	Vietnam	Bach Ma National Park, Hue	V2960 (FU) / –	LC504511	+
A. procera Nees	Malaysia	Lambir Hills National Park, Sarawak	– / Fijridiyanto & Murakami (2009)	AB260854	–
A. pruinosa Nees	Malaysia	Bario, Sarawak	SWK1199 (FU) / –	–	+
A. rehderiana (C.K.Allen) Kosterm. ex Yahara	Vietnam	Bi Doup-Nui Ba National Park, Lam Dong	V4084 (FU) / Mitsuyuki *et al*. (2018)	LC258563	+
A. sesquipedalis Hook.f. & Thoms. ex Hook.f.	Malaysia	Kuala Lumpur	– / Li *et al*. (2006)	AF272247	–
A. sesquipedalis Hook.f. & Thoms. ex Hook.f. var. *cambodiana* Lecomte	Cambodia	Bokor National Park, Kampot	1920 (FU) / –	LC504512	+
A. sesquipedalis Hook.f. & Thoms. ex	Cambodia	Cardamon, Koh Kong	708 (FU) / –	LC504513	+

Hook.f. var. *cambodiana* Lecomte					
A. sesquipedalis Hook.f. & Thoms. ex Hook.f. var. *cambodiana* Lecomte	Cambodia	Koh Kong	4722 (FU) / Mitsuyuki *et al*. (2018)	LC258562	+
A. sesquipedalis Hook.f. & Thoms. ex Hook.f. var. *sesquipedalis*	Myanmar	Taninthayri Nature Reserve, Tanintharyi	MY366 (FU) / –	LC504515	+
A. sesquipedalis Hook.f. & Thoms. ex Hook.f. var. *sesquipedalis*	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1594 (FU) / –	LC504514	+
A. sulcata S.Julia	Malaysia	Bario, Sarwak	SWK1107 (FU) / –	LC504516	+
A. trichocarpa C.K.Allen	China	Daguan, Yunnan	– / Li *et al*. (2006)	AY817116	–
A. tsaii Hu	China	Malipo, Yunnan	– / Li *et al*. 2006; Li *et a*l. 2007; Mitsuyuki *et*	AY817119	–

			al. (2018)		
A. aff. *tsaii*	Vietnam	Bi Doup Nui Ba National Park, Lam Dong	V4477 (FU) / –	LC504508	+
A. sp. 1	Indonesia	Bantimulung Bulusarum, Sulawesi	S72 (FU) / –	LC504523	+
A. sp. 2	Indonesia	Mandor, West Kalimantan	IK9 (FU) / –	LC504522	+
A. sp. 3	Vietnam	Bach Ma National Park, Hue	V2703 (FU) / –	LC504518	+
Litsea accedens Boerl.	Malaysia	Tatau, Bintulu, Sarawak	SWK1827 (FU) / –	LC504525	+
L. accedens Boerl.	Malaysia	Sungai Jelalong, Bintulu, Sarawak	SWK1896 (FU) / –	LC504526	+
L. johorensis Gamble	Malaysia	Lambir Hills National Park, Sarawak	SWK1917 (FU) / –	LC504527	+
L. johorensis Gamble	Malaysia	Lambir Hills National Park, Sarawak	SWK2629 (FU) / –	LC504528	+
L. verticillata Hance	Vietnam	Vu Quang National	V3539 (FU) / –	LC504529	+

		Park, Vinh			
Machilus sp.	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4044 (FU) / –	LC504532	+
Neolitsea aciculata (Blume) Koidz.	Japan	Kyoto	– / Fijridiyanto & Murakami (2009)	AB260884	–
Neolitsea aciculata (Blume) Koidz.	Japan	Iriomote Island, Okinawa	– / Mitsuyuki *et al*. (2018)	LC258523	–
Neolitsea aciculata (Blume) Koidz.	Taiwan	Lienhuachi	– / Mitsuyuki *et al*. (2018)	LC258533	–
N. alongensis Lecomte	Thailand	Phu Kradueng Natioinal Park, Loei	T4432 (FU) / Mitsuyuki *et al*. (2018)	LC258532	+
N. aurata (Hayata) Koidz.	China	Guangxi	–/Li et al. (2007); Mitsuyuki *et al*. (2018)	DQ124270	–
N. aurata (Hayata) Koidz.	Japan	Iriomote Island, Okinawa	– / Mitsuyuki *et al*. (2018)	LC258516	–
N. aurata (Hayata) Koidz.	Japan	Iriomote Iand, Okinawa	– / Mitsuyuki *et al*. (2018)	LC258517	–

N. aurata var. *chekiangensis* (Nakai) Y.C.Yang & P.H.Huang	China	Zhejiang	– / Li *et al*. (2007); Mitsuyuki *et al*. 2018	DQ124271	–
N. aureosericea Kosterm.	Thailand	Khao Luang National Park, Nakhon Ratchasima	T4050 (FU) / Mitsuyuki *et al*. (2018)	LC258531	+
N. brassii C.K.Allen	Australia	Queensland	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124272	–
N. cambodiana Lecomte	Cambodia	Bokor National Park, Kampot	4578 (FU) / Mitsuyuki *et al*. (2018)	LC258503	+
N. cambodiana var. *glabra* C.K.Allen	China	Guangdong	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124273	–
N. cassia (L.) Kosterm.	Indonesia	Bogor Botanical Garden, Java	– / Fijridiyanto & Murakami (2009)	AB260885	–
N. cassiifolia Merr.	Indonesia	Gede Pangrango National Park, Java	IJ598 (FU) / Mitsuyuki *et al*.	LC258508	+

			(2018)		
N. chrysotricha H.W.Li	China	Yunnan	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124274	–
N. chuii Merr.	China	Yunnan	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124275	–
N. confertifolia (Hemsl.) Merr.	China	Hunan	– / Li *et al*. (2007)	DQ124276	–
N. confertifolia (Hemsl.) Merr.	China	–	– / Mitsuyuki *et al*. (2018)	JF977143.2	–
N. dealbata (R.Br.) Merr.	Australia	Queensland	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124277	–
N. elaeocarpa H.Liou	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1214 (FU) / Mitsuyuki *et al*. (2018)	LC258534	+
N. elaeocarpa H.Liou	Vietnam	Bach Ma National Park, Hue	V2510 (FU) / Mitsuyuki *et al*.	LC258540	+

			(2018)		
N. elaeocarpa H.Liou	Vietnam	Hai Van Pass, Hue	V3058 (FU) / Mitsuyuki *et al*. (2018)	LC258544	+
N. homilantha C.K.Allen	China	Yunnan	– / Li et al. (2007); Mitsuyuki *et al*. (2018)	DQ124278	–
N. javanica (Blume) Backer	Indonesia	Cibodas Botanical Garden, Java	– / Fijridiyanto & Murakami (2009)	AB260886	–
N. javanica (Blume) Backer	Indonesia	Halimun, Java	IJ1464 (FU) / Mitsuyuki *et al*. (2018)	LC258507	+
N. javanica (Blume) Backer	Indonesia	Gede Pangrango National Park, Java	IJ607 (FU) / Mitsuyuki *et al*. (2018)	LC258509	+
N. javanica (Blume) Backer	Indonesia	Gede Pangrango National Park, Java	IJ800 (FU) / Mitsuyuki *et al*. (2018)	LC258511	+
N. kraduengensis	Thailand	Phu Kradueng	T3479 (FU) /	LC258528	+

Tagane & Yahara		National Park, Loei	Mitsuyuki *et al*. (2018)		
N. kwangsiensis H.Liou	China	Hongkong	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124279	–
N. latifolia (Blume) S.Moore	Indonesia	Air Sirah, Padang, W Sumatra	IS778 (FU) / Mitsuyuki *et a*l. (2018)	LC258513	+
N. levinei Merr.	China	Mengla, Yunnan	– / Li *et al*. (2006); Mitsuyuki *et al*. (2018)	AY265401	–
N. lunglingensis H.W.Li	China	Yunnan	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124280	–
N. merrilliana C.K.Allen	Vietnam	Ba Na Nature Reserve, Da Nang	V3111 (FU) / Mitsuyuki *et al*. (2018)	LC258545	+
N. merrilliana C.K.Allen	Vietnam	Vu Quang National Park, Vinh	V3804 (FU) / Mitsuyuki *et al*.	LC258550	+

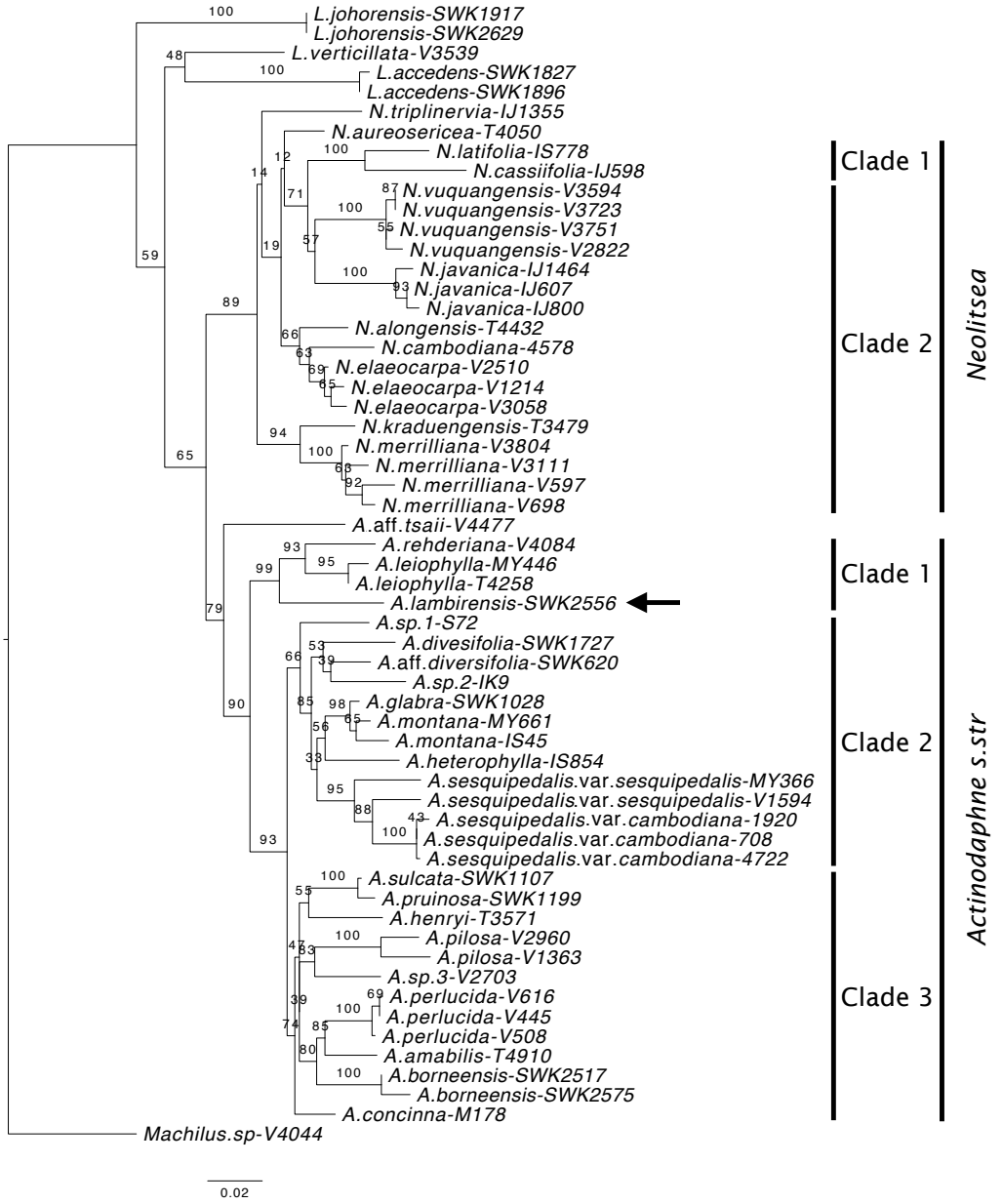
			(2018)		
N. merrilliana C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V597 (FU) / Mitsuyuki *et al*. (2018)	LC258557	+
N. merrilliana C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V698 (FU) / Mitsuyuki *et al*. (2018)	LC258560	+
N. ovatifolia var. *puberula* Y.C.Yang & P.H.Huang	China	Yunnan	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124282	–
N. ovatifolia Y.C.Yang & P.H.Huang	China	Hongkong	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124281	–
N. pallens (D.Don) Momiy. & H.Hara	China	Xizang	– / Mitsuyuki *et al*. (2018)	DQ124283	–
N. phanerophlebia Merr.	China	Guangdong	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124284	–
N. pingbienensis Y.C.	China	Yunnan	– / Li *et al*. (2007);	DQ124285	–

Yang & P.H.Huang			Mitsuyuki *et al*. (2018)		
N. pinninervis Y.C.Yang & P.H.Huang	China	Guangxi	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124286	–
N. polycarpa H.Liou	China	Yunnan	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124287	–
N. pulchella Merr.	China	Guangxi	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124288	–
N. sericea (Blume) Koidz.	China	–	– / Mitsuyuki *et al*. (2018)	FM957817.2	–
N. sericea (Blume) Koidz.	Indonesia	Bogor Botanical Garden, Java	– / Fijridiyanto & Murakami (2009)	AB260887	–
N. sericea (Blume) Koidz.	Japan	Honshu	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124289	–
N. sericea (Blume)	Japan	Fukuoka	– / Mitsuyuki *et al*.	LC258515	–

Koidz.			(2018)		
N. sericea (Blume) Koidz.	Japan	Okinawa	– / Mitsuyuki *et al*. (2018)	LC258518	–
N. sutchuanensis var. *gongshanensis* H.W.Li	China	Yunnan	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124291	–
N. sutchuanensis Yang	China	Sichuan	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124290	–
N. triplinervia Merr.	Indonesia	Gede Pangrango National Park, Java	– / Mitsuyuki *et al*. (2018)	LC258510	–
N. triplinervia Merr.	Indonesia	Halimun Salak National Park, Java	IJ1355 (FU) / Mitsuyuki *et al*. (2018)	LC258506	+
N. umbrosa (Nees) Gamble	Thailand	Khao Luang National Park, Nakhon Ratchasima	– / Mitsuyuki *et al*. (2018)	LC258529	–
N. umbrosa (Nees)	Vietnam	Bi Doup Nui Ba NP,	– / Mitsuyuki *et al*.	LC258552	–

Gamble		Lam Dong	(2018)		
N. undulatifolia (H. Lév.) C.K.Allen	China	Guangxi	– / Li *et al*. (2007); Mitsuyuki *et al*. (2018)	DQ124292	–
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Bach Ma National Park, Hue	V2822 (FU) / Mitsuyuki *et al*. (2018)	LC258542	+
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Vu Quang National Park, Vinh	V3594 (FU) / Mitsuyuki *et al*. (2018)	LC258547	+
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Vu Quang National Park, Vinh	V3723 (FU) / Mitsuyuki *et al*. (2018)	LC258548	+
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Vu Quang National Park, Vinh	V3751 (FU) / Mitsuyuki *et al*. (2018)	LC258549	+
N. wushanica var. *pubens* Y.C.Yang & P.H.Huang	China	Hunan	– / Li et al. (2007); Mitsuyuki *et al*. (2018)	DQ124293	–

N. zeylanica Merr.	Cambodia	Bokor National Park, Kampot	– / Mitsuyuki *et al.* (2018)	LC258504	–



Appendix 2. A MIG-seq ML tree for 31 samples (22 species) of *Actinodaphne*, 21 samples (11 species) of *Neolitsea*, five samples (three species) of *Litsea*, and one sample of *Machilus*, obtained using minimum percentage of samples in a population (r) = 0.5. Branches are labeled with bootstrap values. Voucher specimen ID or GenBank accession number is added after each specimen name. Compared with a MIG-seq tree

obtained using r = 0.025, the resolution was lower in that 38 % (21/55) of the branches were supported by bootstrap values of >90 %, but the position of *A. lambirensis* was not changed. The position of *A. lambianensis* was not changed also in trees obtained using r= 0.1, 0.2, 0.3, and 0.4.

Chapter II

Phylogenetic reconstruction and Species Discovery using Genome-Wide SNP Data: An application of MIG-seq to *Actinodaphne* and *Neolitsea* (Lauraceae)

Abstract

Multiplexed Inter-Simple Sequence Repeats (ISSRs) genotyping by sequencing (MIG-seq) is a method to obtain genome-wide SNPs using a set of ISSRs as a primer set. Here, we examined effectiveness of MIG-seq for phylogenetic reconstruction and species discovery of *Actinodaphne* and *Neolitsea* in Southeast Asia. We compared a MIG-seq tree reconstructed for 25 and 45 species of *Actinodaphne* and *Neolitsea*, respectively, with an ITS tree for 18 and 33 species of two genera. As a result, 119 of 162 (72 %) branches and 26 of 88 (30 %) branches were supported by bootstrap values of 85 %< in MIG-seq and ITS trees, respectively. In the 20 nodes supported by both ITS and MIG-seq trees, bootstrap support was always higher on the MIG-seq tree. In one of two inconsistent cases between the MIG-seq tree and the ITS tree, topologies of the MIG-seq tree agreed with morphological resemblance. In the MIG-seq tree, *Actinodaphne* was separated into two clades: *Actinodaphne* 1 including *A*. aff. *tsaii* 1, and *A*. aff. *tsaii* 2, and *Actinodaphne* 2 including the other 23 spp. *Actinodaphne* 1, *Actinodaphne* 2, and *Neolitsea* were almost equally differentiated. The MIG-seq tree supported sister relationship for 18 pairs of species, and sister species of each pair are distinguished by diagnostic traits. In both genera, morphologically similar species were often not sister to each other, suggesting repeated parallel evolution of leaf traits. On the MIG-seq tree, 6 *Actinodaphne* spp. and 30 *Neolitsea* spp. did not match any described species and are likely to be undescribed species. These results showed that a highly

resolved phylogenetic tree by MIG-seq is effective to discover and deliminate new species.

Keywords:

ITS, next-generation sequencing, parallel evolution, single-nucleotide polymorphism, Southeast Asia, tropical forest

Introduction

Vascular plants are so highly diversified in terrestrial ecosystems that our taxonomic knowledge on vascular plants still remain imperfect. According to an estimate by Bebber et al. (2010), about 70,000 species of vascular plants remain to be described despite continued taxonomic studies since 18th centuries. To discover and describe vascular plant species, DNA sequences of some genes that are generally variable among species have been used as DNA barcodes (CBOL Plant Working Group 2009; Kress et al. 2005). However, variability of the standard DNA barcode regions in vascular plants is lower than the DNA barcode region of animals, and even when three genes are combined, the discriminatory power of plant species is 60 to 93 % (Hollingsworth et al. 2015). In contrast, recent advance in restriction site-associated DNA sequencing (RAD-seq) enabled us to discover a lot of single nucleotide polymorphisms (SNPs) across the genome that provide genetic markers sufficient for reconstructing highly-resolved phylogeny among closely related species (Andrews et al. 2015; Cariou et al. 2013,). RAD-seq is a method of amplifying short DNA sequences neighboring to restriction enzyme cleavage sites across the genome and determining a large amount of amplified sequences using a next generation sequencer. The sequences

determined by this method usually include several hundreds to thousands of SNPs, providing much higher discriminatory power among closely related species than conventional methods (Cariou et al. 2013). Recently this method has been successfully applied to vascular plants and provided phylogenetic trees highly resolved among closely species (Parchman et al. 2018). This method is expected to be useful to discover undescribed species in poorly studied areas as in many areas of Southeast Asian tropics (Middleton et al. 2020). However, application of RAD-seq often requires us time consuming process of DNA purification because tissues of many plant species contain such inhibitors of restriction enzyme reactions as tannins, alkaloids and polyphenols (Abdel-Latif and Osman 2017). In contrast, multiplexed Inter-Simple Sequence Repeats (ISSRs) genotyping by sequencing (MIG-seq; Suyama and Matsuki 2015) is a method in which the digestion step with a restriction enzyme of RAD-seq is replaced with a PCR-based step using a set of ISSRs as a primer set, and can be applied to samples that are difficult to be treated with restriction enzymes. In fact, Binh et al. (2018) applied MIG-seq to *Quercus* (Fagaceae) of Vietnam, reconstructed a highly resolved phylogenetic tree, and discovered and described three new species. The aim of this study is to report the second case where MIG-seq is effectively used for phylogenetic reconstruction and species discovery of vascular plants in Southeast Asia.

The tropical region of Southeast Asia harbors remarkable plant diversity as high as in tropical America (Kreft and Jetz 2007; Middleton et al. 2020; Yahara et al. 2012). However, taxonomic studies on vascular plants of this region remain incomplete, where about 3,000 new species were reported from 2011 to 2017 (Middleton et al. 2020). To fill this gap, Yahara et al. (2012) proposed a project to assess plant diversity in the tropical region of Southeast Asia by collecting and recording all the species of

vascular plants found in many small plots. Since then, his team collected about 40,000 specimens and silica-gel dried leaf samples for DNA sequencing from 167 plots of 100 m × 5 m placed in 56 locations of Southeast Asia (Middleton et al. 2020). Among them, Lauraceae was the most frequently collected family, but identification of species in Lauraceae is difficult due to morphological similarity among many species and low availability of fertile specimens. Here, we show that higyly resolved phylogenetic tree obtained by MIG-seq is powerful to delimitate and discriminate species of Lauraceae even for sterile specimens. Among genera of Lauraceae, we studied *Actinodaphne* Nees and *Neolitsea* Merr. that are distinguished only on the basis of flower morphology (3-merous flowers in *Actinodaphne* vs. 2-merous in *Neolitsea*). Because many species of two genera are similar in vegetative and fruit morphology, it is often difficult to discriminate species and even the two genera for sterile or fruiting specimens. Therefore, these genera are a suitable material for testing the effectiveness of MIG–seq in species delimitation.

The genera *Actinodaphne* and *Neolitsea* (Lauraceae) include approximately 100 each species of evergreen trees that mainly occur in tropical Asia (Rohwer 1993; van der Werff 2001). Both morphological analysis (Liou 1934) and molecular phylogenetic analysis (Rohwer 2000; Chanderbali et al. 2001) have supported that *Actinodaphne* and *Neolitsea* are closely related to *Litsea* Lam. *Actinodaphne* and *Neolitsea* can be distinguished from *Litsea* by leaves that are whorled or clustered in the nodes of branches, and *Actinodaphne* and *Neolitsea* can be distinguished on the basis of flower morphology. Recent molecular phylogenetic studies using classic DNA barcodes including *matK*, *ndhF*, *rpb2*, internal transcribed spacer (ITS) and external transcribed spacer (ETS) suggested that *Neolitsea* is monophyletic (Fijridiyanto and Murakami

2009; Li et al. 2004; Li et al. 2006; Li et al. 2007; Mitsuyuki et al. 2018). On the other hand, it is unclear whether *Actinodaphne* is monophyletic or polyphyletic (monophyletic: Fijridiyanto and Murakami 2009, polyphyletic: Li et al. 2004; Li et al. 2006; Li et al. 2007; Mitsuyuki et al. 2018). The phylogenetic relationships reported by these previous studies were based on relatively few phylogenetically informative characters, which resulted in trees with relatively low resolution, even when the sequences of *matK* and ITS (Li et al. 2004), ITS and ETS (Li et al. 2006; Li et al. 2007), or *rpb2*, *matK*, *ndhF*, and nrITS (Fijridiyanto and Murakami 2009) were combined.

The aim of this study was to determine the phylogenetic relationships among samples widely collected from Southeast Asia, including Cambodia, Indonesia, Laos, Malaysia, Myanmar, Thailand, and Vietnam, and discover and delimitate undescribed species, using both classic DNA barcodes (i.e., ITS) and genome-wide SNPs determined by MIG-seq. We addressed the following specific questions. (1) How high is the resolution of a MIG-seq tree compared to an ITS tree ? (2) Is there any inconsistency between a MIG-seq tree and an ITS tree ? (3) How can we use a highly resolved MIG-seq tree to delimitate and discriminate species?

Materials and Methods

Souce of samples

We detected genome-wide SNPs with MIG-seq for 161 samples (Table 1) that were collected through a series of transect surveys in various locations of Southeast Asia. During these surveys, all the tree species within each 100 m × 5 m plot were collected, regardless of whether they have flowers or fruits (Zhang et al. 2016). Among 161 samples, we examined ITS sequences for 113 samples.

DNA extraction

Approximately 0.8 mm × 0.8 mm piece of silica gel-dried leaf samples were crushed using a QIAGEN TissueLyser and washed three times using 1-mL aliquots of buffer solution (0.1M HEPES, pH8.0; 2 % mercaptoethanol; 1 % PVP; 0.05M ascorbic acid), after which DNA was extracted from the leaf samples using the CTAB method of Doyle and Doyle (1987).

ITS sequencing and analysis

Ribosomal ITS sequences were amplified for 32 samples (22 spp.; Table 1, GenBank IDs: LC260478.1, LC504502.1–LC504532.1) using Tks Gflex DNA Polymerase (Takara Bio, Kusatsu, Japan), previously described primers (ITS-18F: GTCCACTGAACCTTATCATTTAGAGG, ITS-26R: GCCGTTACTAAGGGAATCCTTGTTAG; Rohwer et al. 2009), and the following reaction conditions: 95 °C for 4 min; 25 cycles of 94 °C for 30 sec, 55 °C for 1 min, and 72 °C for 1 min; and 72 °C for 10 min. PCR products were subsequently purified using ExoSAP-IT (Affymetrix). Purified amplification products were sequenced with Applied Biosystems 3730 DNA Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

In addition, ITS sequences were also obtained from the NCBI database (https://ncbi.nlm.nih.gov) for the 34 species of *Actinodaphne* and *Neolitsea* (57 sequences, GenBank ID: LC258500.1–LC258509.1, LC258511.1–LC258514.1, LC258519.1–LC258522.1, LC258524.1–LC258532.1, LC258534.1–LC258564.1) that are studied by Mitsuyuki et al. (2018) and that are duplicated with our MIG-seq data

samples. Therefore, the final ITS dataset included 27 sequences from 20 *Actinodaphne* spp., 56 sequences from 33 *Neolitsea* spp., seven sequences from five *Litsea* spp., and one sequence each from *Machilus* and *Phoebe* as outgroupe (Table 1).

DNA sequences were aligned using MEGA7 (Kumar et al. 2016), and after converting the alignment from fasta format to phylip format using kakusan4 (Tanabe 2011), a maximum-likelihood (ML) phylogenetic tree was constructed using RaxML (Stamatakis 2006) with 1000 bootstrap replicates.

MIG-seq

For 161 samples (81 species), we amplified thousands of short sequences (loci) from each genome using primers designed for MIG-seq (Suyama and Matsuki 2015). The 1st PCR step was conducted to amplify inter-simple sequence repeats regions from genomic DNA using the MIG-seq primer set-1 (Suyama and Matsuki 2015). The 2nd PCR step was performed independently to add individual indices to each sample with indexed primers following the protocol of Suyama and Matsuki (2015) except that the 2nd PCR cycles were performed 20 times instead of 12 times. Then, 3 μl of each 2nd PCR product was pooled as a single mixture library. The mixture was purified and fragments in the size range 350−800 bp were selected by a Pippin Prep DNA size selection system (Sage Science, Beverly, MA, USA). The concentration of size-selected library was measured by a SYBR green quantitative PCR assay (Library Quantification Kit; Clontech Laboratories, Mountain View, CA, USA), using approximately 12 pM of libraries that were used for sequencing on an Illumina MiSeq Sequencer (Illumina, San Diego, CA, USA), with a MiSeq Reagent Kit v3 (150 cycle, Illumina).

