RESEARCH ARTICLE

Molecular phylogeny of *Boesenbergia* (*Zingiberaceae*) reveals shared characters and infrageneric division

L.M. Prince[1], J.D. Mood[2], T. Mandáková[3], H.J. de Boer[4,5,*]

[†] Dedicated in memoriam to Dr. Kai Larsen, Dr. Jan-Frits Veldkamp, Dr. Rosemarie Smith, and Dr. Mary Mendum

Abstract   The classification of *Boesenbergia* (*Zingiberaceae*) has long been a challenge, and recent insights from molecular phylogenetic studies have both raised and resolved a number of questions. Combined morphological and molecular revisions resulted in the description of new species as well as merging of *Haplochorema* and *Jirawongsea* into *Boesenbergia*. This study uses the most complete set of *Boesenbergia* accessions and sister genera to investigate the evolutionary relationships of the genus in a phylogenetic framework. This framework confirms the previously identified clades and the placement of the included accessions within those clades. These clades share being single-flowered and having a basipetalous flowering sequence, characters uniting all taxa in *Boesenbergia*, but also have unique or mostly unique characters that are clade specific. In addition, the phylogeny identifies two species, *B. purpureorubra* and *B. siamensis*, that are hard to place within these clades based on both molecular and morphological data, while the morphological data points to a stronger affiliation with the clade including the type of the genus.

INTRODUCTION

Boesenbergia Kuntze (*Zingiberaceae*: *Zingibereae*) as currently circumscribed is a plant genus including between 83 (WCSP 2020) and 117 species (Zingiberaceae Resource Centre 2023). *Boesenbergia* is usually characterized by having single-flowered inflorescences, distichous bracts, and a basipetalous flowering sequence (Mood et al. 2020). The genus is native to China, the Indian subcontinent, and southeast Asia (Mabberley 2017). Inflorescence structure has been noted by several authors to be an important character for classification of the genus (Sakai & Nagamasu 2006a, Techaprasan et al. 2006, Mood et al. 2014a). There are two types of inflorescence in *Boesenbergia*. One type is commonly referred to as distichous, but actually the bracts overlap on one face of the axis but not on the other (Smith 1987). The other has bracts arranged on one side, and is often short, congested, and concealed within the leaf sheath (Sakai & Nagamasu 2006a). Sakai & Nagamasu (2006a) analyzed this trait and found that many taxa from Indochina had a strict distichous inflorescence, whereas those studied from Borneo had bracts arranged on one side.

The *Zingiberoideae* are distinguished from other subfamilies in *Zingiberaceae* by having the plane of leaf distichy parallel to the rhizome. The tribe *Zingibereae* contains 24 genera (Kress et al. 2002, Ngamriabsakul et al. 2004, Mood et al. 2020), and are distinguished from *Globbeae*, the other tribe of the subfamily, by having trilocular ovaries with axial, basal or free columnar placentation, and a labellum that is usually not connate to the filament (Kress et al. 2002, Ngamriabsakul et al. 2004). Chromosome numbers mostly vary according to, and within, the genus (Ngamriabsakul et al. 2004). The genus *Zingiber* Mill. is the most species-rich in the family, followed by *Curcuma* L. and *Hedychium* J.Koenig (WCSP 2020).

Classification of *Boesenbergia* has long been a challenge, and recent insights from molecular phylogenetic studies have resolved several questions. Phylogenetic studies have frequently found that inflorescence type is a strong character correlating with phylogeny that matches a clade with distichous inflorescences and one with one-sides inflorescences (Techaprasan et al. 2008, Mood et al. 2014a, 2019, 2020). Combined morphological and molecular revisions resulted in the description of eight new species (Techaprasan et al. 2006, 2008, Mood et al. 2013, 2019). Several morphological studies have also described new species (Sakai & Nagamasu 2006b, 2009, Newman 2013, Meekiong & Lim 2014, Mood et al. 2014b, 2016a, b, 2018, Aishwarya et al. 2015, Ly 2017). Studies of species in the genus have contributed to better insights into species distribution, detected new records, assessed IUCN red-list status and increased access to identification keys (Chen & Xia 2011, 2019, Sam 2015, Aishwarya & Sabu 2015, 2021). A further morphological and molecular study reduced the

[1] The Field Museum, Department of Botany, 1400 S Lake Shore Dr., Chicago, IL 60605, USA.
[2] Lyon Arboretum, University of Hawaii, 3860 Manoa Road, Honolulu, HI 96822, USA.
[3] CEITEC – Central European Institute of Technology, and Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic.
[4] Naturalis Biodiversity Center, P.O. Box 9514, 2300 RA Leiden, The Netherlands.
[5] Natural History Museum, University of Oslo, Postboks 1172, Blindern, 0318 Oslo, Norway.
* Corresponding author e-mail: h.de.boer@nhm.uio.no.

recently described genus *Jirawongsea* Picheans. (Picheansoonthon et al. 2008) to *Boesenbergia* with five new combinations (Mood et al. 2014a), and a study published in 2020 reduced *Haplochorema* K.Schum. to *Boesenbergia*, and included seven new combinations (Mood et al. 2020).

Molecular data generated in previous studies have helped to elucidate the phylogenetic relationships within *Boesenbergia* as well as of the genus within the tribe. The nuclear ITS and the chloroplast *trnK* intron have been used in several studies (Mood et al. 2014a, 2016a, b, 2019, 2020), in addition to *matK*, *psbA-trnH* and *petA-psbJ* (Techaprasan et al. 2006), AFLP and SSCP (Techaprasan et al. 2008). Previous partial phylogenetic studies have shown a limited support for monophyly of *Boesenbergia* (Mood et al. 2014a), but the authors have nevertheless argued to maintain *Boesenbergia* as a single genus for nomenclatural parsimony (Mood et al. 2020).

In this study, which builds on previous studies, we investigate *Boesenbergia* in a phylogenetic framework using an expanded taxon sampling. The study aims to test the monophyly of *Boesenbergia*, reassess morphological characters of the genus and included clades, and provide a stable definition of the genus.

MATERIALS AND METHODS

Vouchers

Material for DNA extraction was obtained mainly through our collections supported by vouchers deposited at AAU, ASSAM, BISH, BK, BKF, CAL, CUH, DUKE, E, KEP, KRB, KYO, MBK, QBG, SAR, UPM, US, or from existing specimens in these herbaria (for herbarium abbreviations see Thiers continuously updated). Additional data from previous studies (Mood et al. 2013, 2014a, 2019, 2020) were downloaded from NCBI GenBank. Species were selected to cover the maximum morphological variability so far known in *Boesenbergia* in addition to the maximum geographical coverage available to us, and includes 55 out of 117 species. Two loci were investigated, the plastid DNA *trnK* intron and the nuclear ribosomal ITS region (ITS1-5.8S-ITS2). Sixty-six *Boesenbergia* sequences from previous studies (Mood et al. 2013, 2014a, 2016b, 2018, 2019, 2020) were combined with 66 newly generated *Boesenbergia* sequences for the ingroup data set. Fifty-eight scaffold sequences for taxa outside *Boesenbergia* were downloaded from GenBank, and added to 19 new outgroup sequences to complete the data matrix. The nrITS and *trnK* data matrix included a total of 217 sequences. Species names and their authors, specimen voucher information, and GenBank accession numbers per molecular marker (including 85 new sequences, 41 nrITS and 44 *matK*) are summarized in Appendix 1 with accessions newly generated for this study marked by an asterisk (*).

Voucher to sequence

DNA extraction follows Kress et al. (2002), while amplification and analytical methods follow Mood et al. (2013). Consistent with previously published work, the nuclear ribosomal ITS (nrITS) region was amplified using the plant specific 18S-F and 26S-R primers (Prince 2010). The plastid *trnK* region was amplified in two parts, the first using either 1F (Manos & Steele 1997) and mIR (Kress et al. 2002) or 1F (Manos & Steele 1997) and 1235R (Mood et al. 2018). The second half was amplified using mIF (Kress et al. 2002) and 2R (Steele & Vilgalys 1994). Data were sequenced on an ABI Genetic Analyzer and sequences were verified and edited in Sequencher v. 4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Sequences were manually aligned in Se-Al (Rambaut 1996).

