Evolution of leaf nodulation in Myrsinoideae (Primulaceae)

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GENERAL INTRODUCTION

INTRODUCTION

Symbiotic association between prokaryotes and eukaryotes have been reported across many taxa and their association are vary from temporary to permanent (e.g. Paracer & Ahmadjian 2000). The latter association can be highly specific interaction between the partners especially when the prokaryotic symbionts live within the host. This highly symbiotic interaction is considered obligate because both are depending to each other to survive. In many cases of highly specific microbial interaction such as in insects (e.g. Bauman 2005) and plants (e.g. Perkins & Peters 1993, Miller 1990), the symbionts are primarily maternally transmitted (e.g. Gregory & Crystal 2015) and rarely with mixed mode transmission (e.g. Lemaire et al. 2012). The maternally transmitted symbionts are maintained throughout the host generations in a closed manner and considered to have a long-term symbiotic association (e.g. Miller 1990). In plants, symbiotic association with obligate and maternal inherited symbionts are less documented and so far, reported only in the association between aquatic fern *Azolla* Lam. and cyanobacteria *Nostoc* Vaucher ex Bornet & Flahault, and the bacterial leaf nodulation. However, the latter is unique and differ from the former association by the formation of bacterial nodules along the leaf margin.

The leaf nodulation has been documented in approximately 500 species in the family of Rubiaceae and the subfamily Myrsinoideae of Primulaceae. The nodulated species are found mostly in the Rubiaceae primarily in the three distantly related genera *Pavetta* L. (c. 360 spp.: De Block 2015), *Psychotria* L. (c. 80 spp.; Anderson 2002) and *Sericanthe* Robbr. (c. 11 spp.: Lemaire et al. 2011c). Myrsinoideae, in contrast with Rubiaceae, the leaf nodulation is very rare because the nodulated species are found in about 70 species of *Ardisia* Sw. subgenus *Crispardisia* Mez (see Mez 1902, Sleumer 1988, Stone 1989, Larsen & Hu

1996, Chen & Pipoly 1996, Hu & Vidal 2004), four species in *Amblyanthus* A. DC. and a single species in *Amblyanthopsis* Mez.

However, previous studies on bacterial leaf nodule symbiosis for Myrsinoideae are emphasizing only on *Ardisia* subgenus *Crispardisia* (Lemaire et al. 2011a, Ku & Hu 2014). This is because, *Ardisia* is the largest genus consisting over 1000 species and one of the largest genera in tropical plants (Frodin 2004) within Primulaceae (Stevens 2001 onwards). Moreover, the genus is found predominantly in the understory of moist tropical and subtropical rain forests. Hence, materials for the genus is easily to obtain. For the other two nodulated genera, both are recorded so far from Assam, India and hardly to obtain the materials. Therefore, the presence of bacteria inside the leaf nodules of *Amblyanthopsis* and *Amblyanthus* could not be ascertain.

Although the presence of bacterial symbiont, which was identified as *Burkholderia* Yabuuchi et al. (Yabuuchi et al. 1992) has been studied in the leaf nodulated taxa of subgenus *Crispardisia* but majority of the subgenus members have not been examined. Moreover, only East Asian samples were used to investigate the bacterial leaf nodule symbiosis within the family. Whether the other two nodulated genera were also colonized by *Burkholderia* endosymbionts remain to be identified.

In Myrsinoideae, the recent molecular studies (Lemaire et al.2011a, Ku & Hu 2014) have proven the presence of bacterial symbiont in the leaf nodule, shoot tips, seeds and ovary and these observations agree with previous morphological studies in nodulated taxa of *Ardisia* (e.g. Miller & Donnelly 1987, Miller et al. 1983, Miller 1990). Therefore, bacterial leaf nodule symbiosis in this subfamily was hypothesized to be cyclic, in which the presence of obligate symbionts is vital for the growth and survival of the host. Removal of bacterial symbionts causes abnormal growth and degeneration of the apical meristem into a callus, and finally ceases growth of host plants (reviewed in Miller 1990: see Discussion). Apart from

that, the host-symbionts association in this subfamily also have proven to show high host specificity with one-to-one interaction because the host plant harbors a single bacterium (Lemaire et al. 2011a, Ku & Hu 2014). As consequent, the cyclic and highly specific interaction suggested both partners are co-diversified together, and thus phylogenetic tree of symbionts are expected to mirror host tree. However, this hypothesis is partly confirmed only on limited samples of East Asian *Ardisia* (Ku & Hu 2014).