MIG-seq phylogenetic analysis

Quality control of the raw MIG-seq data was performed as described by Suyama and Matsuki (2015). Briefly, 17 bases of read head (3' end of the first primer sequences) were trimmed from the MiSeq reads using fastx_trimmer, which is part of the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and high-quality reads were filtered using FASTQ Quality Filter in the FASTX-Toolkit with the criterion of q = 30 and p = 40 (q: quality cut-off value, p: percent of bases in sequence that must have quality eaqual to or higher than q). Next, TagDust (Lassmann et al. 2009) was used to remove extremely short reads from both read1 sequences (forward sequences of the second PCR) and read2 sequences (reverse sequences of the second PCR).

After the quality control was complete, the remaining reads were assembled using de novo map pipelines (ustacks, cstacks, sstacks) in stacks ver. 1.48 (Catchen et al. 2011). Homologous sequences (loci) were assembled in each sample using ustacks, with the following settings: minimum depth of coverage (m) = 3, maximum distance allowed between stacks (M) = 2, maximum distance allowed to align secondary reads to primary stacks (N) = 1, and maximum gaps = 2. A catalogue of consensus loci was built for each sample by using ustacks to assemble the loci, allowing only two mismatches between sample loci (n). A list of loci was obtained with following settings: all samples belong to the same population and threshold frequency of haplotype count in a population (r) = 0.025. Finally, the presence or absence of loci in each sample were determined using a haplotype list that was generated using the populations pipeline. The genotypes of the samples at each locus were provided by the populations pipeline output file "haplotypes.tsv". The bach_1.vcf-format file that included the SNP sites of all the

samples was converted to phylip format and used to reconstruct a ML tree in RaxML with 500 times bootstrap replicates. A total of 60,557 loci were used to construct the phylogenetic tree.

Results

MIG-seq phylogenetic tree

The ML tree based on MIG-seq data showed high resolution, with 72 % (119 of 162 branches) of the branches supported by bootstrap values of 85 % or higher (Fig. 1, 2). *Litsea* was placed outside of *Actinodaphne* and *Neolitsea* and was separated into two clusters (Fig. 1). One cluster (*Litsea* 1), which was supported by a bootstrap value of 76%, included *L. accedens* Boerl. (SWK689, SWK1827, and SWK1896), *L. verticillata* Hance (V3539), *L.* sp. 1 (V159 and V4427), *L.* sp. 2 (V2765 and V2972), *L* sp. 3 (V4572), *L.* sp. 4 (V585), and *L.* sp. 5 (V5443), and a second cluster (*Litsea* 2), which was supported by a bootstrap value of only 55 %, included *L. johorensis* Gamble (T2421, T3066, SWK1917, and SWK2629) and *Litsea* sp. 6 (V5761). The monophyly of the clade that included both *Actinodaphne* and *Neolitsea* was supported by a bootstrap value of 100 % and was separated into three lower clades: *Neolitsea* (bootstrap value 84 %), *Actinodaphne* 1 (bootstrap value 100 %), which included *A.* aff. *tsaii* (V4477, T200), and *Actinodaphne* 2 (bootstrap value 100 %), which included all the other *Actinodaphne* spp. (Fig. 2). The two species in *Actinodaphne* 1 were characterized by lanceolate leaves with more than 15 lateral veins (Fig. 3).

Actinodaphne 2 was further separated into two clades, both with bootstrap values of 100 %. Clade 1 included *A. rehderiana* (C.K.Allen) Kosterm. ex Yahara (V4084), *A. leiophylla* (Kurz) Hook.f. (MY446, T4258), and *A. lambirensis* sp. nov

(SWK2556), and the second clade, which included all the other *Actinodaphne* spp.; the latter was further separated into Clades 2–6. Clade 2 included *A. henryi* Gamble (T3571) only. Clade 3 (bootstrap value 86 %) included *A. concinna* Ridl. (M178), *A. sulcata* S.Julia (SW1107), and *A. pruinosa* Nees (SWK1199). Clade 4 (bootstrap value 79 %) included *A.* sp. 1 (V2703) and *A. pilosa* (Lour.) Merr. (V1363, V2960). Clade 5 (bootstrap value 100 %) included *A.borneensis* Meisn. (SWK2517 and SWK2575), *A. myriantha* Merr. (SWK1658), *A. rufescens* Blume (SWK2020), *A. amabilis* Kosterm. (T4910), and *A. perlucida* C.K.Allen (V445, V508, V616). Clade 6 (bootstrap value 100 %) included the remaining *Actinodaphne* spp. In Clade 6, the monophyly of *A. sesquipedalis* Hook.f. & Thomson ex Meisn. from Myanmar (MY366) and *A. sesquipedalis* from Cambodia and Vietnam (708, 4722, 1815, 1920, and V1594) was supported by a bootstrap value of 100 %.

Meanwhile, *Neolitsea* was separated into seven clades, with bootstrap values higher than 85 %. Clade 1 (bootstrap value 100%) included *N. javanica* (Blume) Backer (IJ607, IJ800, and IJ1464) and *N.* sp. 1 (SWK1220). Clade 2 (bootstrap value 100 %) included *N. vuquangensis* Mitsuyuki & Yahara (V2822, V3594, V3751, V3723, and V5617), *N.* sp. 2 (V1677 and V2009), *N.* sp. 3 (T5175), and *N.* sp. 4 (V3561 and V6003). Clade 3 (bootstrap value 100 %) included *N. alongensis* Lecomte (T4432), *N. cambodiana* Lecomte (1656, 4578, and 6305), *N. elaeocarpa* H.Liu (V466, V646, V1214, V1245, V2510, V3035, V3044, V3058, V3730 and V5611), *N. kraduengensis* Tagane & Yahara (T3479 and T4722), *N. merrilliana* C.K.Allen (V597, V698, V2200, V3111, V3748, V3804, V5631, V5646, and V5931), and *N.* spp. 5–8 (IS788; T3760 and T5227; V4208; and T1706 and T2535, respectively). Clade 4 (bootstrap value 100 %) included *N. triplinervia* Merr. (IJ1355) and *N.* spp. 9–18 (V1282; V2704;

V1739, V1932, and V4060; V647, V650, and V885; V5735; 1860 and 6325; IS789; IS910; M48, M251, and M257; and V4250, V4244, V4505, and V4516, respectively). Clade 5 (bootstrap value 99 %) included *N. aureosericea* Kosterm. (T4050)*, N. cuipala* (D.Don) Kosterm. (MY1407)*, N. homilantha* C.K.Allen (V4898, V5063)*, N. polycarpa* Lour. (V4561, V4914), and *N.* spp. 19–24 (T2572; 3085 and 6323; V4550; V5333; 4310 and V4430; V3031; and V5745, V5834, V5842, V5843, V5863 and V5866, respectively). Clade 6 (bootstrap value 87 %) included *N. bokorensis* Yahara & Tagane (1442, 1726, 1730, 3160, 3217, 4124, 4126, 4584, and 6312)*, N. cassiifolia* Merr. (IJ598 and IJ740)*, N. latifolia* S.Moore (IS778), and *N.* spp. 25–29 (V271; M76; T2323; T3893; and IJ1319 and IK1303, respectively). Clade 7 (bootstrap value 100 %) included *N.* sp. 30 (V3276 and V5969) only. *N.* sp. 30, collected from Vu Quang National Park, Central Vietnam, was sister to all the other *Neolitsea* spp. It was also morphologically distinct from all the other *Neolitsea* spp. in that its fruits were seated on well-developed, cup-shaped perianth tubes (*vs*. on slightly enlarged disciform or concave perianth tubes) and long leaves (ca. 25 cm *vs*. usually < 10 cm) (Fig. 4). In regard to these traits, *N.* sp. 30 is similar to many *Actinodaphne* spp. In addition, *N.* sp. 30 is morphologically similar to two Chinese species, *A. kweichowensis* Y.C.Yang & P.H.Huang and *A. omeiensis* (H.Liu) C.K.Allen, in that bud scales are persistent at nodes and surround branch bases (Huang & van der Werff, 2008). The 10 specimens of *N. elaeocarpa* were highly variable in regard to the hairiness of lower leaf surfaces but were clustered into a single clade.

Among 25 species of *Actinodaphne* and 45 species of *Neolitsea* whose positions on the MIG-seq tree were determined, 6 species of *Actinodaphne* and 30 species of *Neolitsea* did not match to any previously described species. In the 6

unknown species of *Actinodaphne*, a species (*A. lambirensis*; SWK 2556) had fruits only, and the other nine species had neither flower nor fruit. Similarly, among the 30 unknown species of *Neolitsea,* one species had both flowers and fruits (*N*. sp. 6, V3760 fl., V 5227 fr.), one species had flowers only (*N*. sp. 18, V4505 and V4516), three species (*N*. sp. 11, V1739; *N*. sp. 17, M 257; *N*. sp. 30, V5969) had fruits, and the remaining 25 species had neither flower nor fruit. The MIG-seq tree supported the sister relationship for 20 pairs of species by bootstrap values higher than 70 % (Table 2). In all the 19 pairs, sister species were distinguished by morphological diagnostic traits. Among them, four pairs were sympatric, three pairs were collected from neighboring areas, and the other 13 pairs were collected from distant areas (Table 2).

According to the taxonomic treatments of Indo-China (Lecomte 1914, Ho 1932) and Thailand (Tanaros et al. 2010), *A.* sp. 1 (V2703) was keyed out as *A. sesquipedalis*, in having large leaf-like scale leaves covering terminal buds and large, narrowly lanceolate leaves with acuminate apices and narrowly cuneate bases. In addition, *A. sesquipedalis* was the only Vietnamese species illustrated by Ho (1999) for which the leaf shape is similar to sample V2703. However, in the MIG-seq tree, *A.* sp. 1 was sister to *A. pilosa* (V1363, V2960) of Clade 4, not to *A. sesquipedalis* of Clade 6, despite the following morphologial differences: *A.* sp. 3 has glabrous leaves but *A. pilosa* have tomentose hairs on its young twigs and leaves (Fig. 5 A, B). *Actinodaphne* sp. 1 is morphologically distinct from *A. sesquipedalis* in that leaves are glabrous and whitish below (vs. brownish or whitish hairy), having 8 to 10 lareral veins (vs. 13–18), and scale leaves covering tereminal buds are 1 cm long × 0.2 cm wide (vs. 2.2–7 cm long × 1.3–3 cm wide; Table 3). Similarly, *A*. sp. 3 (IS811, Fig. 5E) was keyed out as *A. sesquipedalis*, in having large, narrowly lanceolate leaves, with acuminate apices and

narrowly cuneate bases. In the MIG-seq tree, however, *A.* sp. 3 was sister to *A. heterophylla* Blume (IS854; Fig. 5F) from Sumatra, which has wider and hairy leaves, not to *A. sesquipedalis*. *A.* sp. 3 is morphologically distinct from *A. sesquipedalis* in that leaves are glabrous below (vs. hariy), having 10–12 lateral veins, and scale leaves covering terminal buds are lacking (Table 3). *Actinodaphne glabra* Blume (SWK 1048; Fig. 5 C) is also morphologically similar to *A. sesquipedalis* but leaves are glabrous below and have 8 to 10 lateral veins (Table 3); it is sister to *A. montana* Gamble (IS45; Fig, 5D) having leaves smaller than *A. glabra* and *A. sesquipedalis,* and not directly sister to *A. sesquipedalis* (Fig. 1). On the other hand, *A. sesquipedalis* was sister to a clade composed of *A. macrophylla* (Blume) Nees (SWK2533; Fig. 6C, D), *A.* sp. 3 (IS811; Fig. 5E), and *A. heterophylla* (IS854; Fig. 5F) that are morphologically diversified. The specimens of *A. sesquipedalis* are morphologically variable; the specimen from Myanmar (MY366; Fig. 7A, B) has more densely hairy leaves and petioles than the specimens from Cambodia and Vietnam (708, 4722, 1815, 1920, and V1594; Fig. 7C,D). *Actinodaphne rufescens* (SWK2020; Fig. 6A) and *A. macrophylla* (SWK2533; Fig. 6 B) are very similar in their large, narrowly lanceolate leaves with acuminate apices, narrowly cuneate bases, and densely hairy lower leaf surfaces. However, *A. rufescens* was placed in Clade 5, and *A. macrophylla* was placed in Clade 6 (Fig. 1). In regard to leaf traits, *A. rufescens* is very similar to *A. macrophylla* described from Java, but it is difficult to determine either is (or neither is) identical with the type specimen of *A. macrophylla* only by sterile specimens.

In *Neolitsea*, *N. merrilliana* (V3111, V2200, V597, V698, V5646, V3748, V5631, V593, and V3804), *N.* sp. 2 (V2009), and *N.* sp. 14 (6325) are similar in having small, ovate or obovate leaves (Fig. 8). However, *N. merrilliana*, *N.* sp. 2, and *N.* sp. 14

were placed in Clades 3, 4, and 2, respectively. *N. merrilliana* (Fig. 8A, V597) was sister to *N. kraduengensis* (Fig. 8B, T3479), which has narrowly lanceolate leaves; *N.* sp. 2 (Fig. 8C, V2009) was sister to three species with larger leaves, *N.* sp. 3, *N.* sp. 4 (Fig. 8D: V3561), and *N. vuquangeisis*; *Neolitsea* sp. 14 (Fig. 8E: 6325) was sister to *N.* sp. 12 (Fig. 8F: V885), which has relatively long, narrow leaves. *N.* sp. 2 (V1677, V2009) is morphologically distinct from *N. merrilliana* in having larger leaves (7.5 to 13 cm vs. shorter than 7 cm in *N. merrilliana*), having scalariform and flat tertiary veins (vs. reticulate and foveolate tertiary veins). Both *N.* sp. 2 and *N. merrilliana* are distributed in southern Vietnam where *N.* sp. 2 was collected in the elevations from 225 m to 1020 m and *N. merrilliana* was from 1200 m to 1350 m. *Neolitsea* sp. 14 (1860, 6325) distributed in Cambodia is distinguished from *N. merrilliana* distributed in Vietnam in having thinner leaves with scalariform tertiary veins (vs. thicker leaves with reticulate tertiary veins).

Neolitsea sp. 6 and *N.* sp. 7 are similar in having ovate leaves less than 15 cm long and densely hairy below, but two species are not sister; *N.* sp. 6 (T3760, T5227) is sister to a clade composed of *N.* sp. 7 (V4208) and *N. elaeocarpa*. *Neolitsea elaeocarpa* is usually distinct from *N.* sp. 6 and *N.* sp. 7 in having lanceolate leaves but two specimens (V1214 and V3058) of *N. elaeocarpa* have ovate leaves. These two specimens are, however, distinguished from *N.* sp. 7 in having thinner twigs (ca. 1 mm in diameter vs. thicher than 2 mm in *N.* sp. 7) and smaller leaves (mostly shorter than 8 cm vs. longer than 8 cm) as in typical forms of *N. elaeocarpa*. While mature leaves of *N.* sp. 6 and *N. elaeocarpa* are almost glabrous below, leaves of *N.* sp. 7 are densely hairy below even on the second-year branches. *Neolitsea* sp. 7 is also distinct from *N.* sp. 6 and *N. elaeocarpa* in having thicker and more densely hairy twigs. Both *N.* sp. 7 and *N.*

elaeocarpa are distributed in southern Vietnam where *N.* sp. 7 were collected from elevations higher than 1600 m and *N. elaeocarpa* were collected in elevations from 600 m to 1200 m. *Neolitsea* sp. 6 was collected from the elevation of 1760 m in Peninsular Thailand.

ITS-based phylogenetic tree

The ML tree based on ITS sequences showed much lower resolution than the MIG-seq tree except for the monophyly of *Neolitsea* supported by a bootstrap value of 85 %. First, the monophyly of the clade including *Actinodaphne* and *Neolitsea* was supported by a bootstrap value as low as 67 %. Second, a bootstrap support for the monophyly of *Actinodaphne* 2 (*Actinodaphne* species excluding *A. laosensis*) was only 47 %. Third, only 26 of the 88 branches (30 %) had bootstrap values of 85 % or higher (Fig. 9, 10). Among these 26 nodes, two nodes were not supported by the MIG-seq tree (Table 4).

Two inconsistent cases were found in two pairs of sister species: a pair of *L.* sp. 1 and *L.* sp. 5 and another pair of *N.* sp. 4 and *N. vuquangensis* (Table 4). In the former pair, there was only one-base change between the ITS sequences. In the latter pair, there were six base changes between an ITS sequence shared by *N. vuquangensis* V2822 and *N.* sp. 4 and another ITS sequence shared by the other samples of *N. vuquangensis*. On the MIG-seq tree, however, *N. vuquangensis* V2822 was clustered with the other samples of *N. vuquangensis* and the monophyly of *N. vuquangensis* samples was supported by a bootstrap value of 100 %. While V2822 was collected from Bach Ma National Park, the other samples of *N. vuquangensis* and *N.* sp. 4 were collected from Vu Quang National Park, located at ca. 400 km northwest of Bach Ma

National Park, where these two species are sympatric and morphologically distinct: *N. vuquangensis* has dense golden hairs on the lower surface of mature leaves but *N.* sp. 4 has mature leaves glabrescent and greenish below. V2822 collected from Bach Ma National Park is morphologically identical with the samples of *N. vuquangensis* collected from Vu Quang National Park and thus the topology of not ITS tree but MIG-seq tree agrees with morphology. In the other 20 nodes supported also by the MIG-seq tree, bootstrap support on the MIG-seq tree was 100 % except for one case of 96 %. There are six other nodes where consistent topologies were supported by both ITS and MIG-seq trees. While the bootstrap support for those nodes of the ITS tree varied from 40 to 98 %, all six nodes were supported by 100% bootstrap values on the MIG-seq tree.

Discussion

The purpose of this study was to evaluate how effectively MIG-seq can be used for phylogenetic reconstruction and species discovery of vascular plants using *Actinodaphne* and *Neolitsea* in Southeast Asia as a test case. As a result, the resolution of the MIG-seq tree was clearly greater than that of the ITS tree. First, 119 of the 162 branches (72 %) were supported by bootstrap values of 85 % or higher in the MIG-seq tree, but only 26 of the 88 branches (30 %) had bootstrap values of 85 % or higher in the ITS tree. In particular, the monophyly of the clade including *Actinodaphne* and *Neolitsea* was supported by a bootstrap value of 100 % in the MIG-seq tree, but a corresponding bootstrap value in the ITS tree was as low as 67 %. Second, in the 20 nodes supported by both ITS and MIG-seq trees, bootstrap support was always higher on the MIG-seq tree where boootstrap values were 100 % except for one case of 96 %.

Third, in one of two inconsistent cases between the MIG-seq tree and the ITS tree, the monophyly of *N. vuquangensis* was supported by a 100 % bootstrap value in the MIG tree but not supproted in the ITS tree. This inconsistency is resulted because there are six base pair changes between an ITS sequence shared by *N. vuquangensis* V2822 and *N.* sp. 4 and another ITS sequence shared by the other samples of *N. vuquangensis*. A plausible explanation for this inconsistency is that V2822 was originated by the hybridization between *N. vuquangensis* and *N.* sp. 4 and as a result of introgression, V2822 shares ITS sequences with *N.* sp. 4 but has morphology and a genomic SNP profile more similar to *N. vuquangensis s. str.* than *N.* sp. 4. Another inconsisntency for the *L.* sp. 1 and *L.* sp. 5 pair may be due to a lineage sorting of ancestral polymorphism in ITS sequences because there was only one-base change between the ITS sequences of *L.* sp. 1 and *L.* sp. 5. In both cases, it is likely that the result of MIG-seq is more reliable. Based on the overall consistency between the ITS tree and the MIG-seq tree, the higher resolution of the MIG-seq tree, and reliability of the MIG-seq tree in the above two cases, only the MIG-seq phylogeny of *Actinodaphne* and *Neolitsea* is discussed below.

Major phylogenetic relationships among Actinodaphne *and* Neolitsea

In the MIG-seq tree, 25 species of *Actinodaphne* were separated into two major clades (Fig. 1): *Actinodaphne* 1 including *A.* aff.*tsaii*, and *Actinodaphne* 2 including the other 25 *Actinodaphne* spp. The relationships among *Actinodaphne* 1, *Actinodaphne* 2, and *Neolitsea* were unclear, suggesting that the three clades are almost equally differentiated. More genetic markers are needed to determine the sequence of differentiation among these three clades. *Neolitsea* is characterized by 2-merous flowers, whereas both *Actinodaphne* 1 and *Actinodaphne* 2 possess 3-merous flowers. However,

the sister relationship between *Actinodaphne* 1 and *Actinodaphne* 2 was not supported by the MIG-seq tree. Some previous studies using ITS sequences have already suggested that some Chinese species of *Actinodaphne* is not monophyletic with the other species (Li et al. 2004; Li et al. 2006; Li et al. 2007; Mitsuyuki et al. 2018). Further MIG-seq studies on Chinese species are needed to determine whether or not *Actinodaphne* is a monophyletic group.

For *Neolitsea*, *N.* sp. 30 (V3276 and V5969) is sister to all the other species in both MIG-seq and ITS trees. It is notable that *N.* sp. 30 is morphologically similar to many species of *Actinodaphne* and different from the other species of *Neolitsea* in that it bears fruits on well-developed, cup-shaped perianth tubes and possesses lanceolate leaves of larger than 25 cm in length (Fig. 4). These morphological traits may be ancestral states shared by *Actinodaphne* spp. and *N.* sp. 30. For *N.* sp. 30, we collected only fruiting specimens and it remains uncertain whether it bears 2-merous flowers characterizing *Neolitsea*. Further studies on flowering specimens of *N.* sp. 30 is needed to elucidate its systematic position and character evolution.

Repeated evolution of similar leaf shape and hairiness

The MIG-seq tree revealed that similar morphological traits evolved repeatedly both in *Actinodaphne* and *Neolitsea*. First, *A.* sp. 1 of Clade 4 (Fig. 5A), *A. glabra* of Clade 6 (Fig. 5C), *A*. sp. 3 of Clade 6 (Fig. 5E) and *A. sesquipedalis* of Clade 6 are similar in having large, narrowly lanceolate leaves but *A.* sp. 1 was sister to *A. pilosa* (Fig. 5B), *A. glabra* was sister to *A. montana* (Fig. 5D), and *A*. sp. 3 was sister to *A. heterophylla* (Fig. 5F), a taxon from Sumatra that bears wider and densely hairy leaves. Second, *A. rufescens* of Clade 5 (Fig. 6A, B) is morphologically very similar to

A. *macrophylla* of Clade 6 (Fig. 6 C, D) but were placed at different positions in the MIG-seq tree. Third, *N.* sp. 2 of Clade 2 (Fig. 8C) and *N*. sp. 14 of Clade 4 (Fig. 8E) were keyed out as *N. merrilliana* of Clade 3 (Fig. 8A). Fourth, *N*. sp. 6 and *N*. sp. 7 were morphologically similar in densely hairy leaf undersides, but were not sister species (Fig. 2). On the other hand, *A. sesquipedalis* which were highly variable in regards to the hairiness of their lamina (Table 3) were monophyletic in the MIG-seq tree. These examples indicate that parallel evolution in leaf size, shape, and hairiness occurred in both *Actinodaphne* and *Neolitsea*.

The relationship between leaf size and environment is well understood, and previous studies have demonstrated that small leaves are generally associated with harsh environments (Nicotra et al. 2011) and poor nutrient availability (Beadle 1966; Cunningham et al. 1999; Fonseca et al. 2000). The latter conditoin may be relevant to the habitats of *N. merrilliana, N*. sp. 2, and *N*. sp. 14, all of which bear small, rounded leaves and were collected from the shallow-soiled forest floors of tropical montane forests. In contrast, the relationship between leaf shape and environment is less predictable (Nicotra et al. 2011). The parallel evolution of narrowly lanceolate leaves in *A. sesquipedalis*, *A.* sp. 1, and *A*. sp. 3 may be a result of adaptive evolution toward increasing leaf number under the trade-off between leaf size and number (Kleiman and Aarssen 2007). However, the adaptive significance of smaller (narrower) and more numerous leaves remain uncertain. In a species pair from western Sumatra, *A. heterophylla* possesses wider and fewer leaves than *A*. sp. 3, even though both species were collected from similar habitats of wet tropical montane forest in western Sumatra (*A*. sp. 3 at 1166 m elev., *A*. *heterophylla* at 1348 m elev.) and, thus, are unlikely to have adapted to different eco-physiological environments. One potential explanation, as

proposed by Moles and Westoby (2000), is that smaller leaves, which are associated with reduced leaf expansion time, are less vulnerable to herbivory.

Leaf hairiness may also be related to defense against herbivory (Hanley et al. 2007). Dense hair can prevent the movement of insects on leaves (Eisner et al. 1998) and decrease the number of eggs laid by herbivorous insects (Handley et al. 2005). Therefore, the evolution of hair density in *Actinodaphne* and *Neolitsea* may be an example of parallel evolution in defensive traits (Kursar and Corley 2003). Further studies on the relationship between herbivory and leaf shape are needed to elucidate the adaptive significance of diverged leaf form in *Actinodaphne* and *Neolitsea*.

Utility of MIG-seq for new species discovery

The MIG-seq tree supported the sister relationship for 18 pairs of species and in all the 18 pairs, sister species are distinguished by diagnostic morphological traits. In two other pairs of samples collected from different countries, we could not find diagnostic traits to distinguish them. As a result, IS45 collected from Sumatra and MY661 collected from Myanmar were identified as *A. montana* (Fig. 1) and another pair, T2572 from Thailand and 3085 and 6323 from Cambodia, were identified as *N.* sp. 19 (Fig. 2). This result shows that MIG-seq is effective in characterizing and delimitating species by determining sister species pairs and comparing morphological characteristics between them. Among 18 sister species paris (Table 2), four pairs were sympatric (collected in the same locality) and thus those are regarded as four pairs of reproductively isolated species. The other three pairs were collected from neighboring areas: *A. borneensis* and *A. myriantha* were collected from Sarawak, *N.* sp. 7 and *N. elaeocarpa* from southern Vietnam, and *N.* sp. 24 and *N. polycarpa* from northen

Vietnam. Both *borneensis* and *A. myriantha* were collected in lowland rainforests of Sarawak and two localities are only 200 km apart, suggesting that the ranges of the two species are overlapping. On the other hand, these species are distinct in leaves (glabrous, veins flat below vs densely hairy, veins raised below; Table 2). *Neolitsea* sp. 7 and *N. elaeocarpa* were collected from different elevations in the neigboring area, suggesting that these are diverged by adapting to different habitats. *Neolitsea* sp. 24 and *N. polycarpa* were collected from Vu Quang National Park and Hoang Lien National Park, respectively, approximately 300 km apart from each other. Morphological differences between these two species are relatively slight (leaves smaller, not undulate vs. larger, undulate), and thus these can be treated as two subspecies of the same specices. We treated them as two species considering that they are diverged as largely as in four pairs of previously described species in the MIG-seq tree (Fig. 1, 2: *A. rehderiana* vs. *A. leiophylla*, *A. sulcata* vs. *A. pruinosa*, *A. glabra* vs. *A. montana*, and *N. kraduengensis* vs. *N. merrilliana*), although there is no absolute criterion for species discrimination. For the rest 11 pairs collected from distant areas, morphological differences are more distinct, and they are also diverged as largely as in four pairs of previously described species in the MIG-seq tree.