Molecular phylogeny

Iterative analyses were employed due to ambiguity in the sequence alignment, particularly for the ITS data partition (data not shown). In each round, both the *trnK* and the ITS data were realigned for the samples included in the matrix. Gaps were treated as missing data and were not coded as separate characters. Data analyses were conducted under both maximum likelihood (ML) criteria in RAxML (Stamatakis 2014), maximum parsimony (MP) criteria in PAUP* v. 4.0 (Swofford 2002), and Bayesian posterior probability in MrBayes v. 3.2.6 (Huelsenbeck & Ronquist 2001). For ML analyses in RAxML and MP analyses in PAUP* branch support was assessed via parsimony bootstrap (BS) (Felsenstein 1985). ML bootstrap percentages were estimated in RAxML using a bootstrap analysis with 1 000 replicates of the dataset on the CIPRES Science Gateway (Miller et al. 2010). MP bootstrap percentages were estimated in PAUP* using 1 000 replicates of 10 random addition replicates, hold one tree, tree-bisection-reconnection (TBR), saving a maximum of 10 trees per replicate. Bayesian posterior probabilities (PP) were determined in MrBayes v. 3.2.6 (Huelsenbeck & Ronquist 2001) via XSEDE on the CIPRES Science Gateway (Miller et al. 2010) using three replicates of three million generations (sampling every 100 generations), specifying three data partitions (*matK*, *trnK* (excluding *matK*) and ITS). Burn-in for each analysis was established as all trees with average standard deviation of the split frequencies less than 0.01. This resulted in an average burn-in of 39 % for the *Zingiberaceae* (Family-Wide) analyses and 22 % for the *Boesenbergia* (Sister Group Verification) analyses.

The first round of analyses (Family-Wide) included sequences for all *Boesenbergia* samples plus representatives of 34 other *Zingiberaceae* genera to assess monophyly of the genus and to identify potential sister genera of *Boesenbergia* to be included in the second round of analyses. Parsimony analyses were conducted using 1 000 random sequence addition replicates with TBR branch swapping, hold one tree, reconnection limit of eight, saving no more than 100 trees per replicate on both the separate as well as the combined data partitions. Best trees were further searched (save all trees) until all additional trees were saved or a limit of 500 000 trees was reached (and the search was terminated). *Siphonochilus* was designated as the outgroup taxon for these analyses, since it has previously been identified as the first diverging lineage in the family (Kress et al. 2002).

Maximum likelihood (ML) model parameters were selected based upon results of jModeltest v. 2.1.6 (Guindon & Gascuel 2003, Darriba et al. 2012). Analyses of the combined data matrix were conducted. Likelihood model parameters were identical for all three selection criteria (Akaike Information Criterion (AIC; Akaike 1974), Bayesian Information Criterion (BIC; Schwarz 1978), and Decision Theory as applied in Minin et al. (2003)), so a single analysis of 10 random addition replicates was performed. Model parameters were GTR+I+G: base = (0.3372 0.1618 0.1645) nst = 6 rmat = (1.0703 2.2478 0.5000 1.1178 4.4540) rates = gamma shape = 0.4660 ncat = 4 pinvar = 0.3240.

A second round of analyses (Sister Group Verification) included all *Boesenbergia* samples and representatives of the most closely related (potential sister) genera identified in the first round (Family-Wide) analysis (see Table 1 for included sister taxa per analysis). These analyses were conducted to either confirm the best sister group from the first round, or to identify a better candidate. Maximum likelihood, maximum parsimony and Bayesian analyses were conducted as described above. Placement of *Boesenbergia* was evaluated based on the selection of ten different candidate sister groups.

Table 1 Sister group observations for various clades and the maximum parsimony strict consensus tree position of critical species in *Boesenbergia* based on analyses of realigned combined chloroplast *trnK* and nuclear ITS nucleotide data. Branch support values are Maximum Likelihood bootstrap (ML) / Maximum parsimony bootstrap (MP) / Bayesian posterior probability (PP).

Sister genus	Genus monophyly	*B. purpureorubra*	*B. siamensis*	clade I monophyly	clade II monophyly
Borneocola + *Distichochlamys +* *Myxochlamys +* *Scaphyochlamys*	99 / 99 / 1.00	Sister to *B. siamensis*; – / 53 / n/a	Sister to clade II; 82 / 74 / 1.00	73 / 65 / 1.00	94 / 94 / 1.00
Cornukaempferia	100 / 100 / 1.00	Sister to *B. siamensis*; – / unresolved / –	Sister to clade II; 74 / 97 / 1.00	93 / 89 / 1.00	97 / 89 / 1.00
Haniffia	100 / 100 / 1.00	Sister to *B. siamensis*; – / unresolved / –	Sister to clade II; 69 / 68 / –	62 / 64 / 1.00	95 / 95 / 1.00
Kaempferia + *Kedhalia +* *Newmania*	– / 63 / 0.99	Sister to clade I; n/a / unresolved / –	Sister to clade II; 68 / 57 / 1.00	79 / 74 / 1.00	98 / 98 / 1.00
Zingiber	96 / 94 / 1.00	Sister to clade I; 82 / 73 / –	Sister to clade II; 61 / 56 / 0.99	82 / 85 / 1.00	99 / 98 / 1.00

Note. Bootstrap values below 50 % are marked as '–'. Relationships absent from the consensus tree are marked as 'n/a'.

Chromosome preparations and counting

Actively growing young roots were harvested from cultivated plants of *Boesenbergia siamensis* (Gagnep.) Sirirugsa (*J. Mood 3200*; BISH) and *B. purpureorubra* Mood & L.M.Prince (*J. Mood 3106*; BISH), and subsequently pre-treated with ice-cold water for 12 hours, fixed in ethanol/acetic acid (3 : 1, v/v) fixative for 24 hours at 4 °C and stored at -20 °C until further use. Selected root tips were rinsed in distilled water (twice for five minutes) and citrate buffer (10 mM sodium citrate, pH 4.8; twice for five minutes), and digested in 0.3 % (w/v) cellulase, cytohelicase and pectolyase (all Sigma-Aldrich, St Louis, MO, USA) in citrate buffer at 37 °C for 90 minutes. After digestion, individual root tips were dissected on a microscope slide in approximately 10 µl acetic acid and covered with a cover slip. The cell material was then spread evenly using tapping, thumb pressing and gentle flame-heating. Finally, the slide was flash frozen in liquid nitrogen and the cover slip flicked off with a razor blade. Slides were fixed in ethanol-acetic acid (3 : 1) and air-dried. Chromosomes were counterstained with 2 µg/ml DAPI in Vectashield (Vector Laboratories, Peterborough, UK). Preparations were analyzed and photographed using a Zeiss Axioimager Z2 epifluorescence microscope with a Cool-Cube camera (MetaSystems). At least 20 mitotic chromosome spreads were counted from each accession analyzed. Samples and slides are stored at the chromosome lab at CEITEC, Masaryk University, Czech Republic.
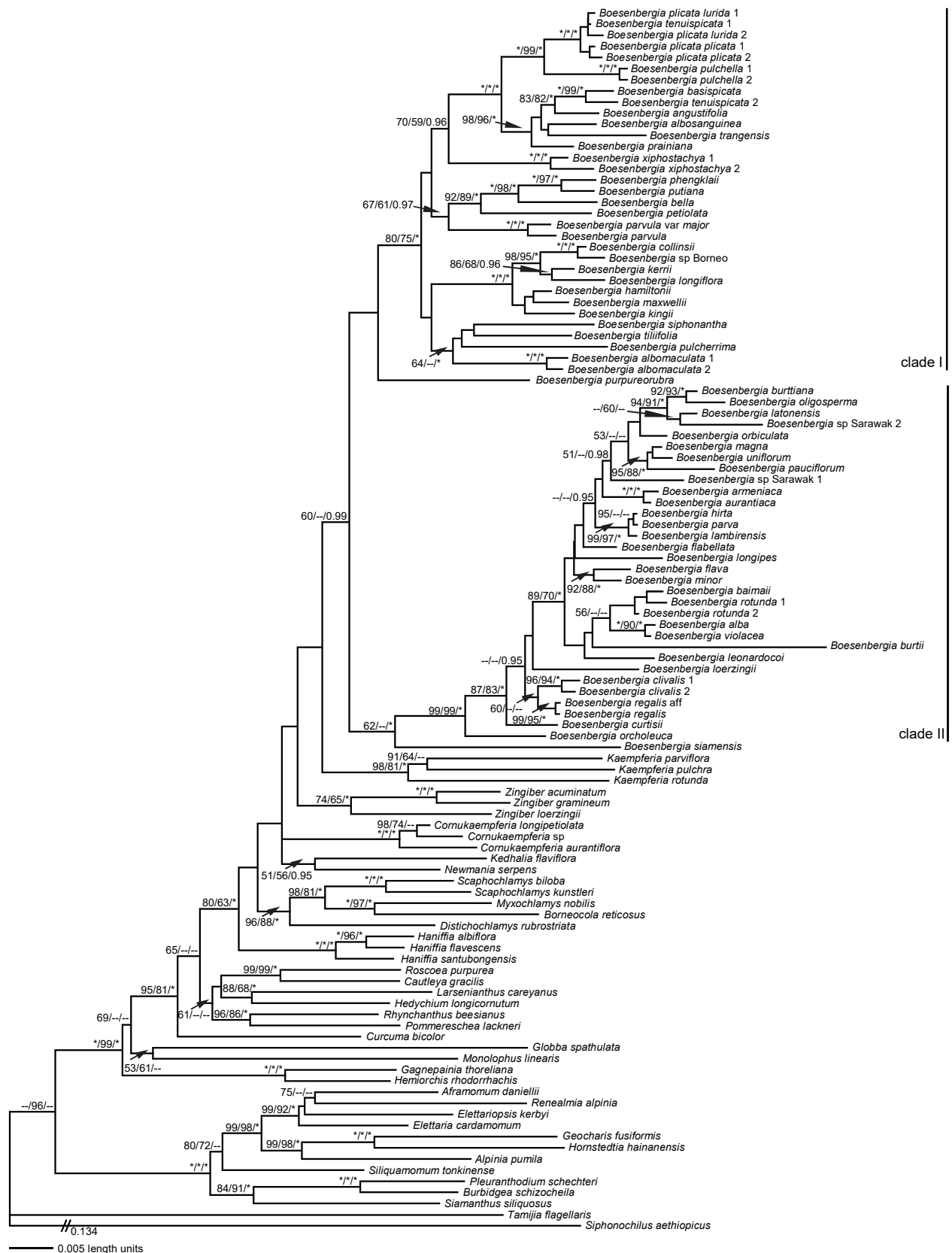
RESULTS