Previous phylogenetic analyses (Ku & Hu 2014) showed that nodulated taxa of *Ardisia* form a monophyletic group and so their bacterial symbionts also do. These results reveal that the leaf nodulation has evolved only once and suggesting a single origin of leaf nodulation throughout the history of symbiotic association between *Ardisia* and its bacterial symbionts. However, emphasizing only on *Ardisia* could not reflect the total picture of evolution of leaf nodulation in Myrsinoideae. Hence, to clarify further evolution of leaf nodulation within this subfamily, current study will include representative species of the other two nodulated genera *Amblyanthus* and *Amblyanthopsis*, which were found and collected from Myanmar recently, as well as the other nodulated taxa of *Ardisia*.

Before it can be assessed the evolution of leaf nodulation within Myrsinoideae, a robust phylogenetic tree needs to be obtained for both hosts and their endosymbionts. For the host, as a first step, it is important to test the monophyly of all three nodulated genera within and among their close relatives. This is because their monophyletic status has never been tested and it is significant to know whether all nodulated genera form monophyletic group or are derived independently within the tree. The information inferred from the host tree would be useful to suggest either the leaf nodulation evolved only once or multiple times within Myrsinoideae. Similarly, the inferred tree of leaf nodulating endosymbionts of Myrsinoideae and Rubiaceae, and other related *Burkholderia* would provide clue to the origin and leaf-nodulating bacteria. A single or multiple occurrence of symbiotic association within

Myrsinoideae can be reveal by comparing the inferred trees of host plants and their endosymbionts. Therefore, the first two chapters in this dissertation will be dedicated on phylogenetic reconstruction of host plant and their endosymbionts, respectively. As for the detection and identification of bacterial symbionts using independent molecular technique (Daniel et al. 2003, Ranjard et al. 2000) will be provided in the second chapter. While information on evolutionary relationship between the endosymbionts and their host will be discuss in the last chapter.

THE DISSERTATION OVERVIEWS

Towards understanding the evolution of leaf nodulation in Myrsinoideae at first will need to obtain a fundamental information such as phylogenetic delimitation of all three nodulated genera and confirmation the presence of symbiotic bacteria in representative nodulated material used in this study.

Therefore, in Chapter 1, a preliminary phylogenetic relationship at generic and subgeneric level of *Ardisia* with related genera will be generated using a single nuclear ribosomal ITS marker. The reconstructed phylogenetic tree will provide the present state of understanding on generic and subgeneric delimitation of *Ardisia* and related genera. Then, the obtained result will be used to infer the phylogenetic status of *Ardisia* particularly. However, the resulted tree may provide insufficient data to infer their relationship including phylogenetic status of all nodulated genera using a single marker.

Hence, Chapter 2 will address the phylogenetic relationship of all three known nodulated genera. A combination of chloroplast *rpL*32-*trn*L and nrITS markers will be used in the analysis to obtain better phylogenetic resolution. On the other hands, phylogenetic trees of endosymbionts will be produced by using four housekeeping genes *atp*D, *gyr*B, *lep*A and *rec*A. Obtaining both trees of host plants and their endosymbionts would provide more

information to infer the occurrence of leaf nodulation in Myrsinoideae either single or multiple origins. Additionally, the culture-independent molecular technique approach will be applied in this study for endosymbionts identification especially that associated with nodulated genera *Amblyanthopsis* and *Amblyanthus*.

Finally, Chapter 3 deals with the understanding of coevolutionary relationship between the endosymbionts and their host plants. To do this, the cophylogenetic analysis will using Indo-Chinese and Malesian nodulated *Ardisia* samples together with the genus *Amblyanthopsis* based on topology- or event-based method JANE 4.0 and TreeMap v3.0β. The reconstructed cophylogenetic analysis will certainly provide additional information to evaluate further the hypothesis of strict-co-speciation in coevolutionary relationship of Myrsinoideae as speculated from previous studies.