In the following 11 cases, a species was sister to a pair of species listed in Table 2: *A. lambirensis* to (*A. rehderiana*, *A. leiophylla*), the monophyly of three species was supported by a bootstrap value of 100 %; *A. concinna* to (*A. sulcata*, *A. pruinosa*), 86 %; *A. rufescens* to (*A. perlucida*, *A. amabilis*), 96 %; *macrophylla to (A.* sp. 3*, A. heterophylla)*, 100 %; *N.* sp. 3 to (*N.* sp. 4, *N. vuquangensis*), 100 %; *N.* sp. 6 to (*N. elaeocarpa*, *N.* sp. 7), 99 %; *N.* sp. 11 to (*N.* sp. 9, *N.* sp. 10), 100 %; *N. triplinervia*, (*N.* sp. 15, *N.* sp. 16), 100 %; *N. aureosericea*, (*N.* sp. 19, *N.* sp. 20), 8 %; and *N.*

homilantha, (*N*. sp. 24, *N. polycarpa*), 80 %. In these cases, we considered them as species to avoid paraphyletic demilitation of a species. For example, while *N*. sp. 6 and *N*. sp. 7 are more similar to each other than to *N. elaeocarpa*, *N*. sp. 7 and *N. elaeocarpa* are found in the same locality and morphologically distinct. The similarity between *N*. sp. 6 collected from Peninsular Thailand and *N*. sp. 7 collected from southern Vietnam are regarded as similarity due to common ancestry (synplesiomorphy). If *N*. sp. 6 and *N*. sp. 7 are treated as a single species, it is paraphyletic. Thus, we treat them as two species. In all 11 cases listed above, we found diagnostic morphological traits characterizing an outside species from a pair of species (data not shown).

As above, using MIG-seq tree and vegetative morphological traits, we could discriminate 25 species of *Actinodaphne* and 45 species of *Neolitsea*. Among them, 6 species (24 %) of *Actinodaphne* and 30 species (65 %) of *Neolitsea* did not match any described species. About 100 each species of *Actinodaphne* and *Neolitsea* have been described until today (Rohwer 1993; van der Werff 2001), but our results indicate that there are more undescribed species of the two genera in Southeast Asia. This results show that MIG-seq is effective for discovering new species from sterile specimens. Similarly, Binh et al. (2018) applied MIG-seq to 19 species of *Quercus* (Fagaceae) collected from Cambodia and Vietnam, reconstructed a highly resolved phylogenetic tree and described three species. In this study, Binh et al. (2018) could describe three new species based on fertile specimens. In this study, however, as many as 6 and 30 species of *Actinodaphne* and *Neolitsea*, respectively, are undescribed, and only two each among them had flowers. This is because trees of Lauraceae do not flower every year (Mase et al. 2020). While traditional taxonomic descriptions of new species have been based on fertile specimens, many tropical species may become extinct before

being named if we continue to follow this traditional procedure (Maddison et al. 2012). To accelerate species discovery and documentation, Mase et al. (2020) recently described three new species of *Machilus* (Lauraceae) based on ITS phylogeny and vegetative morphology. They studied bud morophology and leaf venation carefully to describe new species based on sterile specimens. Further studies by combining such careful observations on vegetative traits with MIG-seq phylogeny are needed to accelerate species discovery in Southeast Asia where more than 400 new species of vascular plants have been described every year (Middleton et al. 2020), and undoubtedly many more species remain to be described.

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Table 1. A list of samples used for sequencing ITS regions and genotyping genome-wide SNPs with MIG-seq.

Species	**Countries**	**Areas**	**Voucher specimens**	**ITS GenBank ID**	**MIG– seq**
Actinodaphne amabilis Kosterm.	Thailand	Khao Luang National Park, Nakhon Ratchasima	T4910 (FU)	LC504502.1	+
A. borneensis Meisn.	Malaysia	Lambir Hills National Park, Sarawak	SWK2517 (FU)	LC504520.1	+
A. borneensis Meisn.	Malaysia	Lambir Hills National Park, Sarawak	SWK2575 (FU)	LC504521.1	+
A. concinna Ridl.	Malaysia	Fraser's Hill, Pahang	M178 (FU)	LC258564.1	+
A. diversifolia Merr.	Malaysia	Tatau, Bintulu, Sarawak	SWK1727 (FU)	LC504503.1	+
A. divesifolia Merr.	Indonesia	Mandor, West Kalimantan	IK9 (FU)	LC504522.1	+
A. glabra Hook f. et Thoms.	Malaysia	Water Catchment Sekawei, Sarawak	SWK1028 (FU)	LC504504.1	+
A. glomerata (Blume) Nees	Malaysia	Watercatchment Camp Ayam, Bintulu, Sarawak	SWK620 (FU)	LC504517.1	+
A. henryi Gamble	Thailand	Phu Kradueng National Park, Loei	T3571 (FU)	LC504507.1	+
A. heterophylla Blume	Indonesia	Airsirah, Padang, Sumatra	IS854 (FU)	LC504524.1	+

A. lambirenis sp. nov	Malaysia	Lambir Hills National Park, Sarawak	SWK2556 (FU)	LC260478.1	+
A. leiophylla (Kurz) Hook. f.	Myanmar	Taninthayri Nature Reserve, Tanintharyi	MY446 (FU)	LC504509.1	+
A. leiophylla (Kurz) Hook. f.	Thailand	Karome Waterfall, Khao Laung National Park, Nakhon Ratchasima	T4258 (FU)	LC504510.1	+
A. macrophylla (Blume) Nees	Malaysia	Lambir Hills National Park, Sarawak	SWK2533 (FU)	–	+
A. montana Gamble	Indonesia	Pinang Pinang, Padang, Sumatra	IS45 (FU)	LC504505.1	+
A. montana Gamble	Myanmar	Taninthayri Nature Reserve, Tanintharyi	MY661 (FU)	LC504506.1	+
A. myriantha Merr.	Malaysia	Tatau, Bintulu, Sarawak	SWK1658 (FU)	–	+
A. perlucida C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V445 (FU)	–	+
A. perlucida C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V508 (FU)	–	+
A. perlucida C.K.Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V616 (FU)	–	+

A. pilosa (Lour.) Merr.	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1363 (FU)	LC504519.1	+
A. pilosa (Lour.) Merr.	Vietnam	Bach Ma National Park, Hue	V2960 (FU)	LC504511.1	+
A. pruinosa Nees	Malaysia	Bario, Sarawak	SWK1199 (FU)	–	+
A. rehderiana Kosterm.	Vietnam	Bi Doup-Nui Ba National Park, Lam Dong	V4084 (FU)	LC258563.1	+
A. rufescens Blume	Malaysia	Lambir Hills National Park, Sarawak	SWK2020 (FU)	–	+
A. sesquipedalis Hook. f. & Thoms. ex Hook. f.	Cambodia	Bokor National Park, Kampot	1815 (FU)	–	+
A. sesquipedalis Hook. f. & Thoms. ex Hook. f.	Cambodia	Bokor National Park, Kampot	1920 (FU)	LC504512.1	+
A. sesquipedalis Hook. f. & Thoms. ex Hook. f.	Cambodia	Koh Kong	4722 (FU)	LC258562.1	+
A. sesquipedalis Hook. f. & Thoms.	Cambodia	Cardamon, Koh Kong	708 (FU)	LC504513.1	+

ex Hook. f.					
A. sesquipedalis Hook. f. & Thoms. ex Hook. f.	Myanmar	Taninthayri Nature Reserve, Tanintharyi	MY366 (FU)	LC504515.1	+
A. sesquipedalis Hook. f. & Thoms. ex Hook. f.	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1594 (FU)	LC504514.1	+
A. sulcata S.Julia	Malaysia	Bario, Sarwak	SWK1107 (FU)	LC504516.1	+
A. aff. *tsaii* 1	Vietnam	Bi Doup Nui Ba National Park, Lam Dong	V4477 (FU)	LC504508.1	+
A. aff. *tsaii* 2	Thailand	Doi Inthanon Nationa Park, Chiang Mai	T200 (FU)	–	+
A. sp. 1	Vietnam	Bach Ma National Park, Hue	V2703 (FU)	LC504518.1	+
A. sp. 2	Indonesia	Bantimulung Bulusarum, Sulawesi	S72 (FU)	LC504523.1	+
A. sp. 3	Indonesia	Airsirah, Padang, Sumatra	IS811 (FU)	–	+
Litsea accedens Boerl.	Malaysia	Watercatchment Camp Ayam, Bintulu, Sarawak	SWK689 (FU)	–	+
L. accedens Boerl.	Malaysia	Tatau, Bintulu, Sarawak	SWK1827 (FU)	LC504525.1	+
L. accedens Boerl.	Malaysia	Sungai Jelalong, Bintulu,	SWK1896 (FU)	LC504526.1	+

		Sarawak			
L. johorensis Gamble	Thailand	Kaeng Krachan National Park, Petchaburi	T2421 (FU)	–	+
L. johorensis Gamble	Thailand	Kaeng Krachan National Park, Petchaburi	T3066 (FU)	–	+
L. johorensis Gamble	Malaysia	Lambir Hills National Park, Sarawak	SWK1917 (FU)	LC504527.1	+
L. johorensis Gamble	Malaysia	Lambir Hills National Park, Sarawak	SWK2629 (FU)	LC504528.1	+
L. verticillata Hance	Vietnam	Vu Quang National Park, Vinh	V3539 (FU)	LC504529.1	+
L. sp. 1	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V159 (FU)	–	+
L. sp. 1	Vietnam	Bi Doup Nui Ba National Park, Lam Dong	V4427 (FU)	LC504530.1	+
L. sp. 2	Vietnam	Bach Ma National Park, Hue	V2765 (FU)	–	+
L. sp. 2	Vietnam	Bach Ma National Park, Hue	V2972 (FU)	–	+
L. sp. 3	Vietnam	Mt. Fansipan, Hoang Lien National Park, Hanoi	V4572 (FU)	–	+
L. sp. 4	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V585 (FU)	–	+

L. sp. 5	Vietnam	Pu Mat National Park, Nghe An	V5443 (FU)	LC504531	+
L. sp. 6	Vietnam	Vu Quang National Park, Vinh	V5761 (FU)	–	+
Machilus sp.	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4044 (FU)	LC504532	+
Neolitsea alongensis Lecomte	Thailand	Phu Kradueng Natioinal Park, Loei	T4432 (FU)	LC258532.1	+
N. aureosericea Kosterm.	Thailand	Khao Luang National Park, Nakhon Ratchasima	T4050 (FU)	LC258531.1	+
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	1442 (FU)	–	+
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	3160 (FU)	–	+
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	3217 (FU)	–	+
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	4124 (FU)	–	+
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	4126 (FU)	–	+
N. bokorensis	Cambodia	Bokor National Park, Kampot	4584 (FU)	–	+

Yahara & Tagane					
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	6312 (FU)	–	+
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	1726 (FU)	LC258500.1	+
N. bokorensis Yahara & Tagane	Cambodia	Bokor National Park, Kampot	1730 (FU)	LC258501.1	+
N. cambodiana Lecomte	Cambodia	Bokor National Park, Kampot	6305 (FU)	–	+
N. cambodiana Lecomte	Cambodia	Bokor National Park, Kampot	1656 (FU)	–	+
N. cambodiana Lecomte	Cambodia	Bokor National Park, Kampot	4578 (FU)	LC258503.1	+
N. cassiifolia Merr.	Indonesia	Gede Pangorango National Park, Java	IJ740 (FU)	–	+
N. cassiifolia Merr.	Indonesia	Gede Pangorango National Park, Java	IJ598 (FU)	LC258508.1	+
N. cuipala (D.Don) Kosterm.	Myanmar	Indawgyi Wildlife Sanctuary, Kachin	MY1407 (FU)	–	+
N. elaeocarpa H.	Vietnam	Hon Ba Nature Reserve,	V1245 (FU)	–	+

Liou		Khanh Hoa			
N. elaeocarpa H. Liou	Vietnam	Hai Van Pass, Hue	V3035 (FU)	–	+
N. elaeocarpa H. Liou	Vietnam	Hai Van Pass, Hue	V3044 (FU)	–	+
N. elaeocarpa H. Liou	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V466 (FU)	–	+
N. elaeocarpa H. Liou	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V646 (FU)	–	+
N. elaeocarpa H. Liou	Vietnam	Vu Quang National Park, Vinh	V3730 (FU)	–	+
N. elaeocarpa H. Liou	Vietnam	Vu Quang National Park, Vinh	V5611 (FU)	–	+
N. elaeocarpa H. Liou	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1214 (FU)	LC258534.1	+
N. elaeocarpa H. Liou	Vietnam	Bach Ma National Park, Hue	V2510 (FU)	LC258540.1	+
N. elaeocarpa H. Liou	Vietnam	Hai Van Pass, Hue	V3058 (FU)	LC258544.1	+
N. homilantha C. K.	Vietnam	Mt. Fansipan, Hoang Lien	V4898 (FU)	–	+

Allen		National Park, Hanoi			
N. homilantha C. K. Allen	Vietnam	Mt. Fansipan Hoang Lien National Park, Hanoi	V5063 (FU)	–	+
N. javanica (Blume) Backer	Indonesia	Halimun Salak National Park, Java	IJ1464 (FU)	LC258507.1	+
N. javanica (Blume) Backer	Indonesia	Gede Pangorango National Park, Java	IJ607 (FU)	LC258509.1	+
N. javanica (Blume) Backer	Indonesia	Gede Pangorango National Park, Java	IJ800 (FU)	LC258511.1	+
N. kraduengensis Tagane & Yahara	Thailand	Phu Kradueng National Park, Loei	T4722 (FU)	–	+
N. kraduengensis Tagane & Yahara	Thailand	Phu Kradueng National Park, Loei	T3479 (FU)	LC258528.1	+
N. latifolia S.Moore	Indonesia	Air Sirah, Padang, W Sumatra	IS778 (FU)	LC258513.1	+
N. merrilliana C.K. Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V2200 (FU)	–	+
N. merrilliana C.K. Allen	Vietnam	Vu Quang National Park, Vinh	V3748 (FU)	–	+
N. merrilliana C.K. Allen	Vietnam	Vu Quang National Park, Vinh	V5631 (FU)	–	+

N. merrilliana C.K. Allen	Vietnam	Vu Quang National Park, Vinh	V5646 (FU)	–	+
N. merrilliana C.K. Allen	Vietnam	Vu Quang National Park, Vinh	V5931 (FU)	–	+
N. merrilliana C.K. Allen	Vietnam	Ba Na Nature Reserve, Da Nang	V3111 (FU)	LC258545.1	+
N. merrilliana C.K. Allen	Vietnam	Vu Quang National Park, Vinh	V3804 (FU)	LC258550.1	+
N. merrilliana C.K. Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V597 (FU)	LC258557.1	+
N. merrilliana C.K. Allen	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V698 (FU)	LC258560.1	+
N. polycarpa H. Liou	Vietnam	Mt. Fansipan, Hoang Lien National Park, Hanoi	V4561 (FU)	–	+
N. polycarpa H. Liou	Vietnam	Mt. Fansipan, Hoang Lien National Park, Hanoi	V4914 (FU)	–	+
N. triplinervia Merr.	Indonesia	Halimun Salak National Park, Java	IJ1355 (FU)	LC258506.1	+
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Vu Quang National Park, Vinh	V5617 (FU)	–	+

N. vuquangensis Mitsuyuki & Yahara	Vietnam	Bach Ma National Park, Hue	V2822 (FU)	LC258542.1	+
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Vu Quang National Park, Vinh	V3594 (FU)	LC258547.1	+
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Vu Quang National Park, Vinh	V3723 (FU)	LC258548.1	+
N. vuquangensis Mitsuyuki & Yahara	Vietnam	Vu Quang National Park, Vinh	V3751 (FU)	LC258549.1	+
N. sp. 1	Malaysia	Bario, Sarawak	SWK1220 (FU)	–	+
N. sp. 2	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1677 (FU)	LC258536.1	+
N. sp. 2	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V2009 (FU)	LC258539.1	+
N. sp. 3	Thailand	Khao Luang National Park, Nakhon Ratchasima	T5175 (FU)	–	+
N. sp. 4	Vietnam	Vu Quang National Park, Vinh	V6003 (FU)	–	+
N. sp. 4	Vietnam	Vu Quang National Park, Vinh	V3561 (FU)	LC258546.1	+
N. sp. 5	Indonesia	Airsirah, Padang, Sumatra	IS788 (FU)	LC258513.1	+
N. sp. 6	Thailand	Khao Luang National Park, Nakhon Ratchasima	T3760 (FU)	LC258529.1	+

N. sp. 6	Thailand	Khao Luang National Park, Nakhon Ratchasima	T5227 (FU)	–	+
N. sp. 7	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4208 (FU)	LC258552.1	+
N. sp. 8	Thailand	Khao Soi Dao Wildlife Sanctuary, Chanthaburi	T1706 (FU)	LC258524.1	+
N. sp. 8	Thailand	Khao Soi Dao Wildlife Sanctuary, Chanthaburi	T2535 (FU)	LC258526.1	+
N. sp. 9	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V1282 (FU)	LC258535.1	+
N. sp. 10	Vietnam	Bach Ma National Park, Hue	V2704 (FU)	LC258541.1	+
N. sp. 11	Vietnam	Hon Ba Nature Reserve, Khanh Haa	V1739 (FU)	LC258537.1	+
N. sp. 11	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V1932 (FU)	LC258538.1	+
N. sp. 11	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4060 (FU)	LC258551.1	+
N. sp. 12	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V647 (FU)	LC258558.1	+
N. sp. 12	Vietnam	Hon Ba Nature Reserve,	V650 (FU)	LC258559.1	+

		Khanh Hoa			
N. sp. 12	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V885 (FU)	LC258561.1	+
N. sp. 13	Vietnam	Vu Quang National Park, Vinh	V5735 (FU)	–	+
N. sp. 14	Cambodia	Kampot	6325 (FU)	–	+
N. sp. 14	Cambodia	Bokor National Park, Kampot	1860 (FU)	LC258502.1	+
N. sp. 15	Indonesia	Airsirah, Padang, Sumatra	IS789 (FU)	–	+
N. sp. 16	Indonesia	Mt. Gadut, Padang, Sumatra	IS910 (FU)	LC258514.1	+
N. sp. 17	Malaysia	Fraser's Hill, Pahang	M251 (FU)	LC258519.1	+
N. sp. 17	Malaysia	Fraser's Hill, Pahang	M257 (FU)	LC258520.1	+
N. sp. 17	Malaysia	Fraser's Hill, Pahang	M48 (FU)	LC258521.1	+
N. sp. 18	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4244 (FU)	LC258553.1	+
N. sp. 18	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4250 (FU)	LC258554.1	+
N. sp. 18	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4505 (FU)	LC258555.1	+
N. sp. 18	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4516 (FU)	LC258556.1	+
N. sp. 19	Thailand	Khao Soi Dao Wildlife	T2572 (FU)	LC258527.1	+

		Sanctuary, Chanthaburi			
N. sp. 19	Cambodia	Bokor National Park, Kampot	3085 (FU)	–	+
N. sp. 19	Cambodia	Bokor National Park, Kampot	6323 (FU)	–	+
N. sp. 20	Vietnam	Mt. Fansipan, Hoang Lien National Park, Hanoi	V4550 (FU)	–	+
N. sp. 21	Vietnam	Mt. Fansipan, Hoang Lien National Park, Hanoi	V5333 (FU)	–	+
N. sp. 22	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4310 (FU)	–	+
N. sp. 22	Vietnam	Bi Doup Nui Ba NP, Lam Dong	V4430	–	+
N. sp. 23	Vietnam	Hai Van Pass, Hue	V3031 (FU)	LC258543.1	+
N. sp. 24	Vietnam	Vu Quang National Park, Vinh	V5745 (FU)	–	+
N. sp. 24	Vietnam	Vu Quang National Park, Vinh	V5834 (FU)	–	+
N. sp. 24	Vietnam	Vu Quang National Park, Vinh	V5842 (FU)	–	+
N. sp. 24	Vietnam	Vu Quang National Park, Vinh	V5843 (FU)	–	+
N. sp. 24	Vietnam	Vu Quang National Park, Vinh	V5863 (FU)	–	+
N. sp. 24	Vietnam	Vu Quang National Park, Vinh	V5866 (FU)	–	+
N. sp. 25	Vietnam	Hon Ba Nature Reserve, Khanh Hoa	V271 (FU)	–	+

N. sp. 26	Malaysia	Fraser's Hill, Pahang	M76 (FU)	LC258522.1	+
N. sp. 27	Thailand	Kaeng Krachan National Park, Petchaburi	T2323 (FU)	LC258525.1	+
N. sp. 28	Thailand	Khao Luang National Park, Nakhon Ratchasima	T3893 (FU)	LC258530.1	+
N. sp. 29	Indonesia	Bukit Bangkirai, Kalimantan	IK1303 (FU)	LC258512.1	+
N. sp. 29	Indonesia	Halimun Salak National Park, Java	IJ1319 (FU)	LC258505.1	+
N. sp. 30	Vietnam	Vu Quang National Park, Vinh	V3276 (FU)	–	+
N. sp. 30	Vietnam	Vu Quang National Park, Vinh	V5969 (FU)	LC504533	+
Phoebe lanceolata (Nees) Nees	Cambodia	Bokor National Park, Kampot	5810 (FU)	–	+

Table. 2 Morphological distinctions between 18 pairs of sister species determined by the MIG-seq tree.

Species A	**ID of species A**	**Species B**	**ID of species B**	**Bootstrap support (%)**	**Morphological distinction**	**Distribution**
A. aff. *tsaii* 1	V4477	*A.* aff. *tsaii* 2	T200	100	Leaves glaucous (lao) vs. not glaucous (sp 13)	Vietnam vs N Thailand
A. rehderiana	V4084	*A. leiophylla*	T4258, MY446	100	Tertiary veins raised below (reh) vs. flat (lei)	Vietnam vs Peninsular Thailand & S Myanmar
A. sulcata	SWK1107	*A. pruinosa*	SWK1199	100	Leaves broader, hariy on midrib below (sul) vs. narrower, glabrous when mature (pru)	Sympatric (Bario, Sarawak)
A. boeneensis	SWK2517, SWK2575	*A. myriantha*	SWK1658	100	Leaves glabrous, veins flat below (sp 5) vs hairy, veins raised below (myr)	Both in Sarawak
A. amabilis	T4910	*A.* sp. 7	V445, V508,	71	Fruits solitary or	Peninsular Thailand

			V616		twined (ama) vs 4-7 fruits clustered (sp 7)	vs S Vietnam
A. glabra	SWK1028	*A. gracilis*	IS45, MY661	100	Leaves larger, glabrous (gla) vs smaller, hairy below (gra)	Sarawak vs Sumatra, Myanmar
A. sp. 3	IS811	*A. heterophylla*	IS854	100	Leaves glabrescent, veins flat (sp 11) vs tomentose, veins raised below (sp 12)	Sympatric (W Java)
N. sp. 1	SWK1220	*N. javanica*	IJ607, IJ800, IJ1464	100	Leaves smaller, petioles curved (sp 1) vs larger, petioles straight (jav)	Sarawak vs W Java
N. sp. 4	V3561, V6003	*N. vuquangensis*	V2822, V3594, V3723, V3751, V5617	100	Leaves not golden-hairy below (sp 4) vs	Sympatric (Vu Quang, N Veitanm)

					golden-hairy (vuq)	
N. kraduengensis	T3479, T4722	*N. merrilliana*	V597, V698, V2200, V3111, V3748, V3804, V5631, V5646, V5931	100	Leaves lanceolate (kra) vs obovate (mer)	NE Thailand vs N and S Vietnam
N. sp. 5	IS788	*N. alongensis*	T4432	100	Leaf apex acuminate (sp 5) vs cuspidate (alo)	Sumatra vs NE Thailand
N. elaeocarpa	V466, V646, V1214, V1245, V2510, V3035, V3044, V3058, V3730, V5611,	*N.* sp. 7	V4208	91	Leaves glabrescent (ela) vs ferruginous hairy (sp 7)	Both in S Vietnam
N. sp. 9	V1282	*N.* sp. 10	V2704	100	Midveins raised above (sp 9) vs flat (sp 10)	N Vietnam vs S Vietnam
N. sp. 12	V647, V650, V885	*N.* sp. 13	V5735	100	Shrub, leaves smaller (sp 12) vs tall tree, leaves	Sympatric (Hon Ba, S Vietnam)

					larger (sp 13)	
N. sp. 15	IS789	*N*. sp. 16	IS910	80	Leaves oblong-lanceolate (sp 15) vs ovate (sp 16)	Parapatric (sp 16 occurs in the higer elevation)
N. sp. 25	V271	*N*. sp. 26	M76	100	Leaves thicker, narrower, not glaucous (sp 25) vs thinner, broader, glaucous (sp 26)	S Vietnam vs Peninsular Malaysia
N. sp. 28	T3893	*N. latifolia*	IS778	100	Leaves smaller, not glaucous (sp 28) vs larger, glaucous (lat)	Peninsular Thailand vs Sumatra
N. cassiifolia	IJ598, IJ740	*N*. sp. 29	IJ1319, IK1303	100	Tertiary veins indistinct (cas) vs raised (sp 29)	Both in W Java; N sp 29 also in Kalimantan

Table. 3 Differences in pubescence and color of leaf undersurface when dried, size of the largest scale leaves covering a terminal bud, and the number of lateral veins among four *A. sesquipedalis*-like species having large (usually larger than 25 cm), narrowly lanceolate leaves with acuminate apices and narrowly cuneate bases.

Species	**Specimens**	**Pubescence and color of leaf undersurface**	**Size of largest scale leaves**	**Lateral veins**
A. sp. 1	V2703	glabrous, whitish	1 cm × 0.2 cm	8–10 pairs
A. *glabra*	SWK1028	glabrous, brownish	1.5 cm × 0.3 cm	8–10 pairs
A. sp. 3	IS811	glabrous, whitish	Lacking	10–12 pairs
A. sesquipedalis	708	moderately yellowish brown hairy, light brownish	2.2 cm × 1.3 cm	15–17 pairs
A. sesquipedalis	1920	sparsely yellowish brown hairy, light brownish	3.5 cm × 2.0 cm	14–16 pairs
A. sesquipedalis	4722	densely whitish hairy, leaf surface color invisible	2.3 cm × 1.4 cm	16–18 pairs
A. sesquipedalis	MY366	Densely orange-brown hairy, leaf surface color invisible	7 cm x 3 cm	13–15 pairs

Table. 4 Comparison of bootstrap values between the ITS tree and the MIG-seq tree. Branches with boot strap values of 85 % or higher on the ITS tree are compared.