Family-wide analyses

The data matrix consisted of 4 159 characters of which 2 852 were constant and 614 potentially parsimony-informative. Although the nrITS region only contributed 895 characters compared to the 3 264 characters from the plastid *trnK* intron (including the *matK* coding region), the number of potentially parsimony-informative characters was nearly evenly split, with 320 from the nuclear genome and 294 from the plastid. Maximum parsimony analysis of the nrITS matrix yielded 19 942 trees of length 1 688 (excluding uninformative characters; tree not shown). The Bayesian maximum clade credibility tree is shown in Appendix 2, with ML bootstrap, MP bootstrap, and Bayesian posterior probabilities values above the branches. The MP strict consensus tree was moderately well resolved, but critical backbone branches had low bootstrap support. Neither *Boesenbergia* nor *Zingiber* were monophyletic, whether analysed under parsimony or likelihood criteria. The parsimony

and likelihood analyses also differed significantly for the support of a few key branches, and the sister relationships of key taxa, namely those of *B. siphonantha* (King ex Baker) M.Sabu, Prasanthk. & Škorničk. and *B. siamensis*. Closely related, monophyletic genera in the tribe *Zingibereae* included *Cornukaempferia* Mood & K.Larsen (100 % ML, 95 % MP, 1.00 PP), *Haniffia* Holttum (89 % ML, 99 % MP, 1.00 PP), *Kaempferia* L. (80 % ML, 54 % MP, 1.00 PP) and *Scaphochlamys* Baker (98 % ML, 95 % MP, 1.00 PP), as well as the genera *Borneocola* Y.Y.Sam, *Distichochlamys* M.F.Newman, *Kedhalia* C.K.Lim, *Myxochlamys* A.Takano & Nagam. and *Newmania* N.S.Lý & Škorničk.

Maximum parsimony analysis of the *trnK* intron (including *matK*) matrix produced 500 000 trees of length 695 (excluding uninformative characters. The strict consensus tree was less resolved than the ITS tree, but lower resolution was concentrated in more terminal node relationships. The backbone branches were more strongly supported than the ITS tree, and all (multi-sample) genera were monophyletic (see Appendix 3 for the Bayesian maximum clade credibility tree). *Boesenbergia* was monophyletic, but branch support varied per analytical method (68 % ML, < 50 % MP, 1.00 PP). *Distichochlamys* + (*Scaphochlamys* + *Borneocola* + *Myxochlamys*) was not monophyletic, whether analysed under parsimony or likelihood criteria. Unlike the ITS results, there were no significant differences in support for relationships between the parsimony and likelihood analyses. Closely related, monophyletic genera in the tribe *Zingibereae* included *Cornukaempferia* (100 % ML, 99 % MP, 1.00 PP), *Haniffia* (94 % ML, 91 % MP, 1.00 PP), *Kaempferia* (81 % ML, 70 % MP, 1.00 PP), and *Zingiber* (93 % ML, 87 % MP, 1.00 PP), as well as the genera *Borneocola*, *Distichochlamys*, *Kedhalia*, *Myxochlamys*, *Newmania* and *Scaphochlamys*.

Combined nrITS and *trnK* MP analyses generated the limit of 500 000 trees (length 2 413 excluding uninformative characters). Results of maximum likelihood analyses of the combined data were consistent with the MP results. The Bayesian maximum clade credibility tree is shown in Fig. 1, with ML, MP and PP values. All *Boesenbergia* samples formed a monophyletic clade, but branch support was inconsistent (60 % ML, < 50 % ML, < 0.99 PP). Branch support was inconsistent for the position of *B. siamensis* (62 % ML, < 50 % ML, 1.00 PP) and lacking for *B. purpureorubra*. Five potential sister clades were identified: *Cornukaemperia* (100 % ML, 100 % BS, 1.00 PP), *Borneocola* + *Distichochlamys* + *Myxochlamys* + *Scaphochlamys* (96 % ML, 88 % MP, 1.00 PP), *Haniffia* (100 % ML, 100 % BS, 1.00 PP), *Kaempferia* + *Kedhalia* + *Newmania* (51 % ML, 56 % BS, 0.95 PP) or *Zingiber* (74 % ML, 65 % BS, 1.00 PP).

Boesenbergia plicata lurida 1
Boesenbergia tenuispicata 1
Boesenbergia plicata lurida 2
Boesenbergia plicata plicata 1
Boesenbergia plicata plicata 2
Boesenbergia pulchella 1
Boesenbergia pulchella 2
Boesenbergia basispicata
Boesenbergia tenuispicata 2
Boesenbergia angustifolia
Boesenbergia albosanguinea
Boesenbergia trangensis
Boesenbergia prainiana
Boesenbergia xiphostachya 1
Boesenbergia xiphostachya 2
Boesenbergia phengklaii
Boesenbergia putiana
Boesenbergia bella
Boesenbergia petiolata
Boesenbergia parvula var major
Boesenbergia parvula
Boesenbergia collinsii
Boesenbergia sp Borneo
Boesenbergia kerrii
Boesenbergia longiflora
Boesenbergia hamiltonii
Boesenbergia maxwellii
Boesenbergia kingii
Boesenbergia siphonantha
Boesenbergia tiliifolia
Boesenbergia pulcherrima
Boesenbergia albomaculata 1
Boesenbergia albomaculata 2
Boesenbergia purpureorubra