REFERENCES

Anderson L. 2002. Relationships and generic circumscriptions in the *Psychotria* complex (Rubiaceae, Psychotrieae). Syst. Geogr. Plants **72**: 167–202.

Bauman P. 2005. Biology bacteriocyte-associated endosymbionts in animals. Curr. Opin. Microbiol. **3**: 270–275.

Chen J. and Pipoly J.J. 1996. Myrsinaceae. *In*: Wu, Raven P.H. (eds.) Fl China. Science, Beijing. **15**: 1–38.

Daniel N.F., Spiegelman G.B., Davis W., Wagner E., Lyons E. and Pace N.R. 2003. Culture-Independent Molecular Analysis of Microbial Constituents of the Healthy Human Outer Ear. J. Clin. Microbiol. **41** (1): 295–303.

De Block P., Razafimandimbison S.G., Janssens S., Ochoterena H., Robbrectht E. and Bremer B. 2015. Molecular phylogenetics and generic assessment in the tribe Pavetteae (Rubiaceae). Taxon **64** (1): 79–95.

Frodin D.G. 2004. History and concepts of big plant genera. Taxon **53** (3): 753–776.

Gregory D.D.H. and Crystal L.F. 2015. Reproductive parasitism: maternally inherited symbionts in a biparental world. Cold Spring Harb. Perspec. Biol. **7**: a017699

Hu C.M. and Vidal J.E. 2004. Myrsinaceae. *In*: Morat, Ph. (ed.) Flore du Cambodge du Laos et de Viêtnam 32. 288 pp. Muséum National D'Histoire Naturelle, Paris.

Ku C. and Hu J.M. 2014. Phylogenetic and cophylogenetic analyses of the leaf-nodule symbiosis in *Ardisia* subgenus *Crispardisia* (Myrsinaceae): evidence from nuclear and chloroplast markers and bacterial *rrn* operons. International Journal of Plant Sciences **17** (1): 92–109.

Larsen K. and Hu C.M. 1996. Myrsinaceae. Fl. Thailand **6** (2): 81–178.

Lemaire B., Smets E. and Dessein S. 2011a. Bacterial leaf symbiosis in *Ardisia* (Myrsinoideae, Primulaceae): molecular evidence for host specificity. Res. Microbiol. **162**: 528–534.

Lemaire B., Vandamme P., Merckx V., Smets E. and Dessein S. 2011b. Bacterial Leaf Symbiosis in Angiosperms: Host Specificity without Co-Speciation. PLoS ONE **6** (9): e24430. Available from https://doi.org/10.1371/journal.pone.0024430.

Lemaire B., Oevelen S.V., De Block P., Verstraete B., Smets E., Prinsen E. and Dessein S. 2011c. Identification of the bacterial endosymbionts in leaf nodules of *Pavetta* (Rubiaceae). Int. J. Syst. Evol. Microbiol. **62**: 202–209. Available from doi:10.1111/j.1574-6941.2012. 01356.x

Lemaire B., Janssens S., Smets E. and Dessein S. 2012. Endosymbiont transmission mode in bacterial leaf nodulation as revealed by a population genetic study of *Psychotria leptophylla*. Appl. Environ. Microbiol. **78** (1): 284–287.

Mez C. 1902. Myrsinaceae. Pflanzenr. [Engler] **9**: 1–437.

Miller I.M., Gardner I.C. and Scott A. 1983. The development of marginal leaf nodules in *Ardisia crispa* (Thunb.) A. DC. (Myrsinaceae). Bot. J. Linean. Soc. **86**: 237–252.

Miller I.M. and Donnelly A.E. 1987. Location and distribution of symbiotic bacteria during floral development in *Ardisia crispa*. Plant Cell Environ. **10**: 715–724.

Miller I.M. 1990. Bacterial leaf nodule symbiosis. Adv. Bot. Res. **17**: 163–234.

Paracer S. and Ahmadjian V. 2000. Symbiosis: an introduction to biological associations: 2nd Edition. pp. 1–291. Oxford University Press, Inc., New York.