Branch (identified by taxa above each branch)	**Bootstrap value**	
	ITS	MIG-seq
L. sp. 1, *L*. sp. 5	94	Not supported
L. verticillata, *L*. sp. 1, *L*. sp. 5	99	100
L. accedens (SWK1827, SWK1896)	100	100
L. johorensis (SWK2629, SWK1927)	96	100
A. pilosa (V1363, V2960)	95	100
A. leiophylla, *A. rehderiana*	85	100
A. leiophylla (T4258, MY446)	99	100
A. sesquipedalis (MY366, V1594, 1920, 708, 4722)	97	100
A. sesquipedalis (1920, 708, 4722)	88	100
A. sesquipedalis (708, 4722)	88	96
A. glomerata, *A*. sp.2, *A. sesquipedalis, A. glabra, A. montana,*, *A. diversifolia*, *A. heterophylla*	95	100
N. javanica (IJ607, IJ800, J1464)	98	100
N. sp. 29 (IK1303, IJ1319)	96	100
N. bokorensis (1726, 1730)	98	100
N. merrilliana (V597, V698, V3111, V3804)	99	100
N. sp. 12 (650, 647, 885)	99	100
N. sp. 17 (M48, M251, M257)	94	100
N. sp. 12, *N*. sp. 18, *N*. sp. 17, *N*. sp. 16, *N. triplinervia*, *N*. sp. 14	88	100
N. sp. 19, *N*. sp. 23, *N. aureosericea*	96	100
N. sp. 8 (–T1706, T2535)	99	100
N. sp. 11 (–V1739, V1932, V4060)	98	100
N. sp. 4, *N. vuquangensis* (–V2822)	95	Not supported
N. vuquangensis (–V3594, V3751)	88	80
Neolitsea	85	84

N. sp. 2, *N*. sp. 4, *N. vuquangensis*	98	100

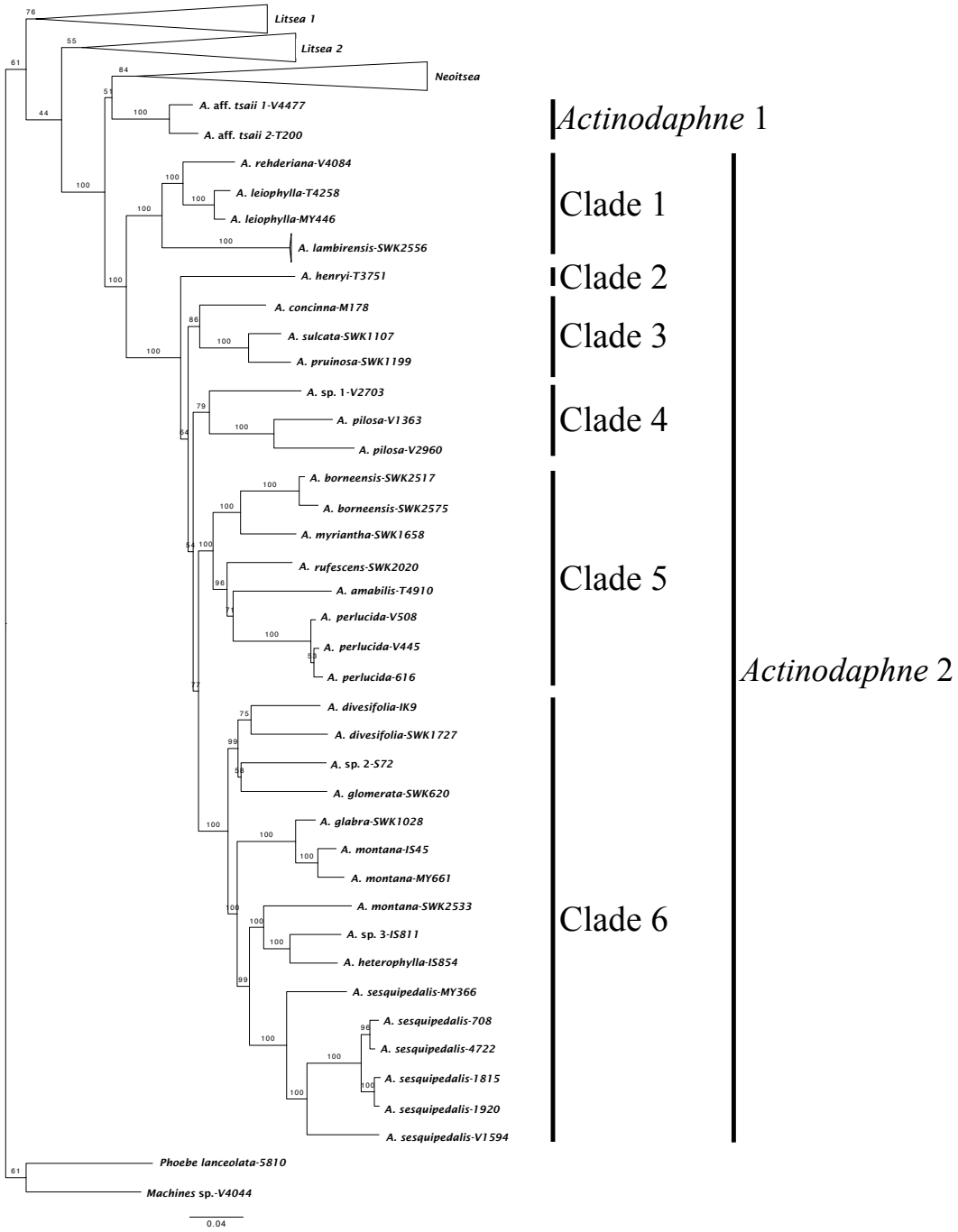

Figure 1. A MIG-seq ML tree for 37 samples (25 species) of *Actinodaphne*, 107 samples (45 species) of *Neolitsea*, 16 samples (nine species) of *Litsea,* and one each sample of *Machilus* and *Phoebe.* Branches are labeled with bootstrap values. The topology for *Neolitsea* is shown in Figure 2. Branches of the following samples are not shown: *Litsea* 1: three samples of *L. accedens* (SWK689, SWK1896 and SWK1827), a

sample of *L. verticillata* (V3539), two samples of *L*. sp. 1 (V159 and V4427), two samples of *L*. sp. 2 (V2972 and V2765), a sample of *L*. sp .3 (V457), a sample of *L*. sp. 4 (V585) and a sample of *L*. sp. 5 (V5443), *Litsea* 2: four samples of *L. johorensis* (T2421, T3066, SWK1917 and SWK2629) and a sample of *L*. sp. 6 (V5751).

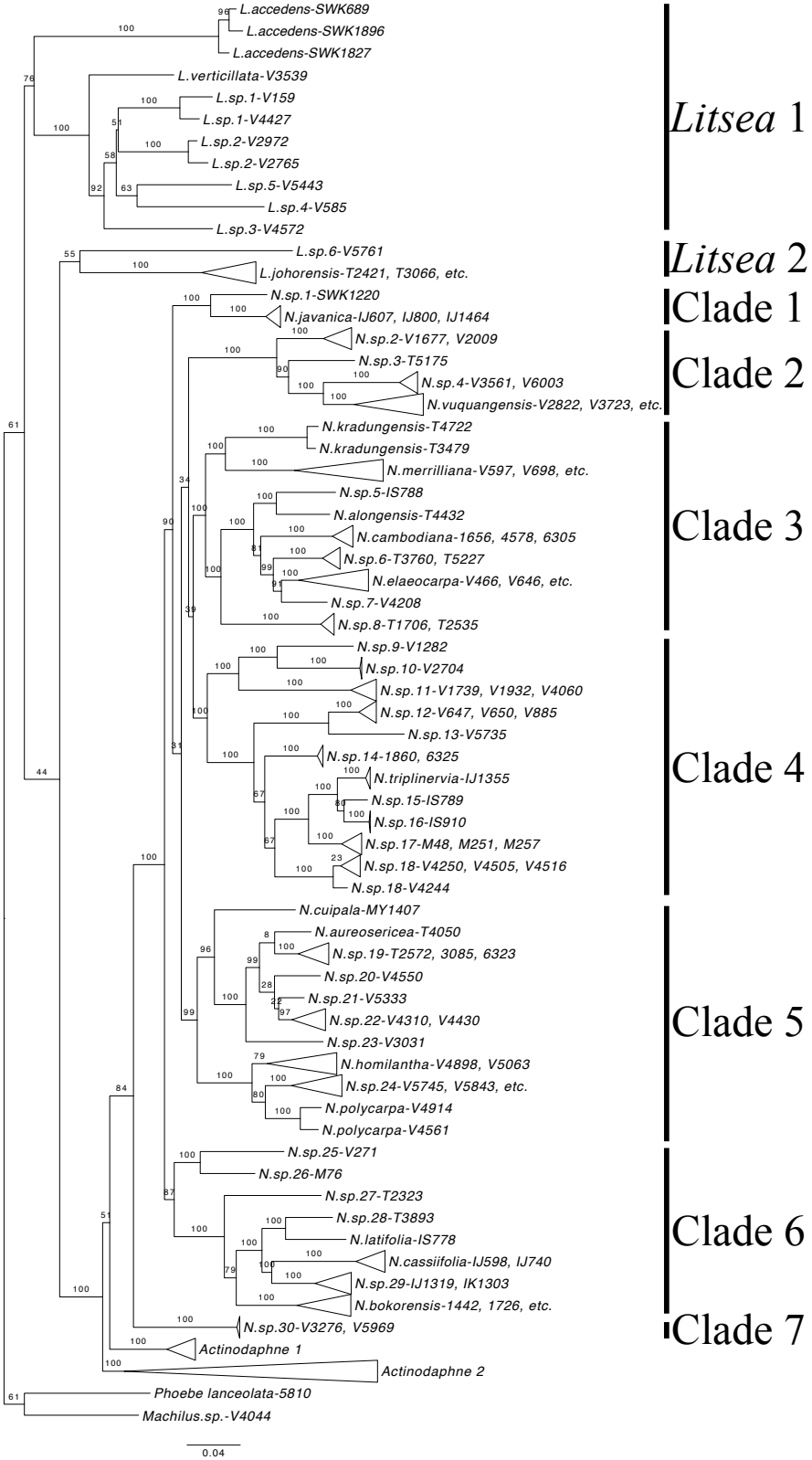

Figure 2. A MIG-seq ML tree for 107 samples (45 species) of *Neolitsea*. Branches are labeled with bootstrap values. Branches of the following samples are not shown: five

samples of *N. vuquangesis* (V2822, V3594, V3751, V3723 and V5617), nine samples of *N. merrilliana* (V597, V698, V2200, V3111, V3748, V3804, V5631, V5646 and V5931), 10 samples of *N. elaeocarpa* (V466, V646, V1214, V1245, V2510, V3035, V3044, V3058, V3730 and V5611), six samples of *N.* sp. 25 (V5745, V5834, V5842, V5843, V5863 and V5866) and nine samples of *N. bokernsis* (1442, 1726, 1730, 3160, 3217, 4124, 4126, 4584 and 6312).

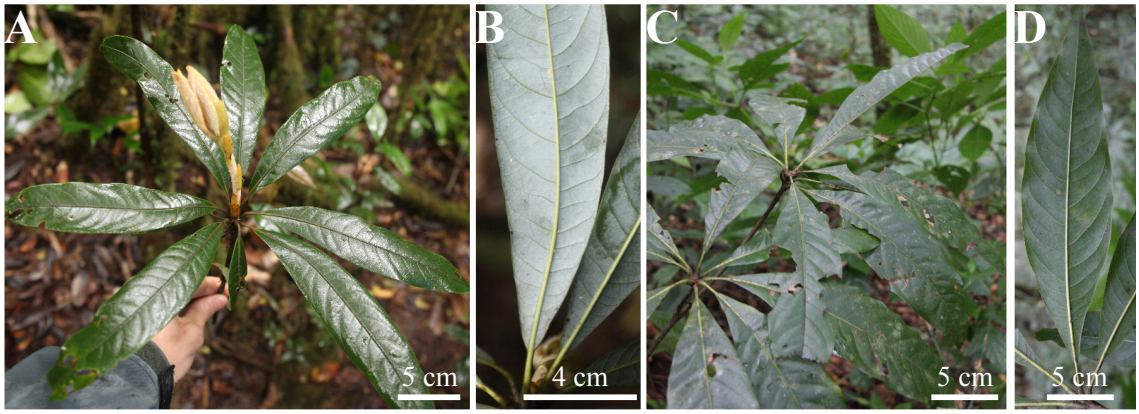

Figure 3. Leafy branch of *A.* aff. *tsaii* 1(V4477; A and B) and *A.* aff. *tsaii* 2 (T200; C and D). A and C: upper surface; B and D: lower surface.

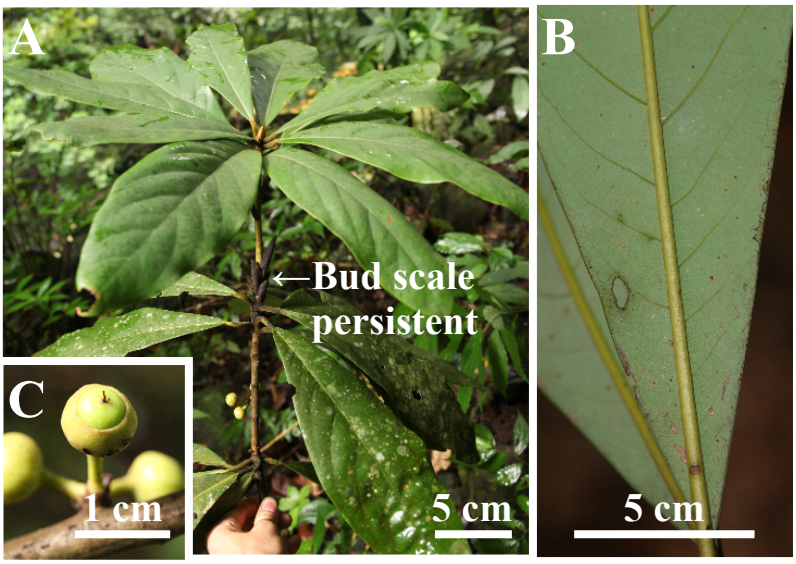

Figure 4. *Neolitsea* sp. 30 (V5969). A: a branch with three whorls of leaves; bud scales are persistent on the second node. B: portion of lower leaf surface. C: young fruits.

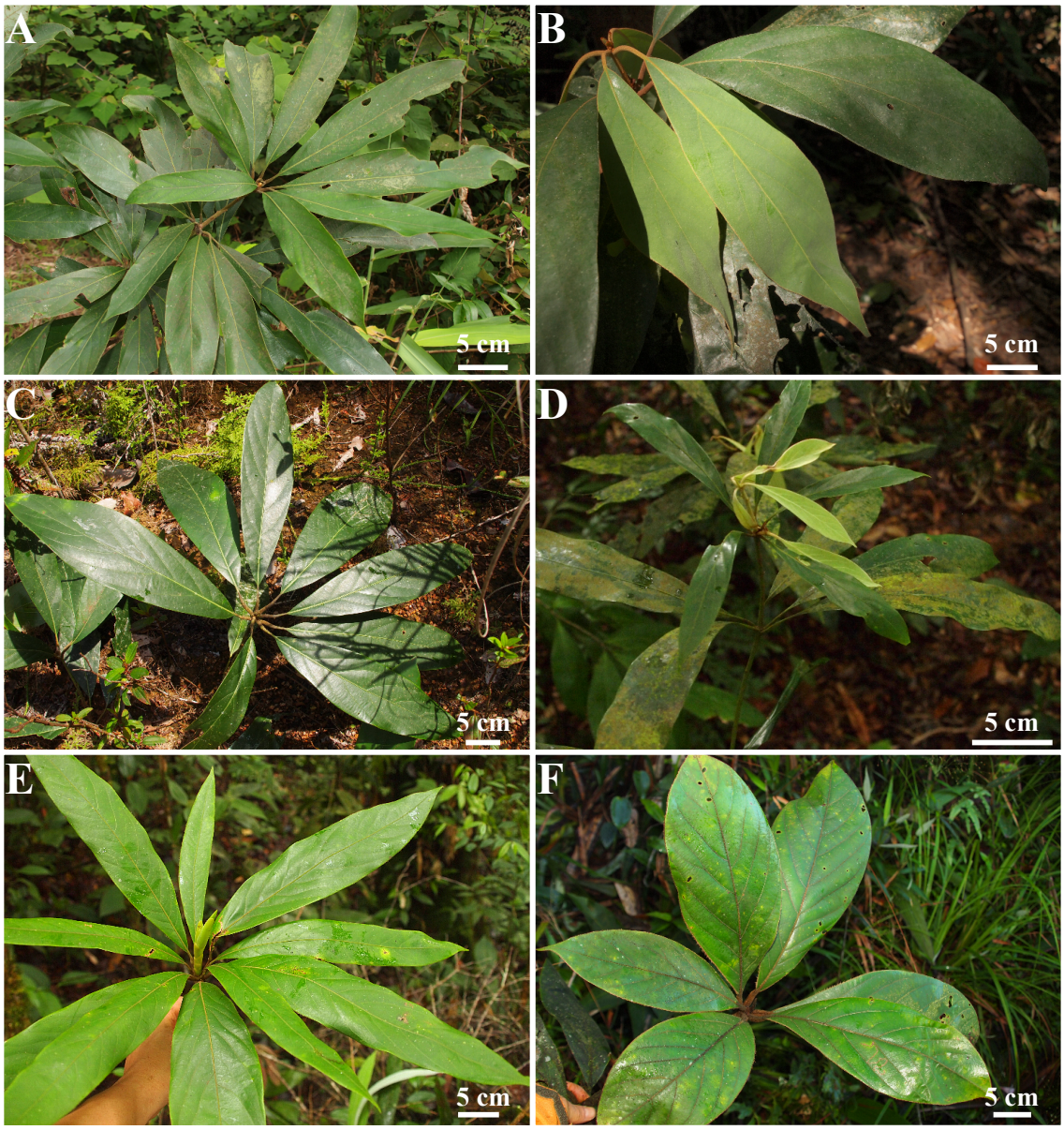

Figure 5. Three non-sister species (A, C, E) that are morphologically similar to *A. sesquipedalis,* and their sister species (B, D, F). *Actinodaphne* sp. 1 (A: V2703) is sister to *A. pilosa* (B: V1363); *A. glabra* (C: SWK1028) is sister to *A. montana* (D: IS45); *A.* sp. 3 (E: IS811) is sister to *A. heterophylla* (F: IS854).

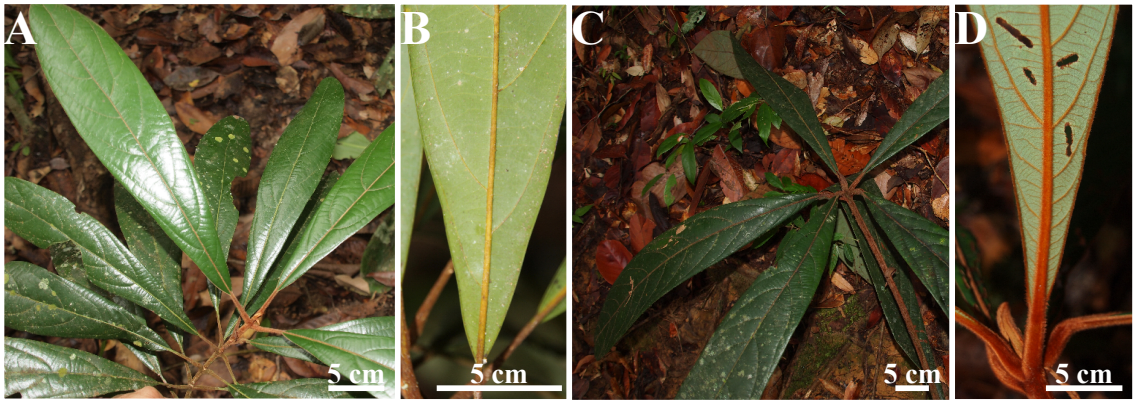

Figure 6. A species morphologically similar to *A. macrophylla.* A and B: *A. rufescens* (SWK2020), A: leafy twig, B: lower leaf surface. C and D: *A. macrophylla* (SWK2533), C: leafy twig, D: lower leaf surface.

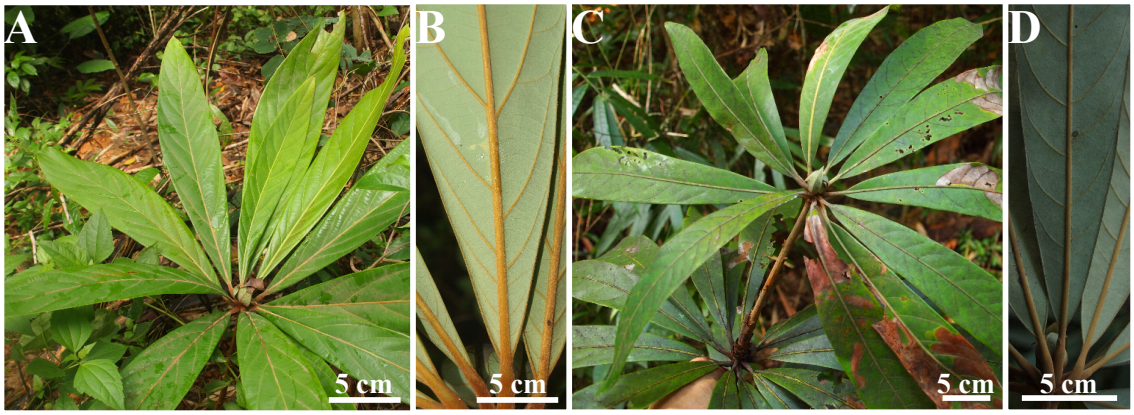

Figure 7. Geographical variation of *A. sesquipedalis* in leaf traits. A and B: *A. sesquipedalis* from Myanmar (MY366), A: leafy twig, B: lower leaf surface. C and D: *A. sesquipedalis* from Cambodia (4722), C: leafy twig, D: lower leaf surface.

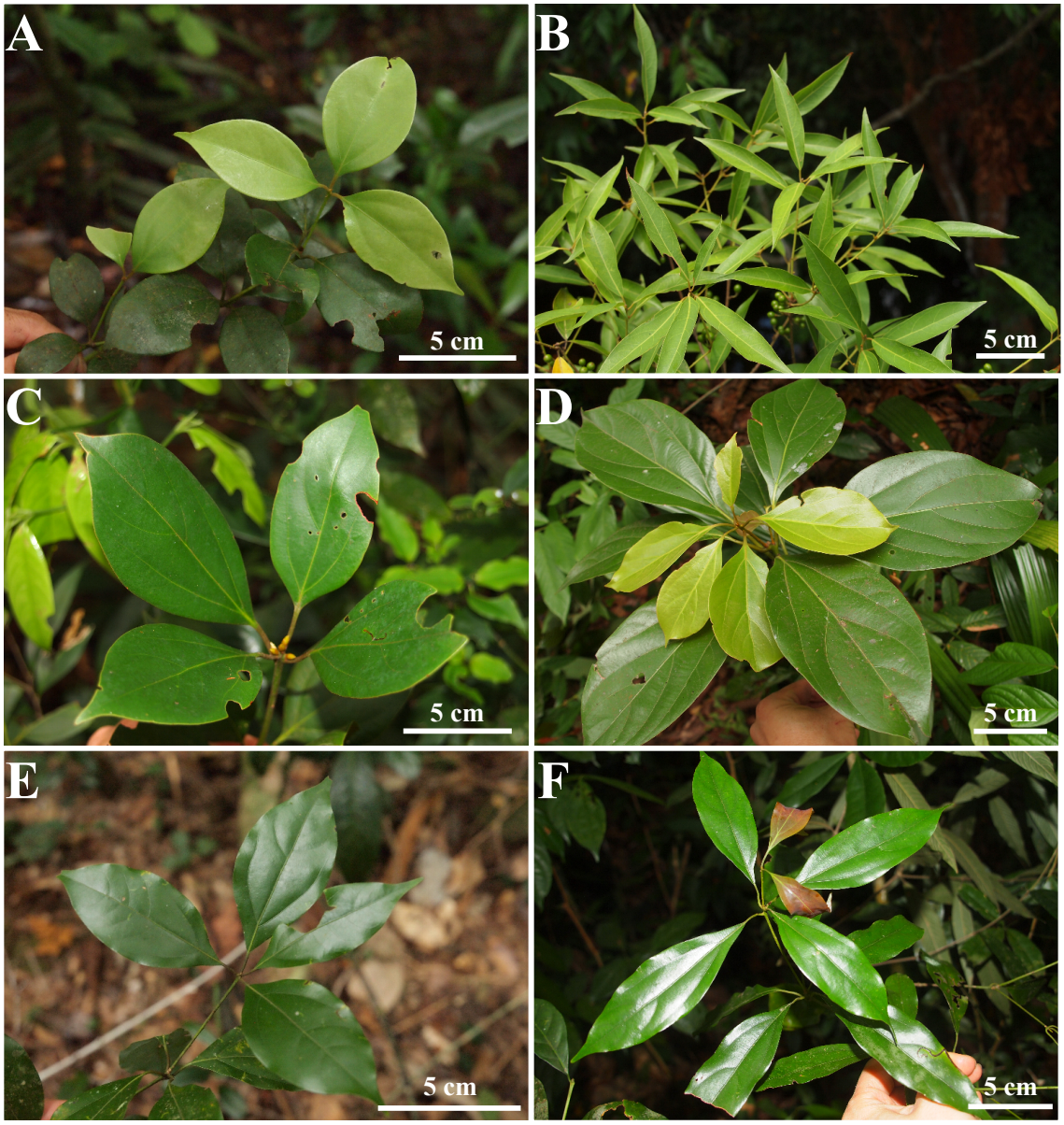

Figure 8. *Neolitsea merrilliana* (A), two non-sister species (C, E) that are morphologically similar to but not sister to *N. merrilliana* in phylogeny, and three related species (B, D, F). *N. merrilliana* (A: V597) is sister to *N. kraduengensis* (B: T3479); *N.* sp. 2 (C: V2009) is sister to *N.* sp. 4 (D: V3561); *N.* sp. 14 (E: 6325) is sister to *N.* sp. 12 (F: V885).

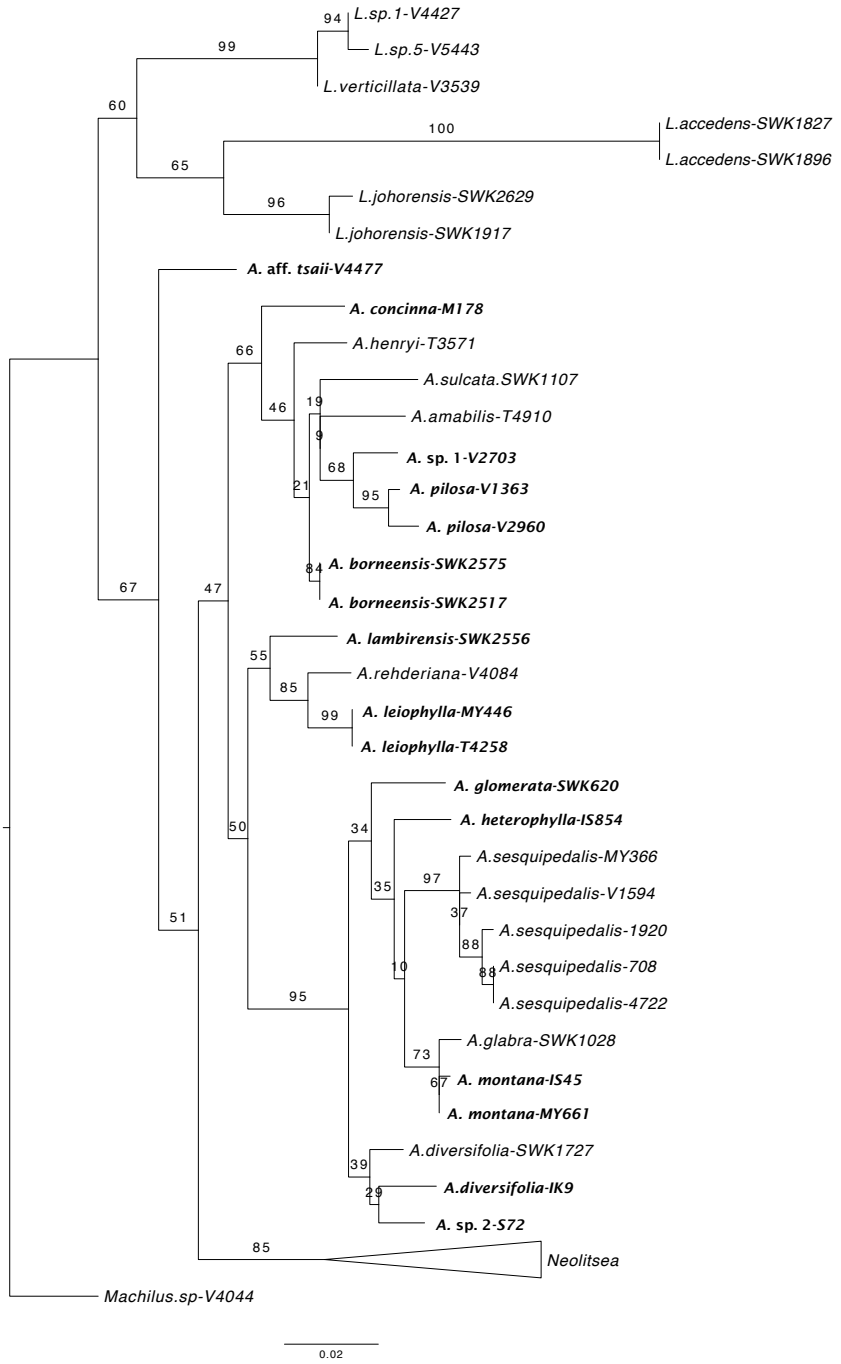

Figure 9. An ITS ML tree for 27 samples (18 species) of *Actinodaphne*, 56 samples (33 species) of *Neolitsea*, seven samples (five species) of *Litsea* and one sample of *Machilus*. Branches are labeled with bootstrap values. The topology for *Neolitsea* is shown in Figure 10.