clade I

Boesenbergia burttiana
Boesenbergia oligosperma
Boesenbergia latonensis
Boesenbergia sp Sarawak 2
Boesenbergia orbiculata
Boesenbergia magna
Boesenbergia uniflorum
Boesenbergia pauciflorum
Boesenbergia sp Sarawak 1
Boesenbergia armeniaca
Boesenbergia aurantiaca
Boesenbergia hirta
Boesenbergia parva
Boesenbergia lambirensis
Boesenbergia flabellata
Boesenbergia longipes
Boesenbergia flava
Boesenbergia minor
Boesenbergia baimaii
Boesenbergia rotunda 1
Boesenbergia rotunda 2
Boesenbergia alba
Boesenbergia violacea
Boesenbergia burtii
Boesenbergia leonardocoi
Boesenbergia loerzingii
Boesenbergia clivalis 1
Boesenbergia clivalis 2
Boesenbergia regalis aff
Boesenbergia regalis
Boesenbergia curtisii
Boesenbergia orcholeuca
Boesenbergia siamensis

clade II

Kaempferia parviflora
Kaempferia pulchra
Kaempferia rotunda
Zingiber acuminatum
Zingiber gramineum
Zingiber loerzingii
Cornukaempferia longipetiolata
Cornukaempferia sp
Cornukaempferia aurantiflora
Kedhalia flaviflora
Newmania serpens
Scaphochlamys biloba
Scaphochlamys kunstleri
Myxochlamys nobilis
Borneocola reticosus
Distichochlamys rubrostriata
Haniffia albiflora
Haniffia flavescens
Haniffia santubongensis
Roscoea purpurea
Cautleya gracilis
Larsenianthus careyanus
Hedychium longicornutum
Rhynchanthus beesianus
Pommereschea lackneri
Curcuma bicolor
Globba spathulata
Monolophus linearis
Gagnepainia thoreliana
Hemiorchis rhodorrhachis
Aframomum daniellii
Renealmia alpinia
Elettariopsis kerbyi
Elettaria cardamomum
Geocharis fusiformis
Hornstedtia hainanensis
Alpinia pumila
Siliquamomum tonkinense
Pleuranthodium schechteri
Burbidgea schizocheila
Siamanthus siliquosus
Tamijia flagellaris
Siphonochilus aethiopicus

0.134

0.005 length units

Fig. 1 Bayesian maximum clade credibility tree of *Boesenbergia* for the combined chloroplast *trnK* and nrITS data showing support values for maximum likelihood (1st, bootstrap values > 50 % shown), maximum parsimony (2nd, bootstrap values > 50 % shown), and Bayesian inference (3rd, posterior probabilities > 0.95 shown, 1.00 shown as *). The branch to *Siphonochilus* was trimmed, and the length is provided below the branch.

Sister group verification

From the family-wide analyses, we identified the smallest possible, modestly supported group (80 % ML, 63 % MP, 1.00 PP) that included *Boesenbergia* to suggest potential sister genera. However, identification of a clear sister group was not possible due to poor bootstrap and posterior probability values. This may be due, in part, to ambiguity in the nucleotide sequence alignments, particularly for the ITS data matrix. Reanalysis of the original data matrix alignment, but with fewer distant relatives, was also unsuccessful in identifying a clear sister genus to *Boesenbergia*. The Bayesian maximum clade credibility tree of the combined data set (Appendix 4) does identify monophyletic, well-supported genera. The relationships of the genera to each other and to *Boesenbergia*, however, are poorly supported. The different reanalyses of the original matrices failed to identify a strong sister genus candidate.

Boesenbergia relationships

Two well-supported clades of *Boesenbergia* taxa are retrieved in the combined analyses (Fig. 1). The *Boesenbergia* s.str. clade, clade I, (80 % ML, 75 % MP, 1.00 PP) includes its type *B. pulcherrima* (Wall.) Kuntze and distichous flowering taxa; and the other, clade II, includes accessions of one-sided flowering *Boesenbergia* as well as the former genus *Haplochorema* (99 % ML, 99 % MP, 1.00 PP). In addition, two accessions of *Boesenbergia* outside these two clades are not resolved, *B. siamensis* and *B. purpureorubra*. The placement of *B. siamensis* was unaffected by sister-group choice, always falling sister to clade II, but the placement of *B. purpureorubra* varied, either falling sister to *B. siamensis* or sister to clade I (Table 1). The chromosome counts of taxa in clade II are 2n = 24, but counts are available only for two *Boesenbergia* taxa, *B. rotunda* (L.) Mansf. and *B. ochroleuca* (Ridl.) Schltr. The chromosome count of *B. siamensis* is 2n = 24 (Fig. 2). Taxa in clade I have mostly 2n = 20 chromosomes though this ranges from 18 to 36 (Goldblatt & Johnson 1991, Eksomtramage & Boontum 1995, Eksomtramage et al. 2002, Mood et al. 2018, 2019). *Boesenbergia purpureorubra* had a chromosome count of 2n = 20 (Fig. 2). Morphology places these taxa confidently within *Boesenbergia*, but the molecular phylogenetic framework with these markers does not provide conclusive support for monophyly of the genus, or clear relationships within the genus beyond the two clades mentioned above.

DISCUSSION

The current molecular framework resolves several evolutionary relationships within *Boesenbergia*. Determining the sister genus as well as confirming the monophyly and relationships within the genus will require a larger nuclear genomic dataset from, for example, target capture sequencing or other genomics approaches that are tolerant of degraded DNA from herbarium vouchers. The expanded sampling compared to previous studies confirms the previously retrieved *Boesenbergia* clades I and II (Mood et al. 2019, 2020) and resolves the placement of many species in these clades. The current molecular phylogenetic framework enables investigation of morphological traits, both in terms of evolutionary relationships and classification. Previous morphological studies have shown that all *Boesenbergia* share being single-flowered and having a basipetalous flowering sequence, even though these characters are by no means unique to the genus (Mood et al. 2013, 2014a, b, 2016a, b, 2018, 2019, 2020). Trilocular ovaries are a shared character of the tribe *Zingibereae*, and Mood et al. (2020) showed that the ovaries in *Haplochorema* (now *Boesenbergia*) are trilocular although these sometimes lack developed septa. Characters shared by *Boesenbergia* in clade I are the presence of an androecial cup, the absence of an anther crest, and possessing mostly saccate labellae, although these can range from flat to clearly saccate, and having an inflorescence with distichously arranged bracts. The species are mostly deciduous, although *B. angustifolia* (Hallier f.) Schltr., *B. basispicata* K.Larsen ex Sirirugsa, *B. pulchella* (Ridl.) Merr. and *B. tiliifolia* (Baker) Kuntze are evergreen. Chromosome numbers are mostly 2n = 20 but range from 18–36 (Goldblatt & Johnson 1991, Eksomtramage & Boontum 1995, Eksomtramage et al. 2002, Mood et al. 2018, 2019). Characters shared by *Boesenbergia* in clade II are the absence of an androecial cup, flat labellae and the presence of an anther crest, although *B. armeniaca* Cowley and *B. regalis* Kharuk. & Tohdam appear to lack an anther crest. Leaves are mostly evergreen, but deciduous in *B. curtisii* (Baker) Schltr., *B. macropoda* Merr., *B. ochroleuca* and *B. rotunda* (L.) Mansf. The chromosome counts of taxa in clade II are 2n = 24. The phylogenetic analyses suggest *B. siamensis* is more closely related to clade II, and it shares a chromosome count of 2n = 24. However, in contrast to most taxa in clade II, it lacks an anther crest, has a saccate labellum,
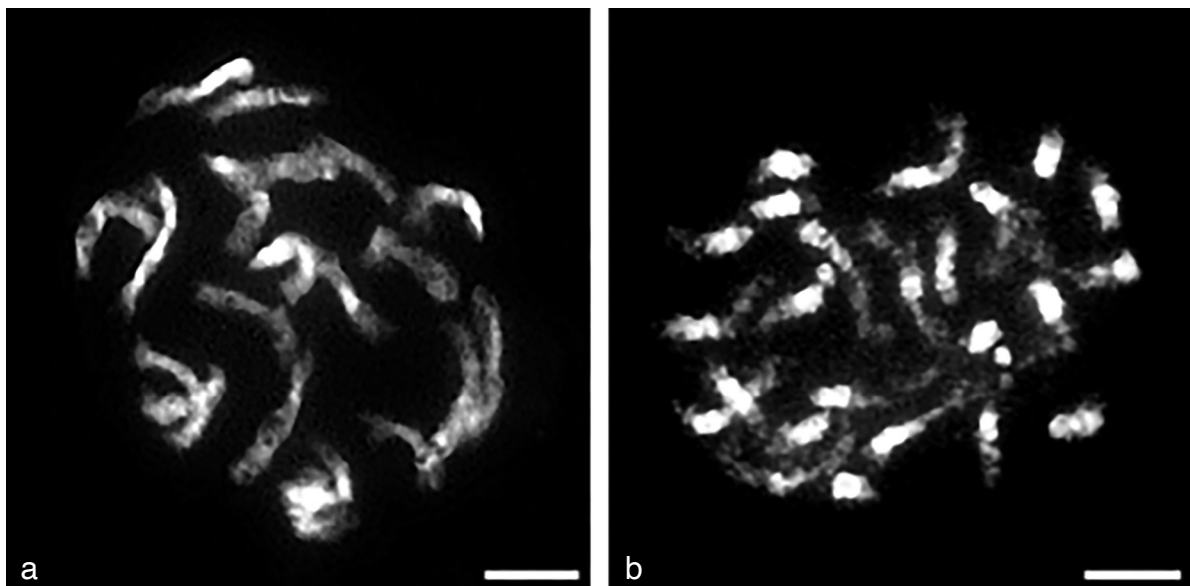
Fig. 2 Mitotic chromosomes of (a) *Boesenbergia purpureorubra* (2n = 20) and (b) *B. siamensis* (2n = 24). Chromosomes were counterstained with DAPI. — Scale bars = 10 μm.