Perkins S.K. and Peters G.A. 1993. The *Azolla-Anabaena* Symbiosis-Endophytes Continuity in the *Azola* Life Cycle is Facilitated by Epidermal Trichomes.1. Partitioning of the Endophytic Anabaena into Developing Sporocarps. New Phytology **123**: 53–64.

Ranjard L., Poly F. and Nazaret S. 2000. Mini review: Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Res. Microbiol. **151** (3): 167–177.

Sleumer H. 1988c. A revision of the genus *Ardisia* Sw. (Myrsinaceae) in New Guinea. Blumea **33**: 115–140.

Stevens P.F. 2001 onwards. Angiosperm Phylogeny Website. Version 14, July 2017. http://www.mobot.org/MOBOT/research/APweb/.

Stone B.C. 1989. Myrsinaceae. *In*: Ng, F.S.P. (ed.) Tree Flora of Malaya 4. pp. 264–284. Longman, Malaysia.

CHAPTER 1

Phylogenetic study of tropical Asian *Ardisia* and related genera: generic and subgeneric delimitation

ABSTRACT

The most comprehensive phylogenetic analysis of the large tropical genus *Ardisia* (Primulaceae) and related genera based on nucleotide sequences of the nuclear ribosomal internal transcribed spacer (nrITS) region from 187 samples predominantly from East Asia, Indo-China and Malesia was presented. Bayesian and maximum likelihood analyses showed that *Ardisia* was divided into two major clades. Furthermore, *Ardisia* was not monophyletic because at least *Hymenandra* was nested in one major clade. Additionally, *Discocalyx* and *Hymenandra* were also another non-monophyletic genus. *Discocalyx* was split into two groups within Clade II, with one species forming a strongly supported clade with *Fittingia* and two species related to the highly supported clade of *Badula* and *Oncostemum*. Two species of *Hymenandra* formed a clade with *Ardisia* subgenus *Pyrgus* in Clade I, but these species were not closely related. While the nodulated genera *Amblyanthopsis*, *Amblyanthus* and *Ardisia* subgenus *Crispardisia*, all were fall into Clade I and shows non-monophyletic relationship. The phylogenetic tree also indicated that the traditional subgeneric circumscription only partially reflected the phylogenetic relationship within *Ardisia*. The results clearly suggested the need for revising the generic and subgeneric circumscription of *Ardisia* and its relatives.

1.0 INTRODUCTION

Ardisia Sw. is the largest genus within Primulaceae and one of the largest tropical genera with estimates of species diversity from c. 450 to over 1000 species (Stevens 2001 onwards, Frodin 2004). The genus is found predominantly in the understorey of moist tropical and subtropical rain forests, but with some species attaining up to 40 m and reaching the canopy (e.g., *Ar. copelandii* Mez in northern Borneo). Traditionally, the genus was placed in the Myrsinaceae, which is now treated as subfamily Myrsinoideae within an enlarged Primulaceae (e.g., APG III 2009). According to Mez (1902), who proposed one of the most comprehensive classification systems of Myrsinoideae (Mez 1902: as the Myrsinaceae, excluding the herbaceous, traditional Primulaceae), *Ardisia* is placed in the tribe Ardisieae with four genera, while the other tribe Myrsineae consists of 26 genera. The Ardisieae were defined mainly by the fewer to many ovules arranged in several series in the placenta. These Myrsinoid genera were distinguished by habit, ovule number and arrangement, floral merosity, floral sexuality, corolla lobes aestivation and the anther connation (Table 1.0a); however, uncertainties were raised on the classification of *Ardisia* and relatives in later studies. For examples, *Tetrardisia* Mez in the tribe Myrsineae was reduced into a subgenus of *Ardisia* by Larsen & Hu (1994) based on the less significant diagnostic character in ovule numbers. The genus *Afrardisia* Mez is now treated within *Ardisia* because the former is also characterized by axillary inflorescences but has been previously considered as a distinct genus (Taton 1979). Sleumer (1988) questioned the generic delimitation of several related genera defined only by sexual differences. *Sadiria* Mez, which was split from the genus *Pimelandra* by Mez (1902), has been suggested a close relationship to *Ardisia* and differentiated only by a single character, the corolla united above the middle (Hu & Deng 2012). Based on these observations, the taxonomic status of *Ardisia* and its related genera

might not be clear, however, the assumption and their classification has not been molecular phylogenetically tested.