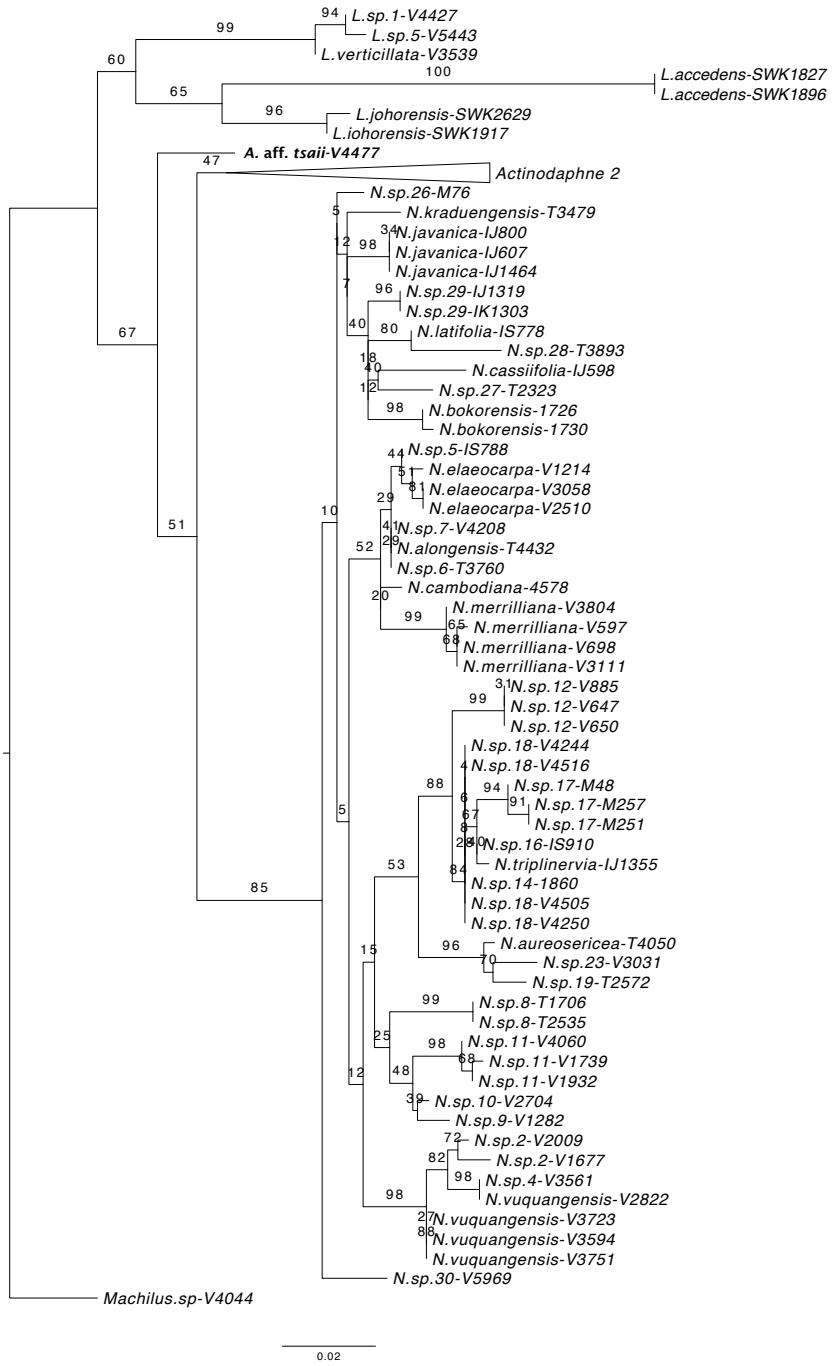

Figure 10. An ITS ML tree for 56 samples (33 species) of *Neolitsea*, seven samples (five species) of *Litsea* and one sample of *Machilus*. Branches are labeled with bootstrap values.

Chapter III

A phylogenetic analysis of *Actinodaphne* (Lauraceae) using multiplexed inter-simple sequence repeats genotyping by sequencing (MIG-seq) and a multivariate analysis of leaf morphological traits revealed 10 undescribed species including two species of *Neoactinodaphne*, a new genus from Vietnam and Thailand

Abstract

A new genus *Neoactinodaphne* Okabe, Tagane & Yahara, including two new species and a variety were described from Vietnam and Thailand. This new genus is characterized by well-developed intervening veins perpendicularly extending between secondary veins. Phylogenetic analyses based on MIG-seq showed that this new genus, having 3-merous flowers with 9 stamens, was sister to but distinct from *Neolitsea*, having 2-merous flowers with 6 stamens. Principal component analysis and a cluster analysis by Unweighted Pair Group Method using arithmetic Average were performed for a total of 67 species of *Actinodaphne* and *Neoactinodaphne* using six leaf traits: maximal number of leaves clustered on the branch top (MLC), midpoint petiole length (PL), midpoint leaf length (LL), midpoint leaf width (LW), midpoint lateral veins (LV), midpoint aspect ratio (AR). *Neoactinodaphne* is placed among species of *Actinodaphne,* showing that *Neoactinodaphne* is difficult to be distinguished from *Actinodaphne* spp by leaf shape. The MIG-seq tree showed that *A. acuminata* was placed not in *Actinodaphne* but in *Litsea*. The MIG-seq tree and morphological observations supported that eight species of *Actinodaphne* (24 %) are considered to be undescribed. Our results showed that phylogenetic analyses using MIG-seq are effective to discover and describe new species if it is combined with morphometric analyses.

Keywords:

Actinodaphne, molecular phylogeny, *Neoactinodaphne, Neolitsea*, new genus, next-generation sequencing, taxonomy

Introduction

Lauraceae are highly diverged in tropical and subtropical evergreen forests of Southeast Asia (Zhu 2006, Raes et al. 2013; Yahara et al. 2016). However, taxonomic studies on Lauraceae of this region remain incomplete, and recent studies reported nine new species of *Actinodaphne* Nees (Julia 2005, Okabe et al. in press), a new genus *Alseodaphnopsis* including three new species (Mo et al. 2017; Li et al. 2020), three new species of *Beilschmiedia* Nees (Nishida 2008, de Kok 2016a, Liu et al. 2013a), a new species of *Caryodaphnopsis* Airy Shaw (Liu et al. 2013b), two new speices of *Cinnamomum* Schaeff (Tagane et al. 2015), two new speices of *Cryptocarya* R. Br. (de Kok 2016b, Zhang et al. in press), three new species of *Endiandra* R. Br. (Arifiani 2001), two new species of *Lindera* Thunb. (Tagane et al. 2015, de Kok 2019), seven new species of *Machilus* Nees (Yahara et al. 2016, de Kok 2019, Mase et al. in press), and two new species of *Neolitsea* Merr. (Mitsuyuki et al. 2018). Further taxonomic studies are needed to elucidate the total diversity of Lauraceae in Southeast Asia. Here, we describe a new genus *Neoactinodaphne* including two new species, *N. hongiaoensis* and *N. langbianensis*, based on the specimen we collected from southern Vietnam. In addition, we show that there are eight undescribed species of *Actinodaphne* among specimens we collected in Southeast Asia.

The genus *Actinodaphne* Nees is a mainly Asiatic group of evergreen trees (Rohwer 1993, van der Werff 2001) close to *Litsea* Lam. and *Neolitsea* (Rohwer 2000, Chanderbali *et al*. 2001). Molecular phylogenetic studies showed that *Actinodaphne* is

unlikely to be monophyletic (Li *et al*. 2004, Li *et al*. 2006, Li *et al*. 2007, Mitsuyuki *et al*. 2018). Recently, Okabe et al. (in press) reconstructed a highly resolved phylogenetic tree of 22 *Actinodaphne* species from Southeast Asia using multiplexed ISSR genotyping by sequencing (MIG-seq; Suyama & Matsuki 2015) and showed that a Vietnamese sample identified as *"Actinodaphne* aff. *tsaii"* is sister not to a clade including the other species of *Actinodaphne* (hereafter designated as *Actinodaphne* s.str.) but to *Neolitsea*. In addition, Okabe et al. (in press) reconstructed another phylogenetic tree using internal transcribed spacer (ITS) sequences for 36 *Actinodaphne* spp., including newly determined sequences for 22 spp. and previously determined sequences for 14 spp. (Li *et al*. 2006, Li *et al*. 2007, Fijridiyanto & Murakami 2009, Mitsuyuki *et al*. 2018). The resulted ITS tree showed that *A.* aff. *tsaii* is close to *A. tsaii* Hu distributed in Yunnan, China.

In this study, we carried out an additional MIG-seq analysis by adding 61 samples that were not examined by Okabe et al. (in press). These new samples include two species that are morphologically similar to *A.* aff. *tsaii* (a sample from Northern Thailand and another sample from Southern Vietnam). The results supported that the clade composed of *A.* aff. *tsaii* and the other two samples was sister not to *Actinodaphne s.str.* but to *Neolitsea*. We also carried out an additional field work in the habitat of *A.* aff. *tsaii* because the specimen studied by Okabe et al. (in press) was in a vegetative state. Consequently, we could collect specimens of *A.* aff. *tsaii* having male and female flowers and young fruits. Using these fertile specimens, we conducted morphological comparison of *A.* aff. *tsaii* and the two similar samples with 63 previously described species of *Actinodaphne* (Ho 1934, Huang & van der Werff 2008, Tanaros et al. 2010, Dao 2017). Those samples did not match any described species. In addition, those species were sister to *Neolitsea*, but they had 3-merous flowers with 9

stamens (*Neolitsea* has 2-merous flowers with 6 stamens).

Based on the results of MIG-seq analysis and morphological comparison, we here describe *A.* aff. *tsaii* as *Neoactinodaphne hongiaoensis* sp. nov. under a new genus *Neoactinodaphne*. In addition, we describe two new species and a new variety of *Neoactinodaphne*: *N. hongiaoensis* var. *inthanonensis*, *N. langbianensis.* Our new MIG-seq tree also showed that eight operational taxonomic units (OTUs) are distinct from 63 previously described species of *Actinodaphne*, suggesting that these are eight undescribed species.

Taxonomy

To validate the names of *Neoactinodaphne hongiaoensis* and *N. langbianensis*, we first describe these two new species under a new genus *Neoactinodaphne*. Then, we document Materials and Methods, followed by Results and Discussion.

1. New genus.

Neoactinodaphne Okabe, Tagane & Yahara, **gen. nov.**

Type. *Neoactinodaphne hongiaoensis* Okabe, Tagane & Yahara (described below).

Diagnosis. The new genus *Neoactinodaphne* is close to *Neolitsea* Merr. and *Actinodaphne* Nees, but is distinguished from *Neolitsea* by 3-merous flowers with 9 stamens (vs. 2-merous flowers with 6 stamens), and from *Actinodaphne*, by intervening veins perpendicularly extending between secondary veins.

Desciption. Evergreen tree up to 20 m tall. Terminal buds perulate with imbricate scales, bud scales ovate to broadly ovate, margin ciliate, outside densely appressed brown hairy outside, and inside glabrous. Current year twigs densely covered with yellowish-brown hairs, old twigs dark brown, glabrescent. Leaves alternate, 5–9 clustered at branch

nodes; blades lanceolate, narrowly elliptic-lanceolate, or oblanceolate, apex acuminate or attenuate, base attenuate, acute, or short cuneate, margin entire, flat or recurved when dry, green, grayish green, or olive green adaxially, glaucous abaxially, densely covered with yellowish-brown hairs abaxially at least when young, midrib distinctly raised on both surfaces, secondary veins 7–17 pairs, prominent on both surfaces, intervening veins between secondary veins 3–6 pairs, perpendicular to broadly ascending, tertiary veins scalariform, slightly prominent adaxially, prominent abaxially; petiole terete or flat only above, hairy. Inflorescence umbellate or shortly paniculate, in leaf axils or on twigs after leaves fallen; male inflorescence 4–16-flowered, bracts 4, imbricate, densely sericeous outside, glabrous inside, caducous. Perianth segments 6, elliptic, hairy both sides. Flowers unisexual and reproductive system monoecious. In male flowers, fertile stamens 9, filaments villous, anther 4-celled, filaments of 1st and 2nd whorls eglandular, of 3rd whorls 2-glandular at base. In female flowers, staminodes 9, stigma shield-shaped. Fruits globose, black when dry, glabrous.

Etymoloty. *Neoactinodaphne* alludes to the morphological resemblance to *Actinodaphne.*

Distribution and habitat: *Neoactinodaphne* includes two species, distributed northern Thailand and southern Vietnam. Both species grow in montane evergreen forests dominated by Fagaceae and Lauraceae.

1. New species. ***Actinodaphne hongiaoensis*** Okabe, Tagane & Yahara, **sp. nov.**

var. *hongiaoensis*

Figure 1and 2

Diagnosis. *Actinodaphne hongiaoensis* is similar to *A. sikkimensis* and *A. tsaii* in having a midrib raised on the adaxial surface, distinct intervening veins perpendicularly extending between lateral veins and villous fillaments, but distinguished from these species in having less than 12 pairs of lateral veins (vs. 12–18 pairs in *A. hongiaoensis*) and oblong fruits (vs. globose in *A. hongiaoensis*). *Actinodaphne hongiaoensis* is also similar to *A. omeiensis* in having densely hairy young twigs, lanceolate leaf blade with usually 12 or more pairs of lateral veins, and 1–4 cm long petiole, but distinct from them in having a midrib raised adaxially (vs. sunken in *A. omeiensis*) and distinct intervening veins perpendicularly extending between lateral veins.

Type. VIETNAM. Lamdong Province: Hon Giao, 12°11'30.89"N, 108°42'42.86"E, alt. 1862 m, 21 January 2020, with male and female flowers and young fruits, *Yahara et al. V11345* (holotype KYO!, isotype DLU!, FU!, KAG).

Description. Tree 12 m tall, GBH 7.7–10.2 cm. Terminal buds ellipsoid, 5–9 mm long, perulate with imbricate scales, bud scales ovate to broadly ovate, 2–7 mm long, apex acuminate or short emarginate, margin ciliate, outside densely appressed brown hairy outside, and inside glabrous. Current year twigs densely covered with yellowish-brown hairs, old twigs dark brown, glabrescent. Leaves alternate, 7–9 clustered at branch nodes; blades narrowly elliptic-lanceolate to oblanceolate, 10.8–17.7 x 3.8–5.8 cm, apex acuminate, base acute to short cuneate, margin recurved when dry, green to grayish green adaxially, glaucous abaxially, densely covered with yellowish brown hairs when young, glabrous on both surfaces when mature, midrib distinctly raised on both surfaces,

secondary veins 10–18 pairs, prominent on both surfaces, intervening veins between secondary veins 1–6, perpendicular, prominent on both surfaces, tertiary veins scalariform, slightly prominent adaxially, prominent abaxially; petiole 0.7–2 cm long, flat to concave adaxially, rounded abaxially, pubescent. Inflorescence umbellate in leaf axils, 4–7-flowered, peduncle to 0.4 mm long, yellow brown hairy; bracts 4, imbricate, semiorbicular to ovate-oblong, 4–5 x 3–5 mm, densely sericeous outside, glabrous inside, caducous. Pedicle 3–7 mm long, densely villous, enlarging to 9 mm long when fruiting. Perianth segments 6, elliptic, ca. 2 x 1.3 mm, densely sericeous both sides, margin ciliate. Flowers unisexual and reproductive system monoecious. In male flowers, fertile stamens 9, filaments ca. 3 mm long, villous, anther ca. 1 mm long, 4-celled, filaments of 1st and 2nd whorls eglandular, those of 3rd whorls 2-glandular at base, glands reniform, stipitate, rudimentary pistil glabrous. In female flowers, staminodes 9, ca. 3 mm long, style ca. 1 mm long, stigma shield-shaped, ca. 0.4 mm in diam. Immature fruits globose, ca. 4 mm long, black when dry, glabrous.

Additional specimens examined. VIETNAM. Lam Dong province, Bidoup-Nui Ba National Park, Hon Giao: 12°11'28.2"N, 108°42'46.8"E, alt. 1807 m, 27 Feb. 2016, *Tagane et al. V4477* [ster.] (DLU, FU); ibid., 12°11'32.16"N, 108°42'41.56"E, alt. 1887 m, 22 Apr. 2019, *Yahara et al. V9860* [ster.] (DLU, FU); ibid, 12°11'30.89"N, 108°42'42.86"E, alt. 1862 m, 21 Jan. 2020, *Yahara et al. V11345* [male fl.] (DLU, FU, KAG), *V11347* [fr.] (DLU, FU, KAG).

Distribution and habitat. Endemic to Vietnam. Only five trees are known in the montane evergreen forest of the type locality.

Phenology. Specimens with male and female flowers and young fruits were collected in January (V11345, V11346, V11347).

Etymology. The specific epithet *hongiaoensis* reflects the area where the type was collected.

GenBank accession No. Yahara et al. V4477. LC504508 (ITS)

var. *inthanonensis* Okabe & Yahara, var. nov.

Figure 3

Diagnosis. This variety is distinguished from var. *hongiaoensis* by lanceolate leaves (vs. elliptic-lanceolate to oblanceolate) and shortly paniculate inflorescence (vs. umbellate).

Type. THAILAND. Chiang Mai, Doi Inthanon, 1700 m altitude, 15 Jan. 1997, with male flowers, *M.Hara 408* (CBM176875)

Description. Tree 20 m tall, DBH 40 cm. Terminal bud ellipsoid, ca. 4 mm long, perulate with imbricate scales, bud scale broadly ovate, orbicular, ovate, 3–10 mm long, apex mucronate apiculate or emarginate, margin ciliate, densely apressed brown hairy outside, glabrous inside. Current year twigs densely covered with yellowish-brown hairs, old twigs grayish brown to dark brown, glabrescent. Leaves alternate, 4–8 clustered at branch nodes; blade lanceolate, oblong elliptic, 10.8–17.7 x 3.8–5.8 cm for adult, 14–19.2 x 4.5–6.8 cm for sapling, apex acute or attenuate, base attenuate or cuneate, margin entire, flat to slightly recurved when dry, grayish green adaxially, yellowish brown

abaxially, glaucous abaxially, glabrous except densely yellowish brown hairy on both surfaces when young, midrib prominent on both surfaces, secondary vein 14–18 pairs, prominent on both surfaces, intervening veins between secondary veins (0–)1–5 at angle of ca. 90 degree from midrib, scalariform, or scalariforming-reticulate, prominent on both surfaces; petilole 1.0–1.8 cm long for adult, 1.5–2.5 cm long for sapling, terete or flat only above, densely yellowish brown hairy when young. Inflorescence umbellate with short pedncle or paniclate in leaf axils or on twigs behind leaves, up to 16–flowered, peduncle to 2–4 mm long, yellowish brown hairy; bracts imbricate, broadly ovate-triangular, 1–2.5 mm, densely appressed yellowish brown hairy outside, glabrous inside, caduceus. Pedicle 2.5–4 mm long, densely yellowish brown hairy. Flowers unisexual. Male flower: up to 17 per inflorescence, perianth segments 6, ovate-oblong, ca. 3 x 2 mm, apex obtuse, margin ciliate, densely appressed hairy outside, pubescent lower 1/2 except near base which is glabrous, fertile stamens 9, filaments ca. 3.5 mm long, villous, anther ca. 1.2 mm long, 4-celled, filaments of 3rd whorls 2-glandular at base, grands reniform, stipetate, rudimentary pistil ca. 2.1 mm long, glabrous. Female flowers not seen. Fruits subglobose, ca. 7 mm in diam., green brown to blackish brown, glabrous, seated on perianth tube, fruiting pedicel 4–6 mm long, densely yellowish brown hairy.

Additional specimens examined. Thailand. Chiang Mai, Doi Inthanon, 1700 m altitude, 15 Jan. 1997, with male flowers, M. Hara 408 (CBM176875); ibid., 28 Dec. 1996, M.Hara 119 (CBM176878); ibid., 24 Dec. 1996, M. Hara 38 (CBM176879) ibid., 18 Jan. 1999, with male flowers, K. Chai-udom 1004 (CBM176876); ibid., 30 Apr. 1999, with fruits, K. Chai-udom 1098 (CBM176877); ibid., 30 Apr. 1999, with fruits, K. Chai -udom 1098 (CBM176874); ibid.

Distribution and habitat. Thailand (endemic to Doi Inthanon). Spradically found in hill evergreen forest at 1700 m alt.

Phenology. Specimens with male flowers were collected in January (M.Hara 408). Specimens with fruits were collected in April (K. Chai-udom 1098).

Etymology. Of Doi Inthanon (type locality).

Note. As far as we examined, var. *inthanoensis* has from 8 to 16 male flowers per inflorescence while var. *hongiaoensis* has 4–7 flowers per inflorescence. However, the number of specimens having male flowers is limited and we are not sure whether this trait is stable and effective for discriminating the two varieties.

Neoactinodaphne langbianensis Okabe, Tagane & Yahara **sp. nov.**

Diagnosis. *Neoactinodaphne hongiaoensis* is distinguished from *N. hongiaoensis* by smaller leaves (7.2–11 x 1.7–3.5 cm vs. 10.8–17.7 x 3.8–5.8 cm in *N. hongiaoensis*), 5–6 clustered at branch nodes (7–9 in *N. hongiaoensis*), and fewer secondary veins (7–12 pairs vs. 10–17 pairs in *N. hongiaoensis*).

Type. VIETNAM. Lamdong Province: Mt. Langbian, in montane evergreen forest near the summit, 12°02'50.32"N, 108°26'24.53"E, alt. 2109 m, 21 December 2018, with male flowers, *Yahara et al. V9599* (holotype KYO!, isotype DLU!, FU!).

Description.

Tree 20 m tall, DBH 40 cm. Terminal buds ellipsoid, ca. 1 mm long, perulate with imbricate scales, bud scales ovate, 2–4 mm long, margin ciliate, outside densely appressed brown hairy, inside glabrous. Current year twigs densely covered with yellowish-brown hairs, old twigs dark brown, pubescent. Leaves alternate, 5–6 clustered at branch nodes; blade lanceolate, 7.2–11 x 1.7–3.5 cm, apex attenuate, base attenuate or acute, margin entire, flat to slightly recurved when dry, olive green adaxially, glaucous abaxially, midrib prominent on both surfaces, secondary veins 7–12 pairs, prominent abaxially, densely covered with yellowish-brown hairs abaxially, intervening veins between secondary veins 3–4, perpendicular to broadly ascending, tertiary veins scalariform, or scalariforming-reticulate, prominent abaxially; petioles 0.7–1 cm long, terete or flat only above, densely covered with yellowish-brown hairs. Inflorescence umbellate in leaf axils or on twigs after leaves fallen, from 4 to 16 male flowers per inflorescence, yellowish brown hairy; 4 bracts imbricate, broadly ovate 2–2.5 mm, yellowish brown hairy both sides, caducous. Perianth segments 6, elliptic, pubescent adaxially, sparsely pubescent abaxially. Male flowers: fertile stamens 9; filaments tomentose, of 3rd whorls each with 2 sessile or shortly stipitate glands at base. Female flowers and fruits not seen.

Additional specimens examined. VIETNAM. Lam Dong province, Bidoup-Nui Ba National Park, Mt. langbian: , 12 02'46.3"N, 108 26'01.5"E, alt. 1905 m, 25 Mar. 2018, *Yahara et al. V7895* [ster.] and *V8040* [ster.] (DLU, FU, KAG); ibid, 12º02'48.13"N, 108º26'06.67"E, alt. 1923 m, 24 June 2018, *Tagane et al. V8960* [ster.] (DLU, FU, KAG).

Distribution. Vietnam (Endemic to Mt. langbian). Individuals are found in the montane

evergreen forest.

Phenology. Specimens with male flowers were collected in December (V9599).

Etymology. Of Mt. langbian (type locality).

Materials and Methods

Field survey

We first discovered the new species *Neoactinodaphne hongiaoensis* during our field survey in Bidoup-Nui Ba National Park, Lam Dong province, Vietnam in February 2016. We set up a small plot of 100 m x 5 m at the altitude of 1807 m in Hon Giao (12°11'28.2"N, 108°42'46.8"E; near the border of Lamdong and Khanh Hoa Provinces) and recorded all the vascular plant species within the plot following the method described by Zhang et al. (2017, 2019), Tagane (2019) and Mase et al. (2020). Because the specimen collected in this survey was in a vegetative state, we carried out additional field surveys in the same location in April 2019 and January 2020. In the third survey, we collected specimens with male and female flowers and young fruits. The 168 samples used for MIG-seq analyses in the study were collected through a series of transect surveys in various locations of Southeast Asia (Tagane 2019), including Khanh Hoa Province adjacent to Lam Dong Province.

We collected *Neoactinodaphne hongiaoensis* var. *inthanonensis* in a 500 m x 300 m plot registered as a plot of Smithonian Forest Global Eearth Observatory Network (https://forestgeo.si.edu/sites/asia/doi-inthanon) in November 2011. We

collected our specimen (T200) from a 6 m tall tree, tagged as ID 0028650 as *Actinodaphne sikkimenensis*.

We first collected the new species *Neoactinodaphne langbianensis* as *Neolitsea* sp. during our field survey in Mt. Langbian at Bidoup-Nui Ba National Park, Lam Dong province, Vietnam in March 2018. Again, we set up a small plot of 100 m x 5 m at the altitude of 1905 m in Mt. Langbian (12 02'46.3"N, 108 26'01.5"E). We recorded three sterile trees of *N. langbianensis* in the plot. In December 2018, we collected flowering specimens of at the altitude of 2109 m (12°02'50.32"N, 108°26'24.53"E) on the way to the peak of Mt. Langbian.

The other specimens used in this study (Table 1) was collected in our field surveys in Myanmar, Thailand, Cambodia, Laos, Vietnam, Taiwan, Japan, Phillipines, Malaysia and Indonesia since 2011. We collected these specimens in 100 m x 5 m plots for plant diversity assessments or neaby these plots (see Zhang et al. 2017, 2019, Tagane 2019. and Mase et al. 2020).