an androecial cup, but like several other species is deciduous. *Boesenbergia purpureorubra* which either shows affinity with *B. siamensis* or clade I, and similarly has a saccate labellum, absence of anther crest, presence of androecial cup, and is deciduous. The only difference is that it has a chromosome count of 2n = 20 like clade I, a distinction from *B. siamensis*.

Previous molecular phylogenetic analyses of *Boesenbergia* (Mood et al. 2014a) show that labellum form is less significant in *Boesenbergia* systematics than inflorescence structure and flowering sequence. This was also used as an argument by Mood et al. (2020) to merge *Haplochorema*, with a flat labellae, into *Boesenbergia*. Hypothesizing about the evolutionary relationships and eventual placement of *B. siamensis* and *B. purpureorubra* in a phylogeny, we could argue that the morphological characters mostly point to affinity of both species with clade I.

Infrageneric divisions of *Boesenbergia* have been used to attempt to classify the species based on morphological characters (Baker 1890, Kuntze 1891, Ridley 1899, 1924, Schumann 1904, Valeton 1918). *Boesenbergia pulcherrima* was published by Wallich in 1829 as *Gastrochilus pulcherrima* Wall. It was not until 1891 that Kuntze showed that *Gastrochilus* D.Don had priority for the orchid genus and that the name *Boesenbergia* should be applied. Later authors, including Schumann (1904) and Valeton (1918) continued to use the name *Gastrochilus* Wall. despite Kuntze's publication. Valeton (1918) used infrageneric divisions including subgenera, sections and subsections. The section *Nudae* Valeton of subgenus *Densiflorae* Valeton as well as the subgenus *Scaphochlamydae* Valeton includes the species found in clade I in the molecular phylogenies, whereas section *Cristatae* Valeton of subgenus *Densiflorae* Valeton includes those of clade II. Subgenus *Scaphochlamydae* Valeton and subgenus *Strobiliformes* Valeton also include species from other ginger genera. Although some of the groups identified by Valeton do correspond to clades retrieved in molecular studies, the relations among them do not. Loesner (1930) used infrageneric divisions with rows (*reihe*), sections and subsections in *Boesenbergia*. The rows I – *Eugastrochilus* Valeton and rows II – *Paragastrochilus* Valeton correspond with clade I and II in the molecular phylogenies, but the lower-level divisions at section and subsection level do not correspond. The current molecular phylogeny provides a framework to understand infrageneric relationships within *Boesenbergia*, including support for two clades comparable to the rows of Loesner (1930). However, the unresolved placement of *B. purpureorubra* and *B. siamensis*, in addition to the weak support for monophyly and the unresolved sister genus relation, does not warrant a new infrageneric classification at this time.

CONCLUSIONS

This study summarizes a decade of research on the systematics and taxonomy of *Boesenbergia* in molecular phylogenetic framework investigating the relations and evolution of morphological characters in the genus. The molecular phylogeny is largely congruent with the cytogenetic and morphological data. The phylogenetic framework confirms the previously identified clades I and II (Mood et al. 2019, 2020) and the placement of the included accessions within those clades. These clades share characters uniting all taxa in *Boesenbergia*, but also have unique or mostly unique characters that are clade specific. In addition, the phylogeny identifies two species, *B. purpureorubra* and *B. siamensis*, that are hard to place within these clades based on either molecular or morphological data, although the morphological data points to a stronger affiliation with clade I. Although significant progress has been made, four key questions warrant further study: the infrageneric relations of *Boesenber-*

gia, the monophyly of the genus, the sister genus relationship, and the placement of *B. purpureorubra* and *B. siamensis*.

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Appendix 1   Species names, specimen voucher information, (Index Herbariorum herbarium code; Thiers continuously updated), and GenBank accession numbers per molecular marker. Newly generated sequences are marked with an asterisk (*). Missing data is indicated with a dash (–).