Uncertainties have been seen also for the subgeneric classification within *Ardisia.* Mez (1902) circumscribed 14 subgenera in the genus based on characteristics of habit, leaf morphology, inflorescence position and floral morphology (Table 1.0b), but subsequent studies have raised questions to the subgeneric classification of *Ardisia*. For example, subgenus *Bladhia* (Thunb.) Mez is readily distinguished by the monocaul, herbaceous or suffrutescent and low creeping habit (rarely reaching up to 3 m as in *Ar. gigantifolia* Stapf) with subopposite or subverticillate leaves along the stem (e.g. Julius & Utteridge 2012, Yang & Dwyer 1989). This feature, however, is attributed also to subgenus *Pyrgus* (Lour.) Mez, especially the leaf arrangement along the stem. The main obvious difference between these two is that the latter has specialized lateral branches with terminal inflorescences in between the subverticillate leaves, whereas the former does not. Regarding the character of inflorescence position, the subgenus *Pimelandra* (A. DC.) Mez is quite distinct by having axillary inflorescences along the lateral branches. Of the subgenera with terminal inflorescences, some have unique characters to easily define them, such as the leaf bacterial nodules in subgenus *Crispardisia* Mez, but several, including *Acrardisia* Mez, *Stylardisia* Mez and *Tinopsis* Mez, are difficult to delimit, and correctly assign species to subgenera without mature flower buds or when in fruit. This is especially true when attempting to distinguish between members of subgenera *Stylardisia* and *Acrardisia*, with the former differentiated only from the latter in having the style exserted from the flower bud prior to anthesis. Likewise, subgenera *Akosmos* Mez and *Tinus* (Burm.) Mez have inflorescences that are referred to as axillary (see Stone 1989) or lateral (see Larsen & Hu 1996) in the upper part of normal leafy branches. To distinguish them further, the morphology of the calyx is required: either the lobes spreading during anthesis as in subgenus *Akosmos* or distinctly

imbricate as in subgenus *Tinus*. For subgenus *Crispardisia*, its monophyly has been confirmed using several East Asian samples (Ku & Hu 2014), but its relationship with majority nodulated taxa of *Ardisia* and another nodulated genera *Amblyanthopsis* Mez and *Amblyanthus* A. DC. have not been examined to further test the phylogenetic status of this subgenus. Given the current circumscriptions, these subgenera have been hypothesized to be paraphyletic and polyphyletic (e.g. Stone 1989, 1990b), and, in addition, it is common to find authors struggling to assign a species to a subgenus with confidence, with several new species described with uncertain or tentative subgeneric placement that has been subsequently shown to be misplaced (e.g. Furtado 1959, Sleumer 1988b, Stone 1989).

In Malesia region, 17 out of 45 currently accepted genera in the subfamily *Myrsinoideae*, are recognized (Ståhl & Anderberg 2004), and nine subgenera of *Ardisia* present viz. *Acrardisia*, *Akosmos*, *Bladhia*, *Crispardisia, Pimelandra*, *Pyrgus*, *Stylardisia*, *Tinopsis* and *Tinus* (Table 1.0b, see Stone 1982). Thus, Malesia region provides an ideal testing ground for assessing problems of generic and subgeneric delimitation in the subfamily Myrsinoideae because it retains the largest diversity of subgenera in Asia.