Review of taxonomic literature

To characterize the new species *Neoactinodaphne hongiaoensis* morphologically, we first applied keys developed in previous taxonomic studies of *Actinodaphne* in Vietnam and surrounding countries including China and Thailand (Ho 1934, Huang & van den Werff 2008, Tanaros et al. 2010, Dao 2017). Then, we compared our specimens of *N. hongiaoensis* with specimen images of morphologically similar species using the JSTOR Global Plants (http://plants.jstor.org/) and Chinese Virtual Herbarium (http://www.cvh.ac.cn/en). We also examined specimens kept in BKF, FOF, KYO HNL and SAR. In Vietnam, the following three species not listed by Ho (1934), Huang & van der Werff (2008), and Tanaros et al. (2010) are recorded (Hô

1999): *A. ellipticibacca* Kosterm., nom. nud., *A. perlucida* C.K.Allen, *A. rehderiana* (C.K.Allen) Kosterm. ex Yahara. We examined the type specimen images of these species using the JSTOR Global Plants, and the specimens of *A. perlucida* and *A. rehderiana* we collected in the vicinity of the type locality of *N. hongiaoensis*. We also examined the image of a specimen K000793062 collected from Laos and annotated as *Actinodaphne laosensis* Kosterm. *nom. nud*., and a specimen CBM176875 collected from Thailand and identified as *Actinodaphne* sp. To confirm that *N. hongiaoensis* is not identical with any species described from Malaysia and Indonesia, we examined type specimen images and/or original descriptions of all the previously described species (64 described species excluding some poorly known species).

Multivaliate analysis of leaf traits

To confirm that *Neoactinodaphne* spp. do not match any of the described species of *Actinodaphne*, we conducted a principal component analysis (PCA) and a cluster analysis using Unweighted Pair Group Method using arithmetic Average (UPGMA). We constructed a matrix of the following nine traits, maximal number of leaves clustered on the branch top (MLC), midpoint petiole length (PL, mm), midpoint leaf length (LL, cm), midpoint leaf width (LW, cm), midpoint lateral veins (LV, pairs), midpoint aspect ratio (AR), petiole pubescence (PP, three levels: glaburous, pubescens and tomentose), venation type of secondary veins (SV, two levels: pinninerved or triplinerved), and venation type of tertiary veins (TV, four levels: scalariform, interveining veins, reticulate and scaraliform-reticulate), for a total of 65 described species of *Actinodaphne* recorded in China, Myanmar, Thailand, Cambodia, Laos, Vietnam, Malaysia, and Indonesia (Kalimantan, Sumatra and Java) and two species of *Neoactinodaphne*: *N. hongiaoensis* and *N. langbianensis*. Because mimimal and

maximal values of leaf length, leaf width, petiole lenght, lateral veins and aspect ratio are highly correlated, we used a midpoit value of each trait for each species. PCA and UPGMA clustering were performed with R ver. 3.6.0, using function “brcomp” and “hclust”, respectively. For those analysis, we excluded three categorical variables (PP, SV and TV).

DNA extraction and MIG-seq analysis

We performed DNA extraction and MIG-seq analysis following a protocol described by Okabe et al. (in press). Briefly, we extracted DNA from a piece of silica gel-dried leaf samples using the CTAB method of Doyle & Doyle (1987). For 168 samples (64 species of *Actinodaphne, Neoactinodaphne, Neolitsea* and outgroups; Table 3), we amplified thousands of short sequences (loci) from each genome using primers designed for MIG-seq following Suyama & Matsuki (2015). We performed quality control of the raw MIG-seq data and assembled the remaining reads using de novo map pipelines (ustacks, cstacks, sstacks) in stacks ver. 1.48 (Catchen *et al*. 2011). Finally, the SNP sites of all the samples file was converted to phylip format and used to reconstruct a ML tree in RaxML with 500 times bootstrap replicates. A total of 60,557 loci were used to construct the phylogenetic tree.

Results

Review of taxonomic literature

To characterize *Neoactinodaphne hongiaoensis*, we first reviewed taxonomic literature of *Actinodaphne* in China, Indo-china, and Thailand. In the first comprehensive taxonomic study of *Actinodaphne*, Ho (1934) enumerated 10 species of China and Indo-china. He keyed out the following four groups.

(1) Species with cymose inflorescences: *A. henryi* and *A. cochinchinensis* (now treated as a synonym of *A. pilosa*; Huang & van den Werff 2008).

(2) Species with glomerate, sessile or subsessile inflorescences, and triplinerved verticillate or pseudoverticillate leaves: *A. obovata.*

(3) Species with glomerate, sessile or subsessile inflorescences, and pinninerved alternate leaves: *A. hongkongensis* (now treated as a synonym of *Neolitsea cambodiana* var. glabra; Huang & van den Werff 2008), *A. ferruginea*.

(4) Species with glomerate, sessile or subsessile inflorescences, and pinninerved verticillate or pseudoverticillate leaves: *A. cupularis*, *A. reticulata*, *A. sesquipedalis*, *A. chinensis* (now treated as *Litsea rotundifolia*; Huang & van den Werff 2008), *A. confertifolia* (now treated as *Neolitsea confertifolia*; Huang & van den Werff 2008). Using the key of Ho (1934), *N. hongiaoensis* was included in this group.

For the 17 species distributed in China, Huang & van der Werff (2008) keyed out the following groups.

(1) Species with leaf blade triplinerved: *A. obovata* and *A. menghaiensis*.

(2) Species with leaf blade pinninerved and bud scale persistent: *A. obscureinervia*, *A. trichocarpa*, *A. koshepangii*, *A. omeiensis*, *A. kweichowensis* and *A. forrestii*.

(3) Species with leaf blade pinninerved and bud scale caducous: the rest nine species, *A. omeiensis* and *A. forrestii* (the last two species were overlapped to (2)). Using the key of Huang & van den Werff (2008), *Neoactinodaphne hongiaoensis* and *N. langbianensis* were included in this group.

For the 11 species in Thailand, Tanaros et al. (2010) keyed out the following groups.

(1) Species with terminal buds covered with large leaf-like scales: *A. glomerata*, *A. sesquipedalis* var. *cambodiana*, *A. sesquipedalis* var. *glabra* and *A*. sp. 1.

(2) Species with perulate terminal buds and inflorescences arranged in a raceme: *A.*

montana and *A. henryi*.

(3) Species with shoot apex with terminal buds perulate and umbels on short peduncles or fasiculate: the rest six species. Using the key of Tanaros et al. (2010), *N. hongiaoensis* was included in this group.

For the 10 species of Vietnam, Dao (2017) keyed out the following group.

(1) Species with branched inflorescences: *A. obovata*, *A. pilosa*, *A. rehderiana*, and *A. elliptibacca.*

(2) Species with simple umbellate inflorescence and alternate leaves: *A. ferruginea*.

(4) Species with simple umbellate inflorescence and verticillate leaves: *A. tonkinense*, *A. perlucida*, *A. sesquipedalis*, *A. forrestii*, and *A. reticulata*. Using the key of Don (2017), *N. hongiaoensis* was included in this group. Among these species, *A. tonkinense* described by Don (2017) is distinct from *N. hongiaoensis* and all the other species in having peduncles 3–10cm long, *A. sesquipedalis* is distinct in having terminal buds covered with large leaf-like scales (Group (1) of Tanaros et al. 2010), and *A. reticulata* is distinct in having finely reticulate veins (vs. scalariform in *A. hongiaoensis* and many other species).

Using the above keys, a total of 14 spp. were keyed out as morphologically similar to *N. hongiaoensis*. Among them, the following eight species were distingueished from *A. hongiaoensis* in having lateral veins less than 10 pairs (vs. 12–18 pairs in *A. hongiaoensis*): *A. amabilis, A. angustifolia, A. glaucina, A. koshepangii*, *A. mushaensis*, *A. paotingensis*, *A. perglabra*, *A. perlucida*, and *A. tsaii*. Finally, we compared *N. hongiaoensis* with the rest five species: *A. acuminata*, *A. cupularis, A. forrestii, A. omeiensis*, and *A. sikkimensis* (Table 2). We also compared *N. hongiaoensis* with *A. tsaii* because the ITS phylogeny showed that *N. hongiaoensis* is sisiter to *A. tsaii* (Okabe et al. in review).

Among the species compared in Table 2, *A. cupularis*, *A. forrestii* and *A. omeiensis* are different from *A. hongiaoensis* in having a midrib sunken on the adaxial surface (vs. distincltly raised in *A. hongiaoensis*) and no distinct intervening veins between secondary veins (vs. distinct intervening veins perpendicularly extending between secondary veins in *A. hongiaoensis*). *Actinodaphne cupularis* and *A. forrestii* have glabrous fillaments, but *A. hongiaoensis* has villous fillaments; hairiness of fullaments is unknown for *A. omeiensis.* While *A. acuminata*, *A. sikkimensis* and *A. tsaii* are different from *A. hongiaoensis* in having less than 12 pairs of lateral veins (vs. 12–18 pairs in *A. hongiaoensis*) and oblong fruits (vs. globose in *A. hongiaoensis*), these three species are similar to *A. hongiaoensis* in having a midrib distinctly raised on the adaxial surface, distinct intervening veins perpendicularly extending between lateral veins, and villous fillaments. Among these three, *A. acuminata* is distinct in that young twigs and young leaves are glabrous (vs. yellowish brown tomentose in *A. hongiaoensis*; white tomentose in *A. sikkimensis*, and grey-brown tomentose in *A. tsaii*).

Among three Vietnamese species not listed by Ho (1934), Huang & van der Werff (2008), and Tanaros et al. (2010), *A. ellipticibacca* is easily distinguished from *A. hongiaensis* in wider elliptic leaves. *Actinodaphne perlucida* and *A. rehderiana* are distributed in the vicinity of the type locality of *A. hongiaoensis* and we observed these species in their havitats. *Actinodaphne perlucida* was collected at an elevation of 1000m on the eastern slope of Son Thai Commune, Khanh Hoa Province., Khanh Vinh District, Khanh Hoa Province, 12º13'00.63"N, 108º44'58.24"E, *Yahara et al. V10005*, DLU, FU); this point is located approximately 6km east of the type locality of *A. hongiaoensis*. It has lanceolate leaves similar to *A. hongiaoensis*, but is distinct in having young leaves white tomentose (vs. yellowish-brown tomentose in *A. hongiaoensis*), a midrib sunken on the adaxial surface, 7－10 pairs of lateral veins, and no distinct intervening veins

extending between lateral veins. *Actinodaphne rehderiana* was common in Mt. Lambian of Bidoup-Nui Ba National Park (Nagahama et al. 2019). It has elliptic leaves wider than *A. hongiaoensis*, white tomentose young leaves (January 19, 2020, 1693 m, *Yahara et al. V11289*, DLU, FU), midribs sunken on the adaxial surface, 7–10 pairs of lateral veins, and no distinct intervening veins extending between lateral veins.

Neoactinodaphne langbianensis is similar to *N. hongiaoensis* in having a midrib raised on both surfaces and distinct intervening veins perpendicularly extending between lateral veins. However, *N. langbianensis* is easily distinguished from *N. hongiaoensis* by its smaller leaves (10.8-17.7 cm x 3.8-5.8 cm in *N. hongiaoensis* vs. 7-11 cm x 1.7-3.5 cm in *N. langbianensis*) and fewer lateral veins (14-18 vs. 7-10).

Multivaliate analysis of leaf traits

We constructed a matrix of the seven leaf traits: maximal number of leaves clustered on the branch top (MLC), midpoint petiole length (PL), midpoint leaf length (LL), midpoint leaf width (LW), midpoint lateral veins (LV), midpoint aspect ratio (AR), petiole pubescence (PP), venation type of secondary veins (SV), and venation type of tertiary veins (TV) for a total of 67 species including 65 described species of *Actinodaphne* and two species of *Neoactinodaphne*: *N. hongiaoensis* and *N. langbianensis* (Appendix). As a result of principal component analysis using six quantitative traits (MLC, PL, LL, LW, LV and AR), the first principal component (PC1) explained 40 % of the variance. In PC1, LL, LW, and PL had larger loadings (-0.58, -0.557, and -0.533, respectively) than the other three traits with loadings less than 2.5 (Table 3). On the other hand, PC2 and PC3 explained additional 29 % and 13 % of the variance, respectively. In PC2, AR, LV and MLC had higher loadings (0.653, 0.506 and 0.487, respectively). In PC3, LV and MLC had higher loadings (0.78 and 0.538

respectively). Contribution of LL, LW, and PL to PC1, and those of AR, LV, and MLC to PC2 are illustrated in a biplot (Fig. 5A). The lower PC1, the longer and wider leaf blade, and the longer petiole. On the other hand, the larger PC2, the higher aspect ratio (the narrower leaf), the more lateral veins, and the more leaves on a node (Fig. 5A). A biplot of PC1 vs. PC3 illustrates that LV and MLC mainly contributed to PC3 (Fig. 5B); the lower PC3, the more larteral veins, and the more leaves on a node.

In UPGMA clustering using the same six traits, *Actinodaphne* spp. and *Neoactinodaphne* spp. are separated into three clusters: cluster A, B and C (Fig.6). The cluster A and C were separated into nine sub clusters and five sub clusters respectively. Cluster B included *A. lecomtei* C. K. Allen and *A. obscurinervia* Y. C. Yang & P. H. Huang that are characterized by 18 to 40 pairs of lateral veins, while the other species have 18 or less laretal veins. To detect key differences that separated cluster A and C, we draw scatter plots among six traits used for UPGMA clustering. In the scatter plot of leaf length and petiole length, Cluster A and Cluster C are well separated, but neither of the two traits can key out the two clusters (Fig. 7). In Fig. 7, it appears that 24 cm of midpoint leaf length can be used as a criterion to distinguish two groups having larger and smaller leaves. Under this criterion, five species of Cluster C are belonged to a smaller-leaved group. For petiole length, the ranges of Cluster A and C are largely overlapping. *Neoactinodaphne hongiaoensis* and *N. langbianensis* were placed at two remote positions in Cluster A: *N. hongiaoensis* is clustered with *A. acuminata* in Cluster A2 and *N. langbianensis* is clustered in Cluster A8. Cluster A2 including *N. hongiaoensis* and *A. acuminata* is characterized by midpoint leaf length below 24 cm and MLC more than eight. However, our results of phylogenetic analysis showed that *A. acuminata* is placed in a clade of *Litsea* (see *MIG-seq phylogenetic tree* section). Cluster A8 including *N. langbianensis*, *A. reticulata* Meisn., *A. sulcata* S. Julia, *A. tsaii*

Hu, *A. cupularis* (Hemsl.) Gamble, *A. sikkimensis* Meisn., *A. koshapangii* Chun ex H.T.Chang, *A. pruinosa* Nees, *A. cuspidata* Gamble, *A. perglabra* Kosterm., *A. concinna* and *A. trichocarpa* has LL below 11cm, PL usually below 11 mm, LV below 10, and MLC below 8.

MIG-seq phylogenetic tree

The maximum likelihood tree based on MIG-seq data showed high resolution, with 75 % (123/165) of the branches supported by bootstrap values of >90 % (Fig.8). *Litsea* was placed outside of *Actinodaphne*, *Neoactinodaphne,* and *Neolitsea*. *Actinodaphne acuminata* was placed in a clade that included *Litsea* sp. 2–5 and *L. brevipes*, not with other *Actinodaphne* spp. (Fig.8). *Litsea magnifica* Gamble was clustered with *Lindera* spp. and outgroups, not with the other species of *Litsea*.

The monophyly of the clade that included *Actinodaphne, Neoactinodaphne* and *Neolitsea* was supported with 100 % bootstrap value. In this clade, the monophyly of *Actinodaphne,* the monophyly of *Neoactinodaphne* and the monophyly of *Neolitsea* were all supported with 100 % bootstrap value. *Neoactinodaphne* was sister to *Neolitsea* and and the monophyly of a clade including these two genera was supported by a bootstrap value of 97 %. *Neoactinodaphne* included *N. hongiaoensis*, *N. hongiaoensis* var. *inthanonensis* and *N. langbianensis*.

Actinodaphne spp. excluding *A. acuminata* were separated into six clades. Clade 1 including *A. lambirensis* Tagane, Yahara & Okabe, *A. rehderiana* (C. K. Allen) Kosterm. ex Dao, and *A. leiophylla* (Kurz) Hook. f. is branched as the base of *Actinodaphne*. Clade 2 included *A. gullavara* (Buch.-Ham. ex Nees) M. R. Almeida, *A.* sp. 1, *A. glomerata* (Blume) Nees, *A. diversifolia* Merr., *A. macrophylla* (Blume) Nees, *A.* sp. 2, *A. heterophylla* Blume, *A. sesquipedalis* Hook. f. & Thomson ex Meisn., *A.*

glabra Blume, and *A. montana* Gamble. Clade 3 included *A. concinna, A. borneensis* Meisn.*, A. semengohensis* S. Julia*, A. myriantha* Merr.*, A.* sp. 3*, A.* sp. 4*, A.* sp. 5*, A.* aff. *amabilis, A. amabilis* Kosterm.*, A. rufescens* Blume*, and A. perlucida* C.K.Allen. Clade 4 included *A*. sp. 6, *A. pilosa* (Lour.) Merr., *A*. sp. 7, *A*. sp. 8, *A. concolor* Nees, *A. bourdillonii* Gamble and *A. henryi* Gamble. The clade 5 included *A. pruinosa* and *A. sulcata*. Clade 6 included *A. obovata* (Nees) Blume only. All species in Clades 1, 3 and 5 were in Cluster A of UPGMA clustering that is composed of species with leaf length less than 24 cm or petiole length less than 25 mm. Similarly, all species in Clade 6 belonged to Cluster C of UPGMA clustering. In Cluster 2, most (5/7) species belonged to Cluster C and the rest two species belonged to Cluster A. In Clade 4, *A. bourdillonii* and *A. henryi* belongd to cluster A and C, respectively (*A. concolor* in Clade 4 was not included in UPGMA clustering due to limited availablity of morphometirical data).

In the MIG-seq tree, eight species did not match to any previously described species. Among them, *A*. sp 1, *A*. sp. 2 and *A*. sp. 7 were treated as undescribed species in the Chapter II. In addition to evidence from phylogenetic positions, the other five species were distinguished from their sister species by the following morphological traits. *Actinodaphne* sp. 3 was sister to *A. myriantha* and *A. semengohensis* but is distinguished from *A. myriantha* by glabrous leaves (vs. densely hairy below in *A. myriantha*) and from *A. semengohensis* by glaucous leaf undersurface (green in *A. semengohensis*). *Actinodaphne* sp. 4 was sister to *A*. sp. 5, *A. amabilis* and *A*. aff. *amabilis* but distinct in triplinerved lateral veins (vs pinninerved in A. sp. 5, *A. amabilis* and *A*. aff. *amabilis*). *Actinodaphne* sp. 5 was different from *A. amabilis* and *A*. aff. *amabilis* in having lateral veins ascending at an angle of 75 degrees from midlib (vs. 45 degree or less in *A. amabilis*). *Actinodaphne* sp. 6 was sister to *A. pilosa* and *A*. sp. 7 but different in having leaves distinctly whitish below (vs. greenish in *A. pilosa* and *A*. sp.

7) and tertiary veins raised on abaxial surface (vs. flat in *A. pilosa* and *A*. sp. 7). *Actinodaphne* sp. 8 was sister to *A. concolor*, *A. bourdillonii* and *A. henryi*, but distinguished from *A. bourdillonii* and *A. heryi* in wider and obovate leaves (vs. narrower, oblong-lanceolate leaves), and from *A. concolor* by hairy petioles (vs. glabrous).

Discussion

In the new MIG-seq analysis described above, we added 61 samples that were not examined in Chapter I (Okabe et al. in press) and Chapter II. Consequently, the resolution of the phylogenetic tree was improved from the previous ones (Chapters I and II) and we could derive three major conclusions. First, a clade including *N. hongiaoensis*, *N. hongiaoensis* var. *inthanonensis* and *N. langbianensis* was sister to *Neolitsea* and this sister relatioship was supported by 97% bootstrap value. In the previous phylogenetic analyses, *N. hongiaoensis* was identified as "*A*. aff. *tsaii*" (V4477), whose phylogenetic position was unstable (Chapter I and II). Based on the new results, we described *Neoactinodaphne* as a new gunus. Second, the new MIG-seq tree showed that "*A. acuminata"* is not a member of *Actinodaphne* but of *Litsea.* Third, we determined the phylogenetic relationship for 34 species of *Actinodaphne* among which eight species are considered to be undescribed.

The discovery of *Neoactinodaphne*

Previous studies showed that *Actinodaphne* is polyphyletic (Li et al. 2004, Li et al. 2006, Li et al. 2007, Mitsuyuki et al. 2018, Okabe et al. in press). In the present study, we described a new genus *Neoactinodaphne* including two new species that were classified as *Actinodaphne* in having 3-merous flowers and imbricated bracts, but were

sister to *Neolitsea* that was characterized by having 2-merous flowers, not to the other species of *Actinodaphne*. The morphological characteristic of *Neoactinodaphne* different from *Actinodaphne* s. str. is the well-developed intervening veins perpendicularly extending between secondary veins. *Actinodaphne tsaii* and *A. sikkimensis* also have this character. Among them, *A. tsaii* is considered to be a species of *Neoactinodaphne* because this species was sister to *N. hongiaoensis* (V4477) on the ITS tree (see Chapter II). *Actinodaphne sikkimensis* is a polymorchic species so that further studies are required to clarify its circumscription and identity. A species that is similar to *N. hongiaoensis* was corrected at 1600 m of Phu Bia, the highest peak of Laos (Kerr, A.F.G., #21007, K 000793062), and annotated as *Actinodaphne laosensis* by Kostermans, but this name is not published. This species is distingueished from *N. hongiaoensis* by its pedicel 14-17 mm long (vs. 3-7 mm long in *N. hongiaoensis*). This species may be an undescribed species close to *N. hongiaoensis.* However, only one specimen has been known until today, and further collecting efforts and studies based on new materials are needed to clarify the identity of *A. laosensis*. *Neoactinodaphne* spp. show disjunct distribution in southan China (Yunnan), northern Thailand (Chaingmai), central Laos (Xiangkhouang) and southern Vietnam (Lamdong). Further studies in Southeast Asia may discover more localities of *Neoactinodaphne* spp.

Phylogenetic position of *Actinodaphne acuminata*

Li et al. (2006, 2007) suggested that *Actinodaphne* is polyphyletic based on a phylogenetic analyses using the ITS and ETS sequences. In particular, they showed that *A. forrestii* was close to *Lindera megaphylla* (Li et al. 2006), and *Neolitsea* was nested with some species of *Actinodaphne* (Li et al. 2007). Our study clarifyied that *A. acuminta* belongs to *Litsea*. Our MIG-seq tree also showed that *Litsea* and *Lindera* were

not monophyletic. Further analyses that include many species of *Litsea* and *Lindera* covering the whole diversity of these genera are required to determine the phylogenetic positions of *A. acuminata* and *A. forrestii* and revise the taxonomy of the species-rich group including *Litsea* and *Lindera*. Our results showed that MIG-seq provides rich and informative polymorphic sequences that enable us to obtain finely resolved phylogenyetic trees among species of *Litsea* and its related genera.

Proportion of undescribed species

Among 34 species of *Actinodaphne*, eight species (24 %) of *Actinodaphne* did not match to any previously described species. We considered these eight units as species based on two criteria. First, fives pecies (*A*. sp. 1, *A*. sp. 3, *A*. sp. 4, *A*. sp. 6, *A*. sp. 8) were sister to some pairs of known species. For example, *A*. sp. 1 was sister to a clade including a pair of species, *A. glomerata* and *A. divesifolia,* that are morphologically distinct from each other and also occurs in the same area (distributed Salawak and Sabah in Malaysisa respectively). *Actinodaphne glomerata* and *A. divesifolia* are considered to be different species because morphologically distinct taxonomic units that occur in the same area (being sympatric) have been treated as different species in botanical literature (Stebbins 1950, van Valen 1976, Petit & Excoffier 2009). In this case, there is strong evidence that *A*. sp. 1 is considered to be a species. If *A*. sp. 1 is not distinguished at the species level, we need to merge two known species.

Second, an undescribed species was sister to but distinct from a described species (*A*. sp. 4 and *A. heterophylla*, *A*. sp. 5 and *A. amabilis*, *A*. sp. 7 and *A. pilosa*). In this case, if a pair of sister taxa are distributed in the same area and are genetically and morphologically differentiated, it is appropriate to regard them as different species

(Stebbins 1950, van Valen 1976, Petit & Excoffier 2009). This is the case for *Actinodaphne* sp. 2 and *A. heterophylla* that are collected in the samle locality of western Sumatra. If a pair of sister taxa are allopatric, as for *A*. sp. 5 and *A. amabilis* distributed in Singapore and Thailand, respectively, there is no objective criterion to determine whether these are different species or different subspecies (or variety) of the samen species. We regarded *A*. sp. 5 and *A. amabilis* as two different species considering that these are genetically well diverged as in other pairs of previously described species and also morphologically well differentiated. There is an intermediate situation where a pair of sister taxa are parapatric. This is the case for *A*. sp. 7 and *A. pilosa* that are distributed in the higher and lower elevations in the same area of southern Vietnam. We regarded them as two different species because these are genetically and morphologically well diverged as in other pairs of previously known species.

It is remarkable that as high as 24 % of species were undescribed. This may be because parallel evolution in morphological traits often took place as shown in Chapter II, and phylogenetically different lineages often shows high morphological similarity. We constructed two types of clustering, a phylogenetic tree based on MIG-seq and a UPGMA tree based on morphological traits, and their topologies did not match. Species of UPGMA Cluster A having smaller leaves and species of Cluster C having larger leaves were both not monophyletic in the MIG-seq tree so that leaf size did not reflect phylogenetic relationship. Parallel evolutions in leaf shape, hairness and character of tertiary veins (reticulate vs scalariform) are also suggested. Further studies of trait evolution using phylogenetic trees are requied to deepen our understanding of morphological divergence and convergence in *Actinodaphne* and *Neoactinodaphne*.

The tropical region of Southeast Asia retains high plant species diversity of

plant comparable to tropical America. However, due to incomplete taxonomic studies, its diversity may be underestimated and even recently more than 400 new species of vascular plants have been described every year (Middleton et al. 2020). In this study, we combined a phylogenyetic analysis based on MIG-seq with a multivaliate analysis of leaf traits using a traits matrix constricted from taxonomic literature and morphological observation. Consequently, we discovered 10 undescribed species including eight species of *Actinodaphne* and a new genus including two new species. This study support the fiew of Middleton et al. (2020) that plant diversity of Southeast Asia is underestimated. Our results also showed that phylogenetic analyses using MIG-seq are effective to discover and describe new species if it is combined with morphometric analyses. Further studies on other taxonomic grouls using this approach are to elucidate plant diversity in Southeast Asia where a huge number of species may remain to be described.

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Table 1. A list of samples used for genotyping genome-wide SNPs with MIG-seq.