| Species name	Voucher - IH Herbarium	GenBank accession no. ITS	trnK
Aframomum daniellii (Hook.f.) K.Schum.	*W.J. Kress 99-6375* (US)	AF478705	AF478805
Alpinia pumila Hook.f.	*W.J. Kress 97-6119* (US)	AF478719	AF478819
Amomum kerbyi (R.M.Sm.) Škorničk. & Hlavatá	*W.J. Kress 96-5746* (US)	AF478746	AF478845
Boesenbergia alba (K.Larsen & R.M.Sm.) Mood & L.M.Prince	*H. Funakoshi s.n.* (MBK)	KF982793	KF982802
Boesenbergia albomaculata S.Q.Tong (1)	*J. Mood M14C10* (BKF)	*MN803339	*MN803381
Boesenbergia albomaculata S.Q.Tong (2)	*J. Murata et al. 20050595K* (BKF)	KU159397	KU159408
Boesenbergia albosanguinea (Ridl.) Loes.	*J. Mood 3291* (BKF)	*MN803340	*MN803382
Boesenbergia angustifolia (Hallier f.) Schltr.	*J. Mood 3448* (SAR)	*MN803341	*MN803383
Boesenbergia armeniaca Cowley	*J. Mood 430* (AAU)	*MN803342	*MN803384
Boesenbergia aurantiaca R.M.Sm.	*J. Mood 524* (AAU)	*MN803343	*MN803385
Boesenbergia baimaii Saensouk & K.Larsen	*J. Mood 3275* (BKF)	–	*MN803386
Boesenbergia basispicata K.Larsen ex Sirirugsa	*J. Mood 3134* (BKF)	*MN803344	*MN803387
Boesenbergia bella Mood & L.M.Prince	*J. Mood 16P07* (BKF)	KY701326	KY701339
Boesenbergia burttiana R.M.Sm.	*J. Mood 3410* (SAR)	*MN803345	*MN803388
Boesenbergia burttii (K.Larsen & Jenjitt.) Mood & L.M.Prince	*H. Funakoshi s.n.* (MBK)	KF982794	KF982803
Boesenbergia clivalis (Ridl.) Schltr. (1)	*L.C. Keat s.n.* (UPM)	KU159398	KU159409
Boesenbergia clivalis (Ridl.) Schltr. (2)	*Sam Yen Yen s.n.* (KEP)	*MN803346	*MN803389
Boesenbergia collinsii Mood & L.M.Prince	*J. Mood 06P14* (BK)	JX992751	JX992812
Boesenbergia curtisii (Baker) Schltr.	*J. Mood 1739* (BISH)	KF982795	KF982804
Boesenbergia flabellata S.Sakai & Nagam.	*S. Sakai 378* (KYO)	AB097226	JF715475
Boesenbergia flava Holttum	*Sam Yen Yen s.n.* (KEP)	*MN803347	*MN803390
Boesenbergia hamiltonii Mood, S.Dey & L.M.Prince	*J. Mood & S. Dey 3017* (CAL)	JX992754	JX992815
Boesenbergia hirta (Ridl.) Merr.	*S. Sakai 366* (KYO)	AB097227	JF715476
Boesenbergia kerrii Mood, L.M.Prince & Triboun	*J. Mood & P. Triboun 2044* (BK)	JX992756	JX992817
Boesenbergia kingii Mood & L.M.Prince	*J. Mood & P. Vatcharakorn 3074* (BK)	JX992792	JX992829
Boesenbergia lambirensis S.Sakai & Nagam.	*S. Sakai 367* (KYO)	AB097224	JF715477
Boesenbergia latongensis Meekiong, Ipor & Ibrahim	*Ooi Im Hin 138* (SAR)	*MN803348	*MN803391
Boesenbergia loerzingii (Valeton) K.Larsen ex M.F.Newman, Lhuillier & A.D.Poulsen	*J. Mood 3446* (KRB)	*MN803349	*MN803392
Boesenbergia longiflora (Wall.) Kuntze	*W.J. Kress 03-7305* (US)	JX992797	JX992831
Boesenbergia longipes (King & Prain ex Ridl.) Schltr.	*J. Mood L12170 T38* (QBG)	*MN803350	*MN803393
Boesenbergia macropoda Merr.	*H. Funakoshi s.n.* (MBK)	*MN803351	*MN803394
Boesenbergia magna (R.M.Sm.) Veldkamp & Mood	*H. Funakoshi s.n.* (MBK)	KU291111	KU291118
Boesenbergia maxwellii Mood, L.M.Prince & Triboun	*J. Mood & P. Triboun 2032* (BK)	JX992800	JX992833
Boesenbergia minor (Baker) Kuntze	*Lim Chong Keat s.n.* (KEP)	*MN803352	*MN803395
Boesenbergia ochroleuca (Ridl.) Schltr.	*J. Mood & P. Vatcharakorn 3116* (BKF)	KU159399	KU159410
Boesenbergia oligosperma (K.Schum.) R.M.Sm.	*P.C. Boyce ZI-847* (SAR)	*MN803353	*MN803396
Boesenbergia orbiculata R.M.Sm.	*P.C. Boyce ZI-1124* (SAR)	*MN803354	*MN803397
Boesenbergia parva (Ridl.) Merr.	*S. Sakai 379* (KYO)	AB097225	JF715478
Boesenbergia parvula (Wall. ex Baker) Kuntze var. *major* Mood & Veldkamp	*J. Mood & P. Vatcharakorn 3100* (BKF)	KX425625	KX432259
Boesenbergia parvula (Wall. ex Baker) Kuntze var. *parvula* (Wall. ex Baker) Kuntze	*J. Mood & P. Chalermglin 3460* (BKF)	KX425638	KX432273
Boesenbergia pauciflora (R.M.Sm.) Veldkamp & Mood	*M.F. Newman 2459* (E)	KU291110	KU291117
Boesenbergia petiolata Sirirugsa	*J. Mood M96P82* (BKF)	KY701330	KY701343
Boesenbergia phengklaii Mood	*J. Mood 16P08* (BKF)	KY701324	KY701337
Boesenbergia plicata (Ridl.) Holttum var. *lurida* (Ridl.) Holttum (1)	*J. Mood & P. Vatcharakorn 3120* (BK)	JX992808	JX992839
Boesenbergia plicata (Ridl.) Holttum var. *lurida* (Ridl.) Holttum (2)	*J. Mood 3379* (BKF)	*MN803355	*MN803398
Boesenbergia plicata (Ridl.) Holttum var. *plicata* (Ridl.) Holttum (1)	*J. Mood & P. Vatcharakorn 3177* (BK)	JX992807	JX992840
Boesenbergia plicata (Ridl.) Holttum var. *plicata* (Ridl.) Holttum (2)	*J. Mood 3102* (BKF)	*MN803356	*MN803399
Boesenbergia prainiana (King ex Baker) Schltr.	*J. Mood 3395* (BKF)	*MN803357	*MN803400
Boesenbergia pulchella (Ridl.) Merr. (1)	*Ooi Im Hin 502* (SAR)	*MN803358	*MN803401
Boesenbergia pulchella (Ridl.) Merr. (2)	*J. Mood 3450* (KRB)	*MN803359	*MN803402
Boesenbergia pulcherrima (Wall.) Kuntze	*J. Mood 3363* (BKF)	*MN803360	*MN803403
Boesenbergia purpureorubra Mood & L.M.Prince	*J. Mood M14P19* (BKF)	*MN803361	*MN803404
Boesenbergia putiana Mood & L.M.Prince	*J. Mood 16P09* (BKF)	KY701325	KY701338
Boesenbergia regalis Kharuk. & Tohdam	*Lim Chong Keat s.n.* (KEP)	*MN803363	*MN803406
Boesenbergia aff. *regalis* Kharuk. & Tohdam	*W.J. Kress 99-6334* (US)	*MN803362	*MN803405
Boesenbergia rotunda (L.) Mansf. (1)	*J. Mood 1764* (BKF)	KY701331	KY701344
Boesenbergia rotunda (L.) Mansf. (2)	*J. Mood 3358* (BKF)	–	*MN803407
Boesenbergia siamensis (Gagnep.) Sirirugsa	*J. Mood 3078* (BKF)	*MN803364	*MN803408
Boesenbergia siphonantha (King ex Baker) M.Sabu, Prasanthk. & Škorničk.	*J. Mood & P. Chalermglin 2056* (BKF)	KU159401	KU159412
Boesenbergia sp. Borneo	*J. Mood 1123* (AAU)	*MN803366	*MN803410
Boesenbergia sp. Sarawak (1)	*P.C. Boyce ZI-779* (SAR)	*MN803365	*MN803409
Boesenbergia sp. Sarawak (2)	*P.C. Boyce s.n.* (SAR)	*MN803367	*MN803411
Boesenbergia tenuispicata K.Larsen (1)	*J. Mood 3394* (BKF)	*MN803368	*MN803412
Boesenbergia tenuispicata K.Larsen (2)	*J. Mood 3292* (BKF)	KU159405	KU159416
Boesenbergia tiliifolia (Baker) Kuntze	*Vinitha et al. 130869* (CUH)	KJ872043	KJ872372
Boesenbergia trangensis K.Larsen	*J. Mood 3382* (BKF)	*MN803369	*MN803413
Boesenbergia uniflora (K.Schum.) Ardiyani & Mood	*J. Mood & Ooi Im Hin 3415* (SAR)	KU291112	KU291119
Boesenbergia violacea (K.Larsen & Triboun) Mood & L.M.Prince	*H. Funakoshi s.n.* (BK)	KF982796	KF982805
Boesenbergia xiphostachya (Gagnep.) Loes. (1)	*J. Mood 3276* (BKF)	*MN803370	*MN803414
Boesenbergia xiphostachya (Gagnep.) Loes. (2)	*J. Mood 11C86* (BKF)	KX425643	KX432277
Borneocola reticosus (Ridl.) Y.Y.Sam	*P.C. Boyce & K. Jeland ZI-139* (SAR)	KY492406	KY492441
Burbidgea schizocheila Hackett	*L86-0649* (BISH)	*MN803330	*MN803371
Cautleya gracilis (Sm.) Dandy	*W.J. Kress 99-6361* (US)	AF478734	AF478833
Caulokaempferia linearis (Wall.) K.Larsen	*S. Dey NU198* (ASSAM)	KF982798	KF982807
Cornukaempferia aurantiiflora Mood & K.Larsen	*J. Mood M96P24* (AAU)	*MN803337	*MN803378
Cornukaempferia longipetiolata Mood & K.Larsen	*J. Mood M97P139* (AAU)	–	*MN803379

Appendix 1 (cont.)