In molecular systematics, both chloroplast DNA (cpDNA) and nuclear ribosomal DNA (nrDNA) are widely employed for investigating taxonomic relationships within and among plant families (e.g. Baldwin et al. 1995, Shaw et al. 2007). Nuclear ribosomal internal transcribed spacer ITS (nrITS) is one of the most important regions that can potentially provide high polymorphism sites in contrast to chloroplast DNA (cpDNA). NrITS is therefore commonly used to compensate for the limitation of cpDNA besides obtaining an independent estimates phylogeny at both generic and species levels (e.g. Alvarez & Wendel 2003, Gruenstaeudl et al. 2009, Martin et al. 2003). In several plant groups, including Primulaceae, nrITS provides greater resolution and higher support of phylogenetic relationship when compared to those in an equivalent sample set of cpDNA sequences (e.g.,

Bone et al. 2012, Hao et al. 2004). The present study uses an nrITS dataset as the first step towards multigene and broader sampling in the Myrsinoideae concentrating on *Ardisia*. The aim of this study is to produce a preliminary phylogenetic tree of *Ardisia* and relatives to test the monophyly of *Ardisia*, examine the relationships of *Ardisia* and its related genera, and to evaluate the current subgeneric classification for *Ardisia* proposed by Mez (1902).

1.1 MATERIALS AND METHODS

TAXON SAMPLING

The sources of plant materials together with vouchers and accession numbers for all taxa subjected to phylogenetic analyses were listed in Table 1.1. The sampling strategy for the phylogenetic analysis is (1) to include representative species from as many subgenera of *Ardisia* and (2) to include as many species from associated genera in the Myrsinoideae from tropical Asia as possible. While for *Conandrium* and *Loheria*, no fresh leaf materials were available and the attempt to extract the DNA from herbarium specimens were not successful hence not included in the analysis. Similarly, obtaining the nrITS sequences for candidate species from subgenus *Tinopsis* and members of *Antistrophe* were unsuccessful; this study only had access to material from herbarium specimens for these two groups. This study also struggled to amplify the DNA using nrITS for some species including *Tapeinosperma* but unsuccessful. Sample identification was done at the Royal Botanic Gardens, Kew (K) and Royal Botanic Garden Edinburgh (E), using relevant literature (Furtado 1959, Hu 2002, Hu & Vidal 2004, Larsen & Hu 1996, Sleumer 1988a, 1988c, Stone 1989, 1990a, 1991), and comparison with herbarium specimens, especially types at K and E, as well as images of types from Global Plants JSTOR (http://plants. jstor.org/) and the BioPortal of Naturalis Biodiversity Center (http://bioportal.naturalis.nl/). During identification process some materials could not be identified down to a species level. Hence, for naming purposes, the abbreviation 'sp. vel aff." is applied to an unidentified material which has some affinity to a known species but it is not identical to it, for examples *Ardisia* sp. vel aff. *breviramea* Merr., *Ar*. sp. vel aff. *livida* Mez. and *Ar*. sp. vel aff. *vidalii* C.M. Hu. While the abbreviation 'cf.' is applied to the materials that most of the diagnostic characters correspond to a given species, but some characters are uncertain, for example *Ar*. cf. *amboinensis* Scheff., *Ar*. cf. *cadieri*

Guillaumin and *Ar*. cf. *tsangii* E. Walker. In *Afrardisia* and *Discocalyx* cases, the specimens could not be identified in comparison to any known species so far and, therefore, the abbreviation 'sp.' is applied after the genus name. Thus, a complete sampling for the nine Malesia subgenera and four relatives was not possible, but a total of 90 samples representing eight subgenera and 59 taxa of *Ardisia* (including ambiguous taxa), and other 14 samples representing eight genera were sequenced for the nrITS region as ingroup samples (Table 1.1). In addition, nine samples of *Embelia* Burm. *f*., *Labisia* Lindl. and *Myrsine* L. (Table 1.1) were used for sequencing as outgroup taxa based on their sister relationship to *Ardisia* and other ingroup taxa (Ståhl & Anderberg 2004). Additional sequences data for *Ardisia* (59 accessions), *Badula* Juss. (5 accessions), *Oncostemum* A. Juss. (7 accessions) *Sadiria* (1 accession) and *Pleiomeris* A. DC. (2 accessions) were obtained from GenBank, in which the former four genera used as ingroup while the latter genus used as outgroup.