Species	Vouture ID	Countries / Regions	Areas
Actinodaphne acuminata (Blume) Meisn.	R406	Japan	Mt. Komi, Iriomote Isl.
A. acuminata (Blume) Meisn.	TWN75	Taiwan	Lienhuachin
A. amabilis Kosterm.	T4910-01	Thailand	Khao Luang NP, Nakhon Ratchasima
A. amabilis Kosterm.	T4910-02	Thailand	Khao Luang NP, Nakhon Ratchasima
A. aff. *amabilis*	T5442	Thailand	Khao Yai NP, Nakhon Ratchasima
A. aff. *amabilis*	T5856	Thailand	Khao Yai NP, Nakhon Ratchasima
A. borneensis Meisn.	SWK2517	Malaysia	Lambir Hills NP, Sarawak
A. borneensis Meisn.	SWK2575	Malaysia	Lambir Hills NP, Sarawak
A. borneensis Meisn.	SWK4039	Malaysia	Tubau Sungai Jelalong, Bintulu, Salawak
A. bourdillonii Gamble	MY1068	Myanmar	Mohnyin Township Indawgy Wildlife Sanctuary, Kachin
A. bourdillonii Gamble	MY1551	Myanmar	Mohnyin Township Indawgy Wildlife Sanctuary, Kachin

A. coninna Ridl.	M178	Malaysia	Fraser's Hill, Pahang
A. coninna Ridl.	M198	Malaysia	Fraser's Hill, Pahang
A. concolor Nees	MY4008	Myanmar	Taninthayri NR, Tanintharyi
A. divesifolia Merr.	B537	Brunei	Kuala Belalong Field Study Centre, Temburong
A. divesifolia Merr.	B791	Brunei	Kuala Belalong Field Study Centre, Temburong
A. divesifolia Merr.	IK9	Indonesia	Mandor, West Kalimantan
A. divesifolia Merr.	SWK1727	Malaysia	Tatau, Bintulu, Sarawak
A. divesifolia Merr.	SWK3966	Malaysia	Tatau, Bintulu, Sarawak
A. glabra Hook f. et Thoms.	P31	Phillippines	Mt. Banahaw, Quezon
A. glabra Hook f. et Thoms.	SWK1028	Malaysia	Water Catchment Sekawei, Sarawak
A. glabra Hook f. et Thoms.	SWK3679	Malaysia	Tatau, Bintulu, Sarawak
A. gllavara (Buch.-Ham. ex Nees) M.R.Almeida	MY1066	Myanmar	Mawbi Township Hlawga Park, Yangon
A. gllavara (Buch.-Ham. ex Nees) M.R.Almeida	MY4418	Myanmar	Taninthayri NR, Tanintharyi

A. glomerata (Blume) Nees	SB229	Malaysia	Kinabatangan NP, Sabah
A. glomerata (Blume) Nees	SWK3809	Malaysia	Tatau, Bintulu, Sarawak
A. glomerata (Blume) Nees	SWK3972	Malaysia	Tatau, Bintulu, Sarawak
A. glomerata (Blume) Nees	SWK4006	Malaysia	Tubau Sungai Jelalong, Bintulu, Salawak
A. glomerata (Blume) Nees	SWK620	Malaysia	Watercatchment Camp Ayam, Bintulu, Sarawak
A. henryi Gamble	L2163	Laos	Dong Hua Sao NPA, Champasak,
A. henryi Gamble	L2683	Laos	Dong Hua Sao NPA, Champasak,
A. henryi Gamble	L650	Laos	Nam Ha NPA, Luang Namtha
A. henryi Gamble	L690	Laos	Nam Ha NPA, Luang Namtha
A. henryi Gamble	T3571	Thailand	Phu Kradueng National Park, Loei
A. henryi Gamble	V9284	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. heterophylla Blume	IS854	Indonesia	Airsirah, Padang, Sumatra
A. lambirensis Tagane, Yahara & Okabe	SWK2556	Malaysia	Lambir Hills NP, Sarawak
A. lambirensis Tagane, Yahara & Okabe	SWK5434	Malaysia	Kuching, Kubah NP, Salawak
A. leiophylla (Kurz) Hook. f.	MY446	Myanmar	Pataw Isl., Kyunsu Township, Tanintharyi

A. leiophylla (Kurz) Hook. f.	T4258	Thailand	Karome Waterfall, Khao Laung National Park, Nakhon Ratchasima
A. leiophylla (Kurz) Hook. f.	T5381	Thailand	Khao Ngon National Park, Krabi
A. macrophylla (Blume) Nees	M229	Malaysia	Tanintharyi, Tanintharyi NR
A. macrophylla (Blume) Nees	SWK2533	Malaysia	Lambir Hills NP, Sarawak
A. montana Gamble	IS45	Indonesia	Pinang Pinang, Padang, Sumatra
A. montana Gamble	MY4370	Myanmar	Tanintharyi NR, Tanintharyi
A. montana Gamble	MY661	Myanmar	Taninthayri NR, Tanintharyi
A. myriantha Merr.	SWK1658	Malaysia	Tatau, Bintulu, Sarawak
A. obovata (Nees) Blume	L664	Laos	Nam Ha NPA, Luang Namtha
A. obovata (Nees) Blume	MY1084	Myanmar	Mohnyin Township Indawgy Wildlife Sanctuary, Kachin
A. perlucida C.K.Allen	V10005	Vietnam	Son Thai, Khanh Hoa
A. perlucida C.K.Allen	V445	Vietnam	Hon Ba NR, Khanh Hoa
A. perlucida C.K.Allen	V508	Vietnam	Hon Ba NR, Khanh Hoa
A. perlucida C.K.Allen	V616	Vietnam	Hon Ba NR, Khanh Hoa
A. pilosa (Lour.) Merr.	L3741	Laos	Bolaven Plateu, Attapeu,

A. pilosa (Lour.) Merr.	V1363	Vietnam	Hon Ba NR, Khanh Hoa
A. pilosa (Lour.) Merr.	V2960	Vietnam	Bach Ma National Park, Thua Thien Hue
A. pruinosa Nees	SWK1199	Malaysia	Bario, Sarawak
A. rehderiana (C.K.Allen) Yahara	V11289	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. rehderiana (C.K.Allen) Yahara	V8095	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. rehderiana (C.K.Allen) Yahara	V8111	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. rehderiana (C.K.Allen) Yahara	V8838	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. rehderiana (C.K.Allen) Yahara	V8926	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. rehderiana (C.K.Allen) Yahara	V9001	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. rehderiana (C.K.Allen) Yahara	V9070	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. rufescens Blume	SWK2020	Malaysia	Lambir Hills N, Sarawak
A. rufescens Blume	SWK3451	Malaysia	Lambir Hills NP, Sarawak
A. rufescens Blume	SWK5437	Malaysia	Kuching, Kubah NP, Salawak
A. semengohensis S.Julia	SWK4877	Malaysia	Kuching, Kubah NP, Salawak
A. sesquipedalis Hook.f. & Thoms ex Meisn.	1920-1	Cambodia	Bokor NP, Kampot

A. sesquipedalis Hook.f. & Thoms ex Meisn.	1920-2	Cambodia	Bokor NP, Kampot
A. sesquipedalis Hook.f. & Thoms ex Meisn.	4722	Cambodia	Cardamon, Koh Kong
A. sesquipedalis Hook.f. & Thoms ex Meisn.	708	Cambodia	Cardamon, Koh Kong
A. sesquipedalis Hook.f. & Thoms ex Meisn.	L1014	Laos	Nam Kading NPA, Bolikhamxay,
A. sesquipedalis Hook.f. & Thoms ex Meisn.	L2759	Laos	Dong Hua Sao NPA, Champasak,
A. sesquipedalis Hook.f. & Thoms ex Meisn.	L284	Laos	Dong Hua Sao NPA, Champasak,
A. sesquipedalis Hook.f. & Thoms ex Meisn.	M342	Malaysia	Fraser's Hill, Pahang
A. sesquipedalis Hook.f. & Thoms ex Meisn.	MY1991-1	Myanmar	Meyik Lampi NP, Bo Cho Island,
A. sesquipedalis Hook.f. & Thoms ex Meisn.	MY1991-2	Myanmar	Meyik Lampi NP, Bo Cho Island,
A. sesquipedalis Hook.f. & Thoms ex Meisn.	MY2121	Myanmar	Meyik Lampi NP, Bo Cho Island,
A. sesquipedalis Hook.f. & Thoms ex Meisn.	MY366	Myanmar	Taninthayri NR, Tanintharyi
A. sesquipedalis Hook.f. & Thoms ex Meisn.	MY4060	Myanmar	Taninthayri NR, Tanintharyi
A. sesquipedalis Hook.f. & Thoms ex Meisn.	V1594	Vietnam	Hon Ba NR, Khanh Hoa
A. sulcata S.Julia	SWK1107	Malaysia	Bario, Sarwak
A. sp. 1	S72	Indonesia	Bantimulung Bulusarum, Sulawesi

A. sp. 2	IS811	Indonesia	Airsirah, Padang, Sumatra
A. sp. 3	SWK4755	Malaysia	Kuching, Kubah NP, Salawak
A. sp. 4	M9	Malaysia	Fraser's Hill, Pahang
A. sp. 5	SGP1	Singapore	Bukit Timah
A. sp. 6	V11286	Vietnam	Bi Doup Nui Ba NP, Lam Dong
A. sp. 7	V10641	Vietnam	Kon Chu Rang, Gia Lai
A. sp. 7	V2703	Vietnam	Bach Ma NP, Thua Thien Hue
A. sp. 8	L1193	Laos	Nam Kading NPA, Bolikhamxay,
Litsea accedens (Blume) Boerl.	SWK1896-1	Malaysia	Sungai Jelalong, Bintulu, Sarawak
L. accedens (Blume) Boerl.	SWK1896-2	Malaysia	Sungai Jelalong, Bintulu, Sarawak
L. accedens (Blume) Boerl.	B815	Brunei	Kuala Belalong Field Study Centre, Temburong
L. accedens (Blume) Boerl.	B85	Brunei	Kuala Belalong Field Study Centre, Temburong
L. accedens (Blume) Boerl.	SWK1827	Malaysia	Tatau, Bintulu, Sarawak
L. accedens (Blume) Boerl.	SWK3803	Malaysia	Tatau, Bintulu, Sarawak
L. accedens (Blume) Boerl.	SWK4030	Malaysia	Tubau Sungai Jelalong, Bintulu, Salawak

L. accedens (Blume) Boerl.	SWK5439	Malaysia	Kuching, Kubah NP, Salawak
L. accedens (Blume) Boerl.	SWK689	Malaysia	Watercatchment Camp Ayam, Bintulu, Sarawak
L. brevipes Kosterm.	V7264	Vietnam	Ba Vi NP, Ha Noi
L. brevipes Kosterm.	V7391	Vietnam	Ba Vi NP, Ha Noi
L. johorensis Gamble	M1008	Malaysia	Pasoh Forest Reserve, Negeri Sembilan,
L. johorensis Gamble	M915	Malaysia	Pasoh Forest Reserve, Negeri Sembilan,
L. johorensis Gamble	M944	Malaysia	Pasoh Forest Reserve, Negeri Sembilan,
L. magnifica (Miq.) Villar	IK1457	Indonesia	Bukit Bangkirai, East Kalimantan,
L. magnifica (Miq.) Villar	M812	Malaysia	Pasoh Forest Reserve, Negeri Sembilan
L. magnifica (Miq.) Villar	P115	Phillippines	Mt. Banahaw, Quezon
L. magnifica (Miq.) Villar	P392	Phillippines	Mt. Banahaw, Quezon
L. magnifica (Miq.) Villar	SWK1917	Malaysia	Lambir Hills NP, Sarawak
L. magnifica (Miq.) Villar	SWK2629	Malaysia	Lambir Hills NP, Sarawak
L. magnifica (Miq.) Villar	SWK4023	Malaysia	Tubau Sungai Jelalong, Bintulu, Salawak
L. magnifica (Miq.) Villar	T2421	Thailand	Pechaburi, Kaeng Krachan

L. magnifica (Miq.) Villar	T3066-01	Thailand	Kaeng Krachan, Pechaburi
L. magnifica (Miq.) Villar	T3066-02	Thailand	Kaeng Krachan, Pechaburi
L. sp. 1	V2765	Vietnam	Bach Ma National Park, Thua Thien Hue
L. sp. 2	V2972	Vietnam	Bach Ma National Park, Thua Thien Hue
L. sp. 2	V7234	Vietnam	Ba Vi NP, Ha Noi
L. sp. 3	V159	Vietnam	Hon Ba Nature Reserve, Khanh Hoa
L. sp. 3	V4427	Vietnam	Bi Doup Nui Ba NP, Lam Dong
L. sp. 4	V6696	Vietnam	Ngoc Linh, Kom Tum
L. sp. 4	V6716	Vietnam	Ngoc Linh, Kom Tum
L. sp. 5	V5443	Vietnam	Pu Mat NP, Nghe An
L. sp. 6	V4572	Vietnam	Hoang Lien NP, Lao Cai
L. sp. 7	MY3431	Myanmar	Mt.Victoria, Chin
L. sp. 7	MY3484	Myanmar	Mt.Victoria, Chin
L. sp. 7	MY3602	Myanmar	Mt.Victoria, Chin
L. sp. 8	V585	Vietnam	Hon Ba NR, Khanh Hoa

L. sp .9	SGP9	Singapore	Bukit Timah
L. verticillata Hance	V3539-1	Vietnam	Vu Quang NP, Vinh
L. verticillata Hance	V3539-2	Vietnam	Vu Quang NP, Vinh
Lindera insignis Blume	IS813	Indonesia	Airsirah, Padang, Sumatra
Lindera salmonea Kosterm., nom. nud.	V1426	Vietnam	Hon Ba NR, Khanh Hoa
Machilus sp	V4044	Vietnam	Bi Doup Nui Ba NP, Lam Dong
Neolitsea elaerocarpa H.Liu	V1245	Vietnam	Hon Ba NR, Khanh Hoa
N. elaerocarpa H.Liu	V3035	Vietnam	Hai Van Pass, Hue
N. elaerocarpa H.Liu	V3044	Vietnam	Hai Van Pass, Hue
N. elaerocarpa H.Liu	V3058	Vietnam	Hai Van Pass, Hue
N. elaerocarpa H.Liu	V466	Vietnam	Hon Ba NR, Khanh Hoa
N. elaerocarpa H.Liu	V646	Vietnam	Hon Ba NR, Khanh Hoa
N.merrilliana C.K.Allen	V10289	Vietnam	Kon Chu Rang, Gia Lai
N .sp. 1	V10225	Vietnam	Kon Chu Rang, Gia Lai
N .sp. 2	V7235	Vietnam	Ba Vi NP, Ha Noi

N .sp. 3	V7193	Vietnam	Ba Vi NP, Ha Noi
N .sp. 3	V7224	Vietnam	Ba Vi NP, Ha Noi
N .sp. 4	P431	Phillippines	Mt. Banahaw, Quezon
N .sp. 4	P432	Phillippines	Mt. Banahaw, Quezon
N .sp. 5	IS788	Indonesia	Airsirah, Padang, Sumatra
N .sp. 6	T5461	Thailand	Khao Yai NP, Nakhon Ratchasima
N .sp. 6	T5552	Thailand	Khao Yai NP, Nakhon Ratchasima
N .sp. 7	V10364	Vietnam	Kon Chu Rang, Gia Lai
N .sp. 7	V10461	Vietnam	Kon Chu Rang, Gia Lai
N .sp. 7	V10526	Vietnam	Kon Chu Rang, Gia Lai
N .sp. 8	V3276	Vietnam	Vu Quang National Park, Vinh
N .sp. 8	V5969-1	Vietnam	Vu Quang National Park, Vinh
N .sp. 8	V5969-2	Vietnam	Vu Quang National Park, Vinh
Neoactinodaphne hongiaoensis Okabe, Tagane & Yahara	V11345	Vietnam	Bi Doup Nui Ba NP, Lam Dong

Neoactinodaphne hongiaoensis Okabe, Tagane & Yahara	V11346	Vietnam	Bi Doup Nui Ba NP, Lam Dong
Neoactinodaphne hongiaoensis Okabe, Tagane & Yahara	V11347	Vietnam	Bi Doup Nui Ba NP, Lam Dong
Neoactinodaphne hongiaoensis Okabe, Tagane & Yahara	V4477	Vietnam	Bi Doup Nui Ba National Park, Lam Dong
Neoactinodaphne hongiaoensis Okabe, Tagane & Yahara	V9860	Vietnam	Bi Doup Nui Ba NP, Lam Dong
Neoactinodaphne hongiaoensis var.*inthanonensis* Okabe & Yahara	T200	Thailand	Doi Inthanon, Chiang Mai
Neoactinodaphne langbiangensis Okabe, Tagane & Yahara	V7895	Vietnam	Bi Doup Nui Ba NP, Lam Dong
Neoactinodaphne langbiangensis Okabe, Tagane & Yahara	V8040	Vietnam	Bi Doup Nui Ba NP, Lam Dong
Neoactinodaphne langbiangensis Okabe,	V8960	Vietnam	Bi Doup Nui Ba NP, Lam Dong

Tagane & Yahara			
Phoebe sp.	V7177	Vietnam	Ba Vi NP, Ha Noi
Phoebe sp.	V7361	Vietnam	Ba Vi NP, Ha Noi

Table 2. Morohological characters in leaf of *Neactinodaphne hongiaoensiss* and similar five species.

Characters	*N. hongiaoensis*	*A. acuminata*	*A. cupularis*	*A. forrestii*	*A. omeiensis*	*A. sikkimensis*	*A. tsaii*
Leaf length (cm)	14.0–18.5	7.5–13*	5.5–13.5*; 8.5–19**	9–27*	12–27*	10–14*	10–15*
Leaf width (cm)	3.9–4.5	1.5–3*	1.5–2.7*; 2–5**	2–5*	2.1–6*	2–4*	2–3.5*
Lateral veins (pair)	12–18	12*	8–13*; 6–12**	11–15*	12–15*	8–12*	8–10*
Petiole length (cm)	0.7–2.0	0.5–2.0*	0.3–0.8*; 0.5–1.0**	< 2*	1.1–3.0*	0.5–1.0*	0.3–0.8*
Midrib on the adaxial suface	raised	raised**	sunken**	sunken*	sunken*	raised**	raised*
Young leaves and twigs	yellowish-brown tomentose	glabrous*	puberulent*	yellow-brown appressed	villous*	white tomentose**	gray-brown tomentose*

				tomentose*			
Intervening veins between secondary veins	distinct	distinct**	not distinct***	not distinct**	not distinct**	distinct **	distinct **
Fillaments	villous	villous at base*	glabrous*	glabrous*	unknown	villous*	villous*
Fruits	globose	oblong*	ovoid*	oblong*	subglobose*	oblong*	oblong*
References	This paper	*Huang & van den Werff (2008), **this paper	*Huang & van den Werff (2008), **Tanaros et al. (2010), *** this paper	*Huang & van den Werff (2008). **this paper	Huang & van den Werff (2008), **this paper	*Tanaros et al. (2010), **this paper	Huang & van den Werff (2008), **this paper

Table 3. Loadings of six traits in *Actinodaphne* and *Neoactinodaphne* on the first three principal components (PC1-3).

Traits	PC1	PC2	PC3
MLC	-0.237	0.472	0.673
PL	-0.517	-0.04	0.243
LL	-0.587	-0.038	-0.139
LW	-0.55	-0.329	-0.165
LV	-0.166	0.492	-0.661
AR	-0.047	0.651	-0.064
Proportion of Variance	0.4024	0.2901	0.1304

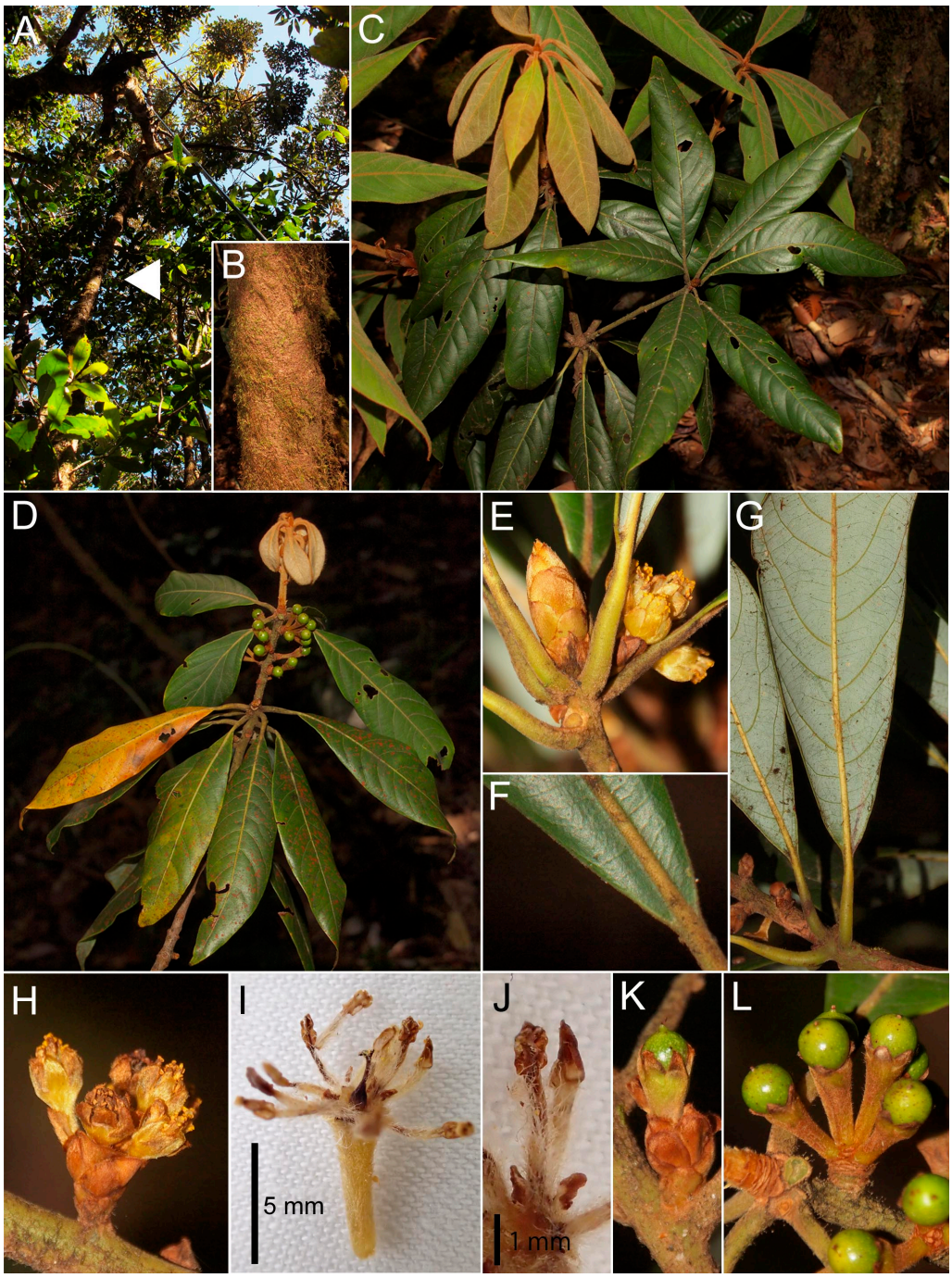

Figure 1. *Neoactinodaphne hongiaoensis* Okabe, Tagane & Yahara. var. *hongiaoensis*.A. Habit.White arrows indicate tree of *N. hongiaoensis*. B. Trunk. C. Leafy twig. D. Branches with infructescence. E. Branch top showing bud scale and male inflorescence. F. Base of lamina showing prominent midrib adaxially. G.

Portion of lower leaf surface. H. Male inflorescence. I. Male flower. J. Stamen of third whorl having 2 reniform stipitate glands. K & L. Young infructescence.

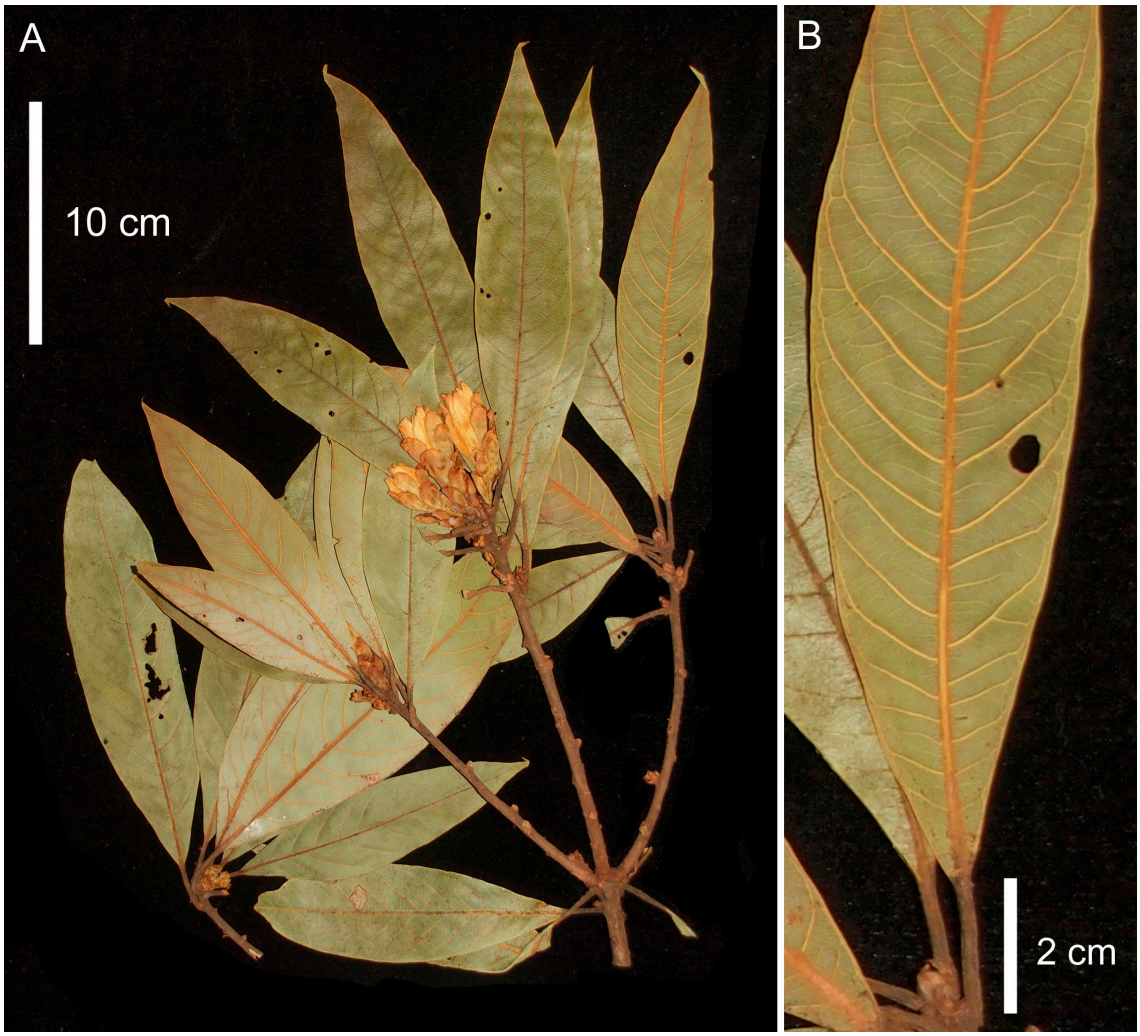

Figure 2. A type specimen of *N. hongiaoensis.* A. type. B. abaxial leaf surface.