Species name	Voucher - IH Herbarium	GenBank accession no.	
		ITS	trnK
Cornukaempferia sp.	*J. Mood M06P06* (BKF)	*MN803338	*MN803380
Curcuma bicolor Mood & K.Larsen	*W.J. Kress 99-6516* (US)	AF478737	AF478837
Distichochlamys rubrostriata W.J.Kress & Rehse	*W.J. Kress 01-6848* (US)	AF478745	AF478844
Elettaria cardamomum (L.) Maton	*J. Mood 2324* (BISH)	*MN803332	*MN803373
Gagnepainia harmandii (Baill.) K.Schum.	*K.J. Williams 00-199* (DUKE)	AF478752	AF478851
Geocharis fusiformis (Ridl.) R.M.Sm.	*J. Mood 1317* (BISH)	*MN803331	*MN803372
Globba spathulata Roxb.	*Hoque Mant-1* (US)	AF478769	AF478870
Haniffia albiflora K.Larsen & Mood	*W.J. Kress 99-6370* (US)	AF478756	AF478855
Haniffia flavescens Y.Y.Sam & Julius	*Julius, Sam & Kueh FRIM 57598* (E)	*MN803335	*MN803376
Haniffia santubongensis S.Y.Wong & P.C.Boyce	*P.C. Boyce & S.Y. Wong ZI-22* (SAR)	*MN803336	*MN803377
Hedychium longicornutum Griff. ex Baker	*W.J. Kress 00-6703* (US)	AF478761	AF478860
Hemiorchis rhodorrhachis K.Schum.	*M.F. Newman 861* (E)	AF478763	AF478863
Hornstedtia hainanensis T.L.Wu & S.J.Chen	*W.J. Kress 97-5769* (US)	AF478766	AF478865
Kaempferia galanga L.	*W.J. Kress 99-6304* (US)	AF478767	AF478868
Kaempferia parviflora Wall. ex Baker	*W.J. Kress 97-6012* (US)	KY701332	AF478866
Kaempferia pulchra Ridl.	*W.J. Kress 94-5371* (US)	KY701333	AF478867
Kedhalia flaviflora C.K.Lim	*C.K. Lim L7995* (SING)	JF825535	JF825540
Larsenianthus careyanus (Benth. & Hook.f.) W.J.Kress & Mood	*W.J. Kress 03-7403* (US)	HM771393	HM771405
Myxochlamys nobilis Nagam. ined.	*H. Nagamasu 8274* (KYO)	AB552948	AB553310
Newmania serpens N.S.Lý & Škorničk.	*Lý 332* (VNM)	JF825536	JF825541
Pleuranthodium schlechteri (K.Schum.) R.M.Sm.	*W.J. Kress 00-6724* (US)	AF478775	AF478876
Pommereschea lackneri Witte	*W.J. Kress 00-6739* (US)	AF478776	AF478877
Renealmia alpinia (Rottb.) Maas	*W.J. Kress 99-6407* (US)	AF478778	AF478879
Rhynchanthus beesianus W.W.Sm.	*W.J. Kress 97-5827* (US)	AF478784	AF478885
Roscoea purpurea Sm.	*W.J. Kress 01-6953* (US)	AF478787	AF478888
Scaphochlamys biloba (Ridl.) Holttum	*W.J. Kress 99-6576* (US)	AF478788	AF478889
Scaphochlamys kunstleri (Baker) Holttum	*W.J. Kress 99-6539* (US)	AF478789	AF478890
Siamanthus siliquosus K.Larsen & Mood	*W.J. Kress 99-6349* (US)	AF478790	AF478891
Siliquamomum tonkinense Baill.	*W.J. Kress 00-6802* (US)	AF478791	AF478892
Siphonochilus aethiopicus (Schweinf.) B.L.Burtt	*GH00-134* (Living collection only)	AF478792	AF478893
Tamijia flagellaris S.Sakai & Nagam.	*Kazuyuki S55* (KYO)	AF478797	AF478898
Zingiber acuminatum Valeton	*A.D. Poulsen 2443* (AAU)	*MN803333	*MN803374
Zingiber gramineum Noronha ex Blume	*W.J. Kress 96-5739* (US)	AF478800	AF478902
Zingiber loerzingii Valeton	*A.D. Poulsen 2451* (AAU)	*MN803334	*MN803375