DNA EXTRACTION, AMPLIFICATION, PURIFICATION AND SEQUENCING

Total genomic DNA was extracted from either silica-gel dried leaves or herbarium specimens using the method described in Doyle & Doyle (1987). The entire nrITS region (ITS1-5.8S-ITS2) was amplified using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GAAGTAAAAGTCGTAACAAGG-3') of White et al. (1990). Polymerase chain reactions (PCR) were performed at 12.5 µL volumes containing 0.4 µM of each primer, 1.25µL of 10X Ex Taq buffer (Takara Bio, Shiga, Japan), 2.5mM MgCl2, 250 µM of each dNTP, 0.2 U Ex Taq DNA Polymerase (Takara Bio) and 1 µL of template DNA. The PCR consisted of one cycle at 95ºC for 5 minutes, 35 cycles of 95 ºC for 30 s, 56 ºC for 30 s and 72 ºC for 3 minutes, and a final extension at 72 ºC for 7 minutes. PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA). Cycle sequencing-reactions were prepared with BigDye Terminator

v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) by following the manufacture's protocol. Cycle sequencing products were purified using the ethanol precipitation method, and Sanger sequencing was performed on an ABI 3500 DNA sequencer (Applied Biosystems).

DNA ALIGNMENT AND PHYLOGENETIC ANALYSES

DNA sequence chromatograms were edited using the DNA Baser Sequence Assembler v.4.36.0 (http://www.dnabaser.com) software. The alignment of sequences was done using the MUSCLE algorithm (Edgar 2004) in MEGA7 software (Kumar et al. 2016), and the extremes of sequences were manually trimmed to build a matrix dataset of uniform length. The most likely substitution model was determined using the corrected Akaike information criterion calculated in the jModelTest (Posada 2008) program. Phylogenetic trees were built using Bayesian inference (BI) and maximum likelihood (ML) methods; in both cases, and gaps were treated as missing data (i.e. they did not contribute any phylogenetic information). BI analysis was run on Mr. Bayes 3.2.5 (Ronquist et al. 2012), assigning the most likely substitution model SYM+I+G to the dataset. Monte Carlo Markov Chains consisted of four iterations during 10 million generations. Twenty-five percent of generations were discarded as burn-in. For ML analysis, RaxML v.8.0.26 (Stamatakis 2008) as implemented in raxmlGUI (Silvestro & Michalak 2012) was done on ML search using 10000 rapid bootstraps under GTR gamma model.

1.2 RESULTS

In total 113 nrITS sequences of 90 samples from *Ardisia*, 14 samples from eight related genera, and nine samples from three outgroup genera were identified in this study and preserved in DDBJ (Table 1.1). The aligned nrITS sequence matrix for 187 samples including outgroups yielded 657 bp in length and 379 sites were variable, 276 sites of which were potentially parsimony informative. The topology of BI tree is largely congruent to ML tree and, therefore, only the former is presented in Figure 1.2. The ingroup were divided into two major groups, here called Clade I (posterior probability of BI (PP) = 1, bootstrap support of ML (BS) = 58%) and Clade II (PP = 0.94 / BS < 50%). Clade I included species of the genera *Amblyanthopsis*, *Amblyanthus, Hymenandra*, *Sadiria* and members from the following *Ardisia* subgenera: *Bladhia* (in part), *Crispardisia*, *Pyrgus* and *Tinus*. Other genera, viz *Afrardisia*, *Badula*, *Discocalyx*, *Fittingia*, *Oncostemum*, *Tetrardisia* and *Systellantha*, all fall into Clade II, together with some members from subgenera *Acrardisia*, *Akosmos*, *Bladhia* (in part), *Pimelandra* and *Stylardisia* of *Ardisia*. The phylogenetic tree indicated that *Ardisia* was not monophyletic because members of the genus not only scattered across the two clades with other genera. At least *Discocalyx* and *Hymenandra* were also another non-monophyletic genus. *Discocalyx* was split into two groups within Clade II, with one species forming a strongly supported clade with *Fittingia* (PP = 1 / BS = 100%) and two species related to the highly supported (PP = 0.99 / BS = 94%) clade of *Badula* (PP = 1 / BP = 100%) and *Oncostemum* (PP = 1 / BP = 89%). Two species of *Hymenandra* formed a clade with *Ardisia* subgenus *Pyrgus* in Clade I (PP = 0.91 / BP = 87%), but these species were not closely related. *Afrardisia* form a monophyletic clade (PP = 1 / BS = 100%) in Clade II. *Tetrardisia* and *Systellantha* were moderately (PP = 1 / BS = 65%) supported as sister genera in Clade II. *Amblyanthopsis* form a strongly supported clade with some members of *Ardisia* subgenus *Crispardisia* (PP = 1 / BS = 94%) in Clade I. While *Sadiria longistyla* Ze H. Wang & H.