Figure 3. A type specimen of *N. hongiaoensis* var. *inthanonensis*

Figure 4. A type specimen of *N. langbianensis*

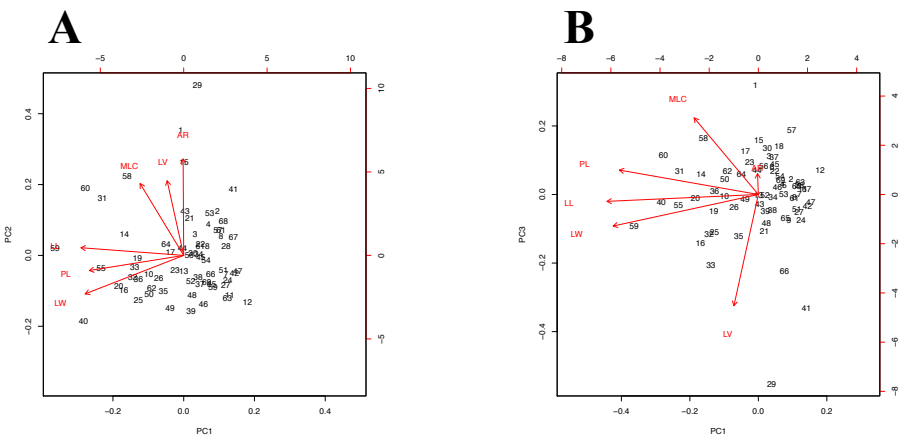

Figure 5. Biplots of the principal component analysis (PCA) using six traits (Maximum leaf cluster: MLC, Petiol length: PL, Leaf length: LL, Leaf width: LW, Lateral veins pairs: LV and Aspect ratio in leaf: AR) of 65 *Actinodaphne* spp. and two *Neoactinodaphne* spp. A. PC1 vs. PC2. B. PC1 vs. PC2

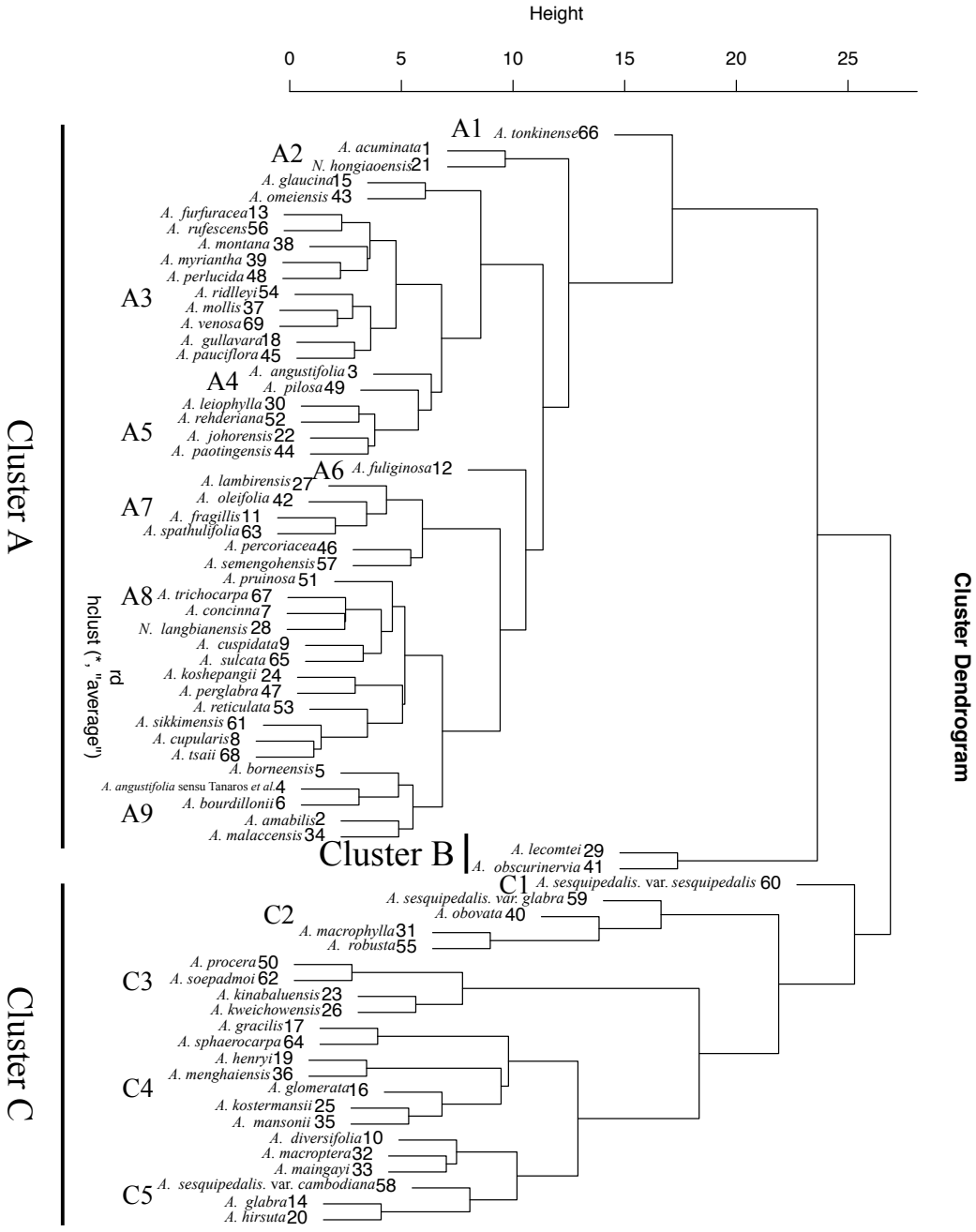
Height
0
5
10
15
20
25
Cluster A
Cluster B
Cluster C
Cluster Dendrogram
hclust (*, "average")
rd
A1
A. tonkinense 66
A2
A. acuminata 1
N. hongiaoensis 21
A. glaucina 15
A. omeiensis 43
A3
A. furfuracea 13
A. rufescens 56
A. montana 38
A. myriantha 39
A. perlucida 48
A. ridlleyi 54
A. mollis 37
A. venosa 69
A. gullavara 18
A. pauciflora 45
A4
A. angustifolia 3
A. pilosa 49
A5
A. leiophylla 30
A. rehderiana 52
A. johorensis 22
A. paotingensis 44
A6
A. fuliginosa 12
A7
A. lambirensis 27
A. oleifolia 42
A. fragillis 11
A. spathulifolia 63
A. percoriacea 46
A. semengohensis 57
A. pruinosa 51
A8
A. trichocarpa 67
A. concinna 7
N. langbianensis 28
A. cuspidata 9
A. sulcata 65
A. koshepangii 24
A. perglabra 47
A. reticulata 53
A. sikkimensis 61
A. cupularis 8
A. tsaii 68
A. borneensis 5
A. angustifolia sensu Tanaros et al. 4
A. bourdillonii 6
A9
A. amabilis 2
A. malaccensis 34
A. lecomtei 29
A. obscurinervia 41
C1
A. sesquipedalis. var. sesquipedalis 60
A. sesquipedalis. var. glabra 59
A. obovata 40
C2
A. macrophylla 31
A. robusta 55
C3
A. procera 50
A. soepadmoi 62
A. kinabaluensis 23
A. kweichowensis 26
C4
A. gracilis 17
A. sphaerocarpa 64
A. henryi 19
A. menghaiensis 36
A. glomerata 16
A. kostermansii 25
A. mansonii 35
C5
A. diversifolia 10
A. macroptera 32
A. maingayi 33
A. sesquipedalis. var. cambodiana 58
A. glabra 14
A. hirsuta 20

Figure 6. Unweighted Pair Group Method using arithmetic Average (UPGMA) clustering using six traits (Maximum leaf cluster: MLC, Petiol length: PL, Leaf length: LL, Leaf width: LW, Lateral veins pairs: LV and Aspect ratio in leaf: AR) of 65 *Actinodaphne* spp. and two *Neoactinodaphne* spp.

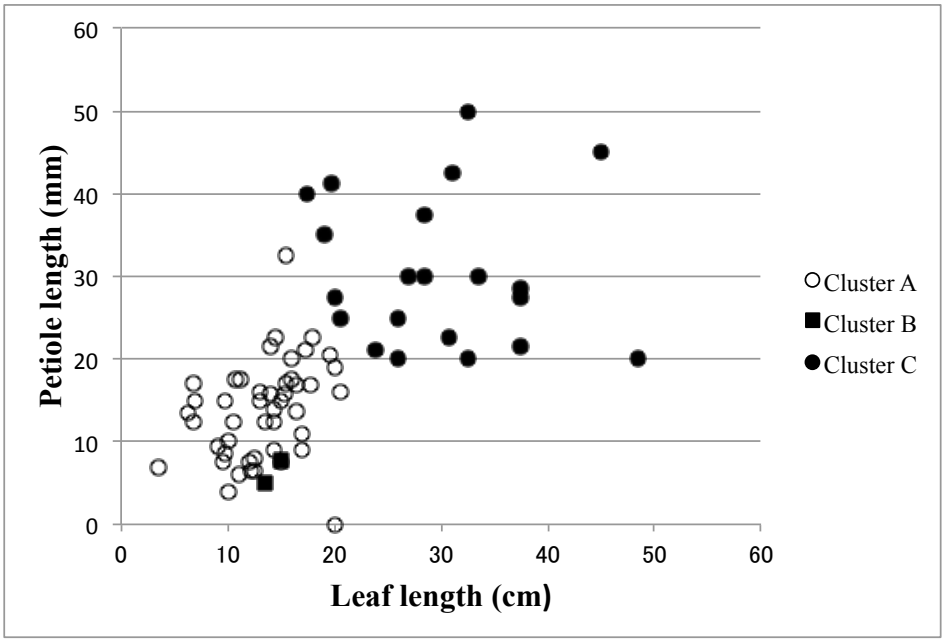

Figure 7. A scatter plot of leaf length and petiole length of 65 *Actinodaphne* spp. and two *Neoactinodaphne* spp.

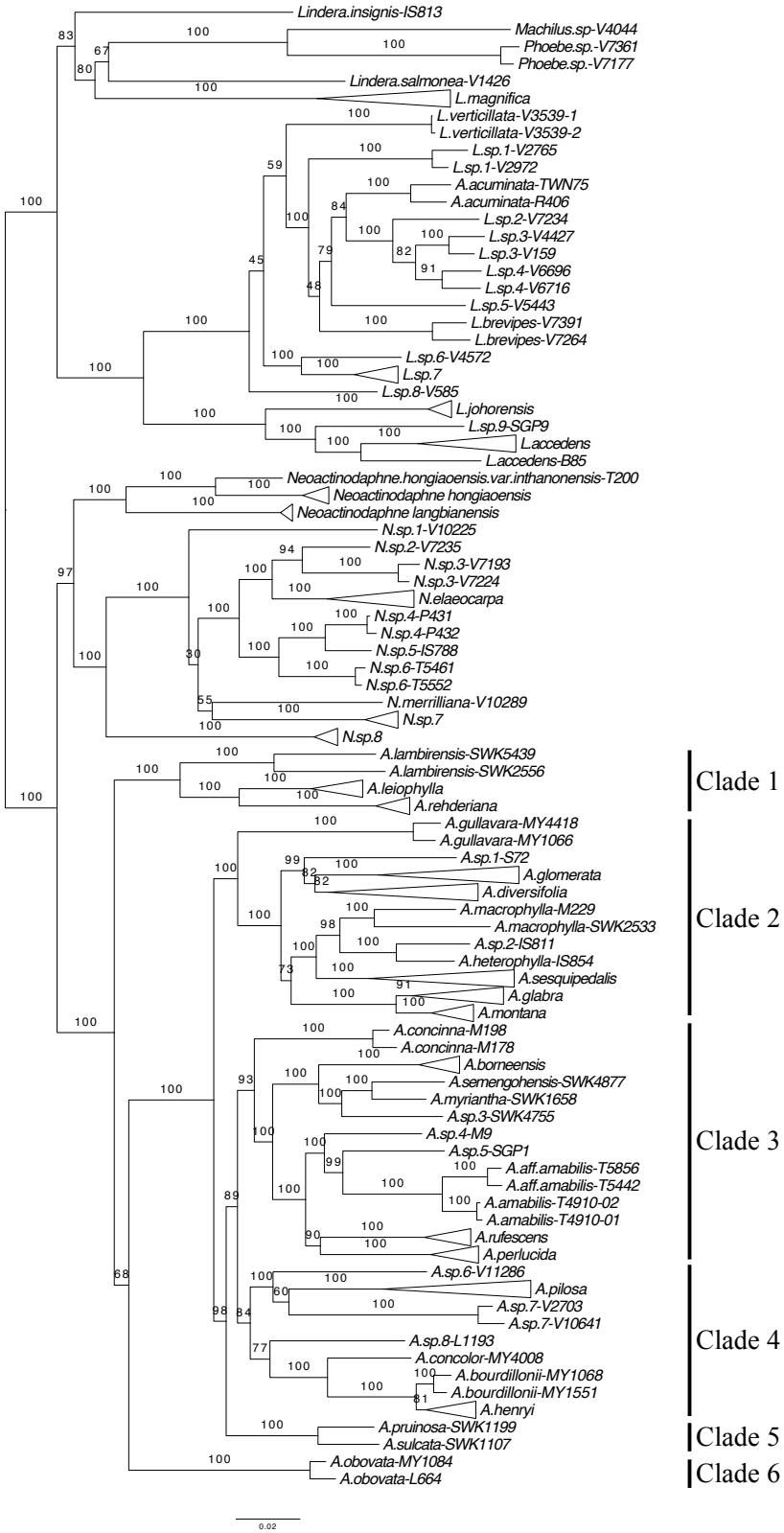
Lindera.insignis-IS813
Machilus.sp-V4044
Phoebe.sp.-V7361
Phoebe.sp.-V7177
Lindera.salmonea-V1426
L.magnifica
L.verticillata-V3539-1
L.verticillata-V3539-2
L.sp.1-V2765
L.sp.1-V2972
A.acuminata-TWN75
A.acuminata-R406
L.sp.2-V7234
L.sp.3-V4427
L.sp.3-V159
L.sp.4-V6696
L.sp.4-V6716
L.sp.5-V5443
L.brevipes-V7391
L.brevipes-V7264
L.sp.6-V4572
L.sp.7
L.sp.8-V585
L.johorensis
L.sp.9-SGP9
L.accedens
L.accedens-B85
Neoactinodaphne.hongiaoensis.var.inthanonensis-T200
Neoactinodaphne hongiaoensis
Neoactinodaphne langbianensis
N.sp.1-V10225
N.sp.2-V7235
N.sp.3-V7193
N.sp.3-V7224
N.elaeocarpa
N.sp.4-P431
N.sp.4-P432
N.sp.5-IS788
N.sp.6-T5461
N.sp.6-T5552
N.merrilliana-V10289
N.sp.7
N.sp.8
A.lambirensis-SWK5439
A.lambirensis-SWK2556
A.leiophylla
A.rehderiana
A.gullavara-MY4418
A.gullavara-MY1066
A.sp.1-S72
A.glomerata
A.diversifolia
A.macrophylla-M229
A.macrophylla-SWK2533
A.sp.2-IS811
A.heterophylla-IS854
A.sesquipedalis
A.glabra
A.montana
A.concinna-M198
A.concinna-M178
A.borneensis
A.semengohensis-SWK4877
A.myriantha-SWK1658
A.sp.3-SWK4755
A.sp.4-M9
A.sp.5-SGP1
A.aff.amabilis-T5856
A.aff.amabilis-T5442
A.amabilis-T4910-02
A.amabilis-T4910-01
A.rufescens
A.perlucida
A.sp.6-V11286
A.pilosa
A.sp.7-V2703
A.sp.7-V10641
A.sp.8-L1193
A.concolor-MY4008
A.bourdillonii-MY1068
A.bourdillonii-MY1551
A.henryi
A.pruinosa-SWK1199
A.sulcata-SWK1107
A.obovata-MY1084
A.obovata-L664
Clade 1
Clade 2
Clade 3
Clade 4
Clade 5
Clade 6
0.02

Figure 8. A MIG-seq ML tree for nine samples (three species) of *Neoactinodaphne*, 94 samples (34 species) of *Actinodaphne*, 22 samples (10 species) of *Neolitsea*, 38 samples (14 species) of *Litsea,* two samples (two species) of *Lindera,* two samples (a species) of *Phoebe* and a sample of *Machilus* (a species). Branches are labeled with bootstrap values. Voucher specimen ID is added after each specimen name.

Appendix. Matrix of the seven leaf traits: maximal number of leaves clustered on the branch top (MLC), midpoint petiole length (PL), midpoint leaf length (LL), midpoint leaf width (LW), midpoint lateral veins (LV), midpoint aspect ratio (AR), petiole pubescence (PP), venation type of secondary veins (SV), and venation type of tertiary veins (TV) for a total of 67 species including 65 described species of *Actinodaphne* and two species of *Neoactinodaphne.* PP is categorized into three lebel: 0: glabrous, 1: pubescens and 2: tomensose.

Species	MLC	PL	MinPL	MaxPL	LL	MinLL	MaxLL	LW	MinLW	MaxLW	LV	MinLV	MaxLV	AR	MinAR	MaxAR	PP	SV	TV
A. acuminata	15	17.5	10	30	11.3	7.5	15	2.3	1.5	3	12.5	10	15	5.0	5.0	5.0	0	pinninerved	reticulate
A. amabilis	5	12.5	10	15	14.3	9.5	19	2.5	2	3	8.0	6	10	5.7	4.8	6.3	1	pinninerved	scalariform
A. angustifolia	5	19.0	18	20	20.0	15	25	3.8	3.5	4	5.0	5	5	5.3	4.3	6.3	0	pinninerved	scalariform
A.	6	9.0	5	13	17.	9	25	3.7	2.3	5	8.5	7	10	4.7	3.9	5.0	1	pinninerve	scalariform

angustifolia sensu *Tanaros et al.*					0													d	
A. borneensis	5	9.0	8	10	14.3	3.5	25	5.3	2	8.5	5.0	3	7	2.7	1.8	2.9	0	pinninerved	scalariform
A. bourdillonii	7	11.0	10	12	17.0	14	20	4.8	4.5	5	7.0	6	8	3.6	3.1	4.0	0	pinninerved	scalariform
A. concinna	5	8.5	7	10	9.8	6.5	13	3.5	2	5	7.0	6	8	2.8	3.3	2.6	1	pinninerved	scalariform
A. cupularis	6	6.5	3	10	12.3	5.5	19	3.3	1.5	5	9.5	6	13	3.8	3.7	3.8	1	pinninerved	reticulate
A. cuspidata	5	10.	9	11	10.	9	11	3.8	3.6	4	7.0	6	8	2.0	2.5	2.8	1	pinninerve	scalariform

		0			0													d	
A. diversifolia	5	22.5	20	25	30.8	30	31.5	8.5	8	9	6.5	6	7	3.6	3.8	3.5	2	pinninerved	scalariform
A. fragilis	4	15.0	10	20	6.9	3.8	10	2.8	1.8	3.8	5.5	5	6	2.5	2.1	2.6	0	pinninerved	scalariform-reticulate
A. fuliginosa	5	7.0	5	9	3.5	2.5	4.5	2.0	1.5	2.5	4.0	4	4	1.8	1.7	1.8	1	pinninerved	reticulate
A. furfuracea	6	16.9	8	25	17.8	10	25	6.4	3.8	8.9	8.0	7	9	2.8	2.6	2.8	1	pinninerved	scalariform
A. glabra	7	27.5	25	30	37.5	30	45	8.3	6.35	10.16	8.0	8	8	4.5	4.7	4.4	0	pinninerved	scalariform
A. glaucina	9	16.0	12	20	20.5	13	28	3.3	2.5	4	10.0	10	10	6.3	5.2	7.0	1	pinninerved	scalariform

A. glomerata	7	20.0	18	30	26.0	11	41	14.0	5	23	11.5	10	13	1.9	2.2	1.8	1	pinninerved	scalariform
A. gracilis	7	25.0	20	30	20.5	17	24	5.5	4	7	6.0	6	6	3.7	4.3	3.4	0	triplinerved	scalariform
A. gullavara	6	15.9	13	19	15.2	7.6	23	3.5	2.5	4.4	4.5	4	5	4.4	3.0	5.2	1	pinninerved	scalariform
A. henryi	6	30.0	20	40	28.5	17	40	8.4	3.7	13	10.5	9	12	3.4	4.6	3.1	2	pinninerved	scalariform
A. hirsuta	5	28.6	25.4	31.75	37.5	30	45	10.8	8.89	12.7	6.0	5	7	3.5	3.4	3.5	1	pinninerved	scalariform
A. johorensis	5	20.0	15	25	16.0	11	21	3.5	2.5	4.5	6.5	4	9	4.6	4.4	4.7	1	pinninerved	reticulate
A.	6	32.	30	35	15.	13	18	4.8	4.6	5	6.5	6	7	3.2	2.8	3.6	2	pinninerve	scalariform

kinabaluensis		5			5													d	
A. koshepangii	4	6.0	5	7	11.0	9	13	4.0	3	5	8.0	7	9	2.8	3.0	2.6	1	pinninerved	reticulate
A. kostermansii	5	25.0	15	35	26.0	21	31	11.3	7	15.5	9.0	7	11	2.3	3.0	2.0	2	pinninerved	scalariform
A. kweichowensis	5	35.0	30	40	19.0	11	27	6.6	3.2	10	9.5	6	13	2.9	3.4	2.7	1	pinninerved	scalariform
A. lambirensis	4	17.0	17	17	6.8	6.8	6.8	2.8	2.8	2.8	8.0	6	10	2.5	2.4	2.4	0	pinninerved	scalariform-reticulate
A. lecomtei	6	13.5	7	20	15.0	10	20	2.3	1.5	3	35.0	30	40	6.7	6.7	6.7	0	pinninerved	reticulate

A. leiophylla	7	21.5	15	28	14.0	10	18	4.0	2.5	5.5	6.0	5	7	3.5	4.0	3.3	0	pinninerved	scalariform
A. macrophylla	12	37.5	25	50	28.5	15	42	8.8	5	12.5	15.5	14	17	3.3	3.0	3.4	2	pinninerved	scalariform
A. macroptera	4	21.5	20	23	37.5	35	40	10.3	8.5	12	8.5	8	9	3.7	4.1	3.3	1	pinninerved	scalariform
A. maingayi	5	20.0	18	22	32.5	30	35	10.8	10	11.5	13.0	11	15	3.0	3.0	3.0	2	pinninerved	scalariform
A. malaccensis	7	12.5	5	20	13.5	7	20	5.0	3	7	10.0	8	12	2.7	2.3	2.9	1	pinninerved	scalariform
A. mansonii	4	21.0	20	22	23.9	22.3	25.5	8.7	8	9.3	9.0	8	10	2.8	2.8	2.7	0	pinninerved	scalariform
A.	6	30.	20	40	27.	15	39	9.0	6	12	7.5	7	8	3.0	2.5	3.3	0	triplinerve	scalariform

menghaiensis		0			0													d	
A. mollis	6	15.9	13	19	14.0	10	18	5.1	2.54	7.62	4.0	4	4	2.8	3.9	2.4	2	pinninerved	scalariform
A. montana	5	15.0	10	20	15.0	11	19	5.5	4.5	6.5	8.5	7	10	2.7	2.4	2.9	1	pinninerved	scalariform
A. myriantha	4	17.0	16	18	15.5	13	18	7.3	6.5	8	6.0	5	7	2.1	2.0	2.3	0	pinninerved	scalariform
A. obovata	5	50.0	30	70	32.5	15	50	13.8	5.5	22	6.5	6	7	2.4	2.7	2.3	2	triplinerved	scalariform
A. obscurinervia	5	5.0	5	5	7.8	6.5	9	1.9	1.5	2.3	22.0	18	26	4.1	4.3	3.9	1	triplinerved	reticulate
A. oleifolia	4	12.	5	20	6.8	4	9.5	2.3	1.5	3	8.0	6	10	3.0	2.7	3.2	0	pinninerve	reticulate

		5																d	
A. omeiensis	6	20.5	11	30	19.5	12	27	4.0	2.1	6	13.5	12	15	4.9	5.7	4.5	0	pinninerved	scalariform
A. paotingensis	7	21.0	17	25	17.3	14	20.5	5.0	3.5	6.5	8.0	7	9	3.5	4.0	3.2	2	pinninerved	scalariform
A. pauciflora	6	13.8	11	17	16.5	10	23	4.4	2.54	6.35	5.5	5	6	3.7	3.9	3.6	1	pinninerved	scalariform
A. percoriacea	5	17.5	10	25	10.8	5.5	16	5.5	2.5	8.5	5.5	4	7	2.0	2.2	1.9	0	pinninerved	scalariform
A. perglabra	4	4.0	3	5	10.0	7	13	3.0	2.5	3.5	6.5	6	7	3.3	2.8	3.7	0	pinninerved	reticulate
A. perlucida	4	17.5	15	20	16.0	11	21	6.5	4	9	8.0	7	9	2.5	2.8	2.3	1	pinninerved	scalariform

A. pilosa	5	22.5	15	30	18.0	12	24	8.5	5	12	6.0	5	7	2.1	2.4	2.0	2	pinninerved	scalariform
A. procera	5	41.3	25	57	19.7	8.9	30.5	9.7	6.6	12.7	6.5	6	7	2.8	1.3	2.4	0	triplinerved	intervening veins
A. pruinosa	4	12.5	10	15	10.5	7.5	13.5	3.3	2.5	4	8.0	7	9	3.2	3.0	3.4	1	pinninerved	scalariform
A. rehderiana	5	22.5	20	25	14.5	12	17	5.3	4	6.5	7.5	7	8	2.8	3.0	2.6	0	pinninerved	scalariform
A. reticulata	7	7.5	7	8	15.0	8	22	3.5	2	5	11.0	10	12	4.3	4.0	4.4	0	pinninerved	reticulate
A. ridlleyi	6	15.0	10	20	13.0	10	16	4.0	3	5	7.0	6	8	3.3	3.3	3.2	1	pinninerved	scalariform
A. robusta	7	42.	25	60	31.	22	40	10.	7.5	14	11.	7	15	2.9	2.9	2.9	2	pinninerve	scalariform

		5			0			8			0							d	
A. rufescens	7	16.9	8	25	16.5	7.6	25	5.1	2.54	7.62	7.0	7	7	3.3	3.0	3.3	2	pinninerved	scalariform
A. semengohensis	7	15.0	10	20	8.5	7.5	9.5	2.2	1.8	2.5	5.0	4	6	4.5	4.2	3.8	1	pinninerved	scalariform
A. sesquipedalis. var. *cambodiana*	10	30.0	20	40	33.5	22	45	6.0	3	9	10.5	9	12	5.6	7.3	5.0	1	pinninerved	scalariform
A. sesquipedalis. var. *glabra*	8	45.0	35	55	45.0	30	60	13.8	9	18.5	14.0	12	16	3.3	3.3	3.2	0	pinninerved	scalariform

A. sesquipedalis . var. *sesquipedalis*	11	25.5	15	36	48.5	33	64	10.0	7	13	11.0	10	12	4.9	4.7	4.9	2	pinninerved	scalariform
A. sikkimensis	6	7.5	5	10	12.0	10	14	3.0	2	4	10.0	8	12	4.0	5.0	3.5	1	pinninerved	intervening veins
A. soepadmoi	6	40.0	20	60	17.5	14.5	20.5	6.8	4.5	9	7.0	6	8	2.6	3.2	2.3	1	pinninerved	scalariform
A. spathulifolia	5	13.5	12	15	6.3	5	7.5	3.3	2.5	4	5.5	5	6	1.9	2.0	1.9	0	pinninerved	reticulate
A. sphaerocarpa	7	27.5	15	40	20.0	15	25	5.5	3.5	7.5	9.0	8	10	3.6	4.3	3.3	0	triplinerved	scalariform

A. sulcata	5	8.0	6	10	12.5	10	15	5.5	4	7	8.5	7	10	2.3	2.5	2.1	1	pinninerved	scalariform
A. tonkinense	3	0.0	0	0	20.0	15	25	6.5	5	8	11.0	10	12	3.1	3.0	3.1	1	pinninerved	scalariform
A. trichocarpa	5	7.5	5	10	9.5	5	14	2.2	1.4	3	8.0	6	10	4.3	3.6	4.7	1	pinninerved	scalariform-reticulate
A. tsaii	6	6.5	6	7	12.5	10	15	2.8	2	3.5	9.0	8	10	4.5	5.0	4.3	2	pinninerved	intervening veins
A. venosa	5	16.0	12	20	13.0	9.5	16.5	4.5	3	6	5.5	4	7	2.9	3.2	2.8	1	pinninerved	scalariform
N. hongiaoensis	9	14.0	10	18	14.3	10.8	17.7	4.8	3.8	5.8	16.0	14	18	3.0	3.2	2.8	2	pinninerved	intervening veins
N.	6	9.5	7	12	9.0	7	11	2.6	1.7	3.5	8.5	7	10	3.5	2.8	3.1	1	pinninerve	intervening veins

langbianensis																		d	