— 0.01 length units
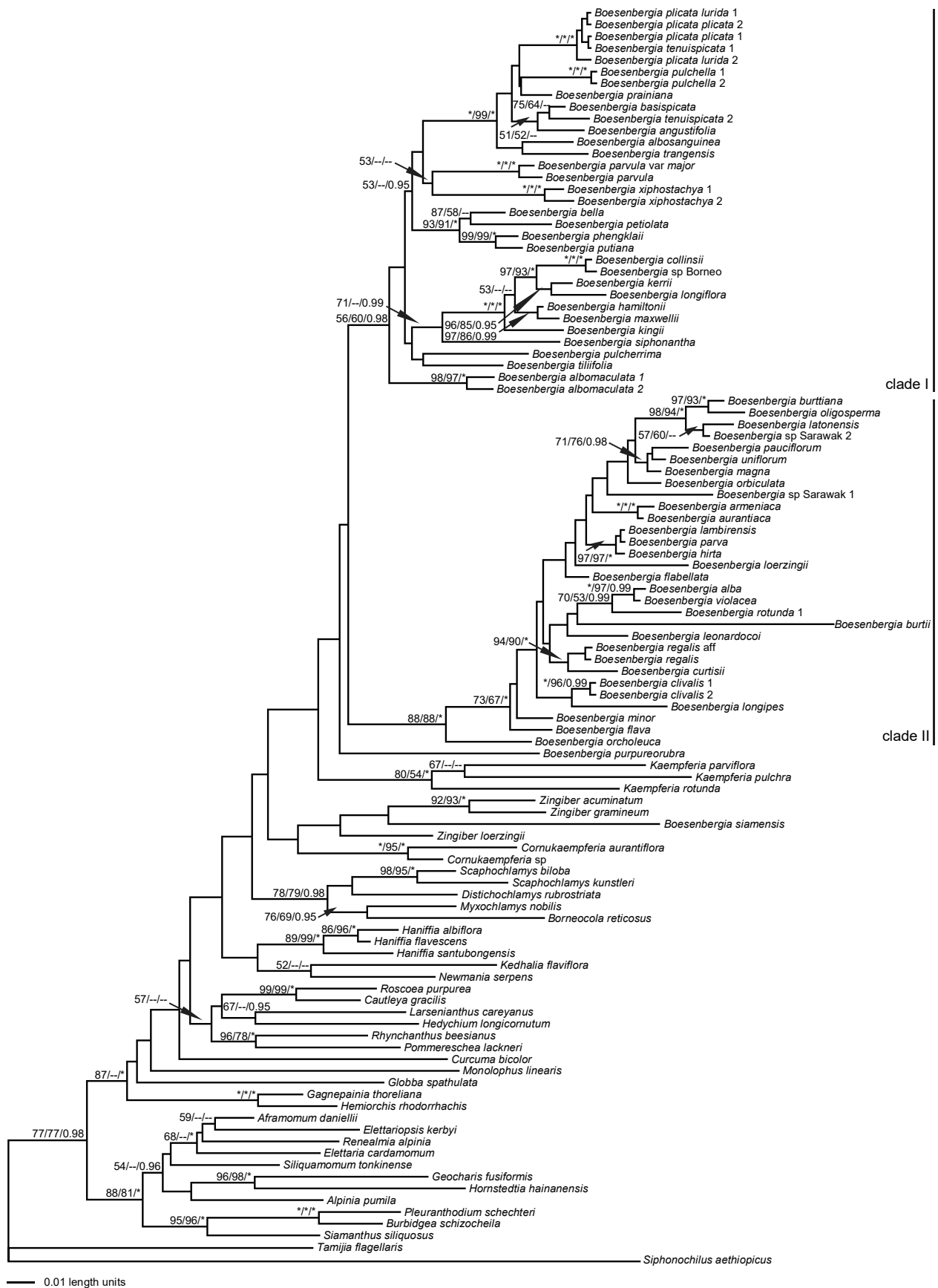
Appendix 2 Bayesian maximum clade credibility tree of *Boesenbergia* for nrITS data showing support values for maximum likelihood (1st, bootstrap values > 50 % shown), maximum parsimony (2nd, bootstrap values > 50 % shown), and Bayesian inference (3rd, posterior probabilities > 0.95 shown, 1.00 shown as *).
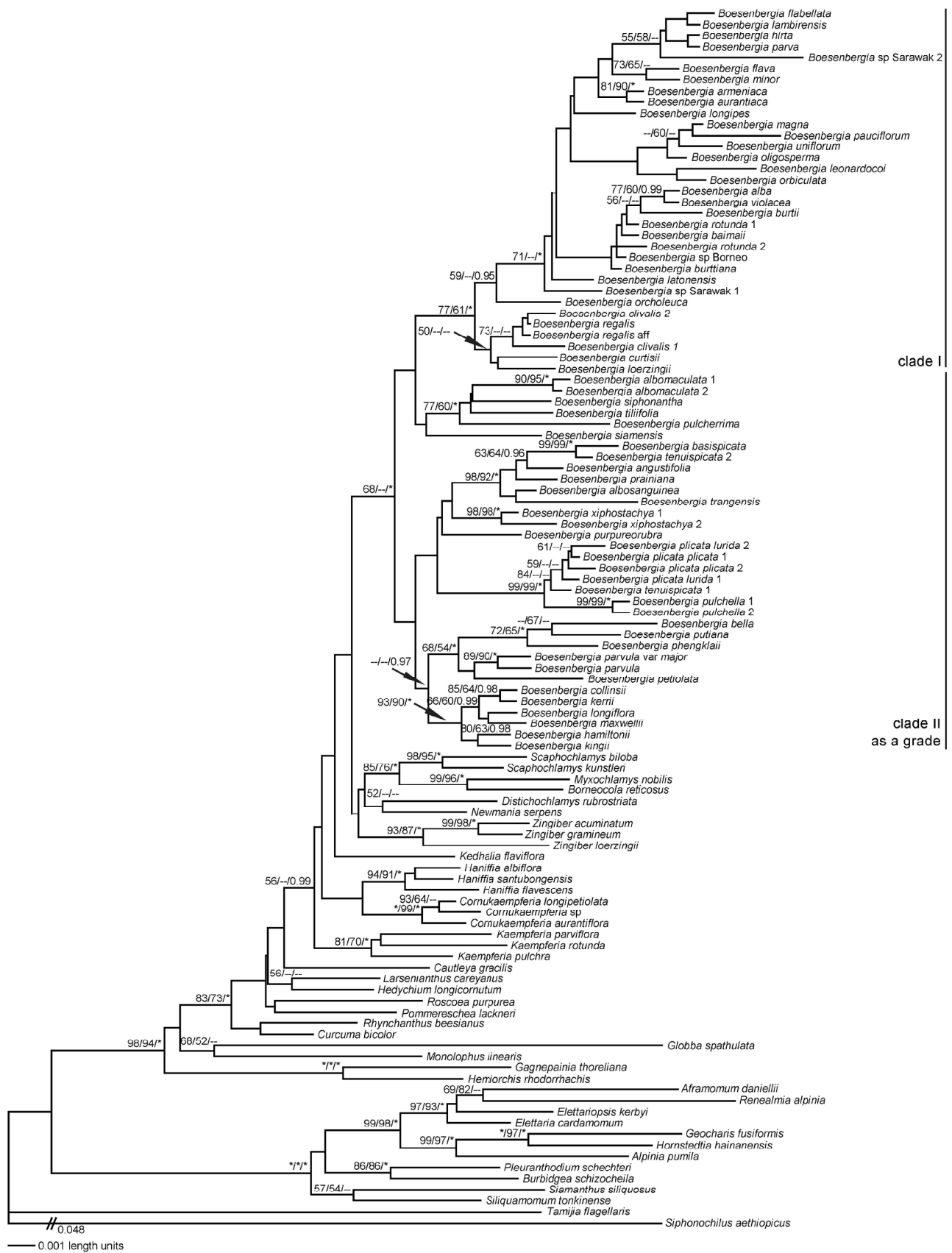
Appendix 3 Bayesian maximum clade credibility tree of *Boesenbergia* for chloroplast trnK data showing support values for maximum likelihood (1st, bootstrap values > 50 % shown), maximum parsimony (2nd, bootstrap values > 50 % shown), and Bayesian inference (3rd, posterior probabilities > 0.95 shown, 1.00 shown as *). The branch to *Siphonochilus* was trimmed, and the length is provided below the branch.
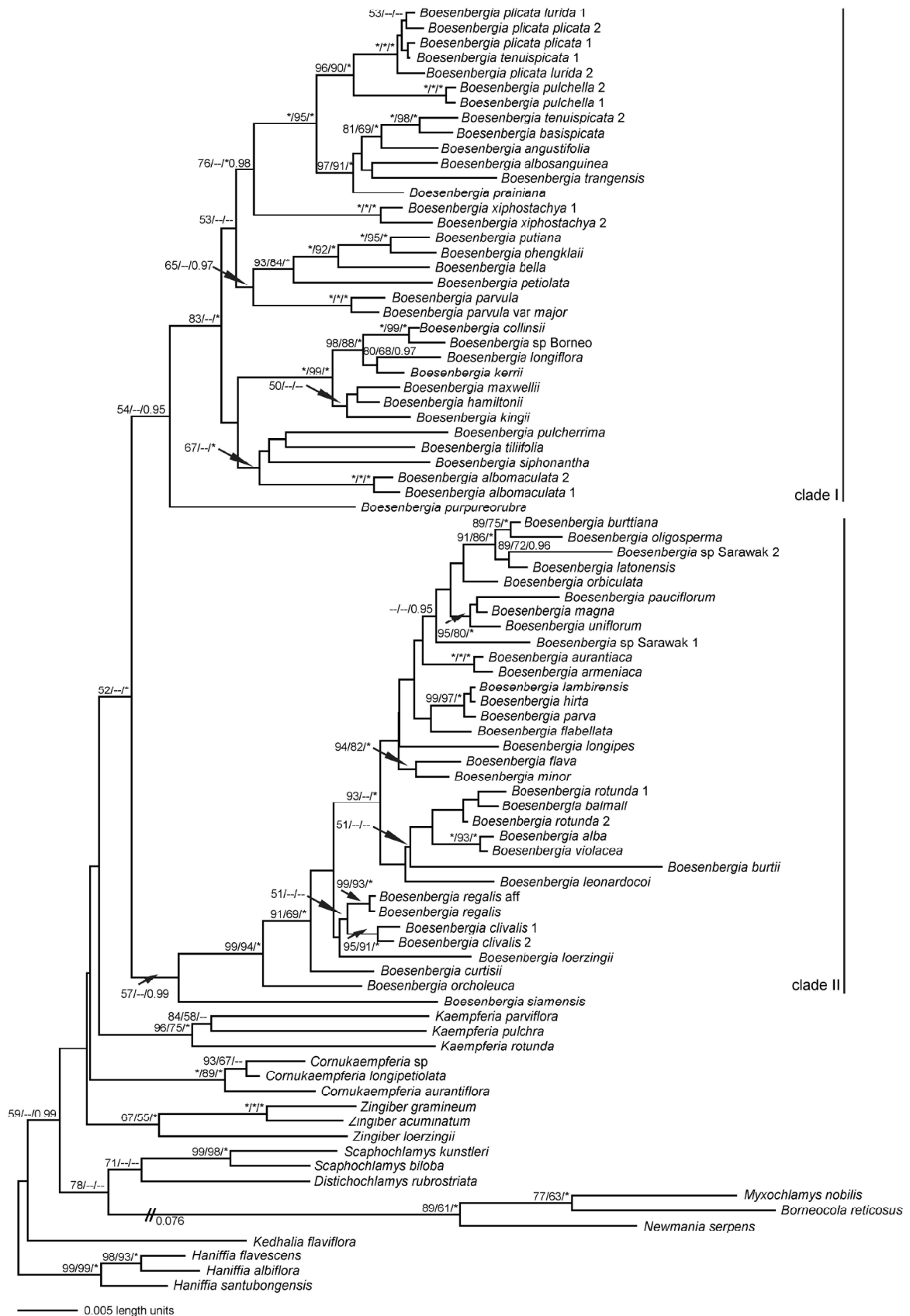
Appendix 4 Bayesian maximum clade credibility tree of *Boesenbergia* and closely related genera relationships for combined plastid trnK and nuclear ITS data. Numbers above branches show support values for maximum likelihood (1st, bootstrap values > 50 % shown), maximum parsimony (2nd, bootstrap values > 50 % shown), and Bayesian inference (3rd, posterior probabilities > 0.95 shown, 1.00 shown as *). The branch to *Myxochalmys + Borneocola + Newmania* was trimmed, and the length is provided below the branch.