Peng (Wang et al. 2018) formed a strongly supported clade with *Amblyanthus* (PP = 1/ BS = 100%) and is moderately (PP = 0.96 / BS = 64%) supported as a sister clade to members subgenus *Pyrgus* and *Tinus* of *Ardisia*.

The phylogenetic tree also did not support the subgeneric classification of *Ardisia*, initially circumscribed by Mez (1902) and now widely accepted. In fact, all subgenera, were able to include in this study, were polyphyletic with members split, mixed within various clades across the two major clades, or were not detected as a monophyletic group due to basal polytomy.

1.3 DISCUSSION

GENERIC DELIMITATION

The present study is by far the most comprehensive phylogenetic investigation among *Ardisia* and its relatives in tropical Asia based on nrITS sequence data from 176 samples from a wide range of Malesian to East Asian species, including 66 species that have never been previously sequenced. With the exception of *Antistrophe*, *Conandrium*, *Loheria* and *Tapeinosperma*, the majority of Malesian genera were included in the analyses. For the first time, *Ardisia* was recovered as non-monophyletic and paraphyletic with several other genera sitting within the major clades. Present results outline a potential taxonomic question in Asian *Ardisia* and; two potential solutions reflecting the phylogenetic relationships are discussed.

A potential solution is to incorporate the genera appeared in the Clade I and Clade II (Figure 1.2) into an enlarged *Ardisia*. The genera to be potentially incorporated into *Ardisia* are *Amblyanthopsis*, *Amblyanthus, Badula, Discocalyx*, *Fittingia*, *Hymenandra*, *Oncostemum*, *Sadiria*, *Systellantha* and *Tetrardisia*, many of which were formerly treated in *Ardisia* (see Mez 1902). This potential solution, however, would make *Ardisia* a very large and more heterogeneous genus, for example not only by consisting of taxa with unisexual and bisexual flowers, but also by including quite diversified taxa in the molecular phylogeny.

Another potential solution is to split *Ardisia* into smaller groups incorporating smaller genera or a part of genus into one of them, for example, by recognizing relatively large genera or tribes representing the major clades (Clade I and Clade II in Figure 1.2), or by recognizing further smaller genera representing distinct clades in the molecular phylogeny. This study analyzed multiple samples from *Hymenandra* and *Discocalyx*, and they are not monophyletic in the molecular phylogeny (Figure 1.2). Some samples of these genera formed

distinct clades with some *Ardisia*, which might be recognized as distinct group (for example, the "*Pyrgus* clade" formed by *Ardisia* and *Hymenandra* in Figure 1.2). Multiple samples also used for *Afrardisia* which was moved into *Ardisia* based on ovule number by Taton (1979) but treated as distinct genus in Ståhl & Anderberg (2004). According to the molecular phylogeny (Figure 1.2), *Afrardisia* samples only formed a distinct clade which might be treated as a distinct group. Similarly, multiple samples were used for *Badula* and *Oncostemum*, and molecular phylogeny reveals each genus form a highly distinct clade which might be treated as distinct groups as currently accepted in Ståhl & Anderberg (2004). Based on the inferred phylogenetic tree, both *Badula* and *Oncostemum* show affinities with *Discocalyx* (*D. schlechteri* and *D.* sp., Figure 1.2), and especially *Badula* because the genus *Discocalyx* was formerly treated as a section under *Badula* by de Candolle (1844) later raised as distinct genus by Mez (1902). *Tetrardisia*, consistently with tetramerous flowers, has an affinity with *Systellantha*, and these two genera are recovered as sister taxa in a strongly supported clade.

Although the current molecular phylogeny raised questions on present classification of *Ardisia* and its related genera, the molecular phylogeny is still not enough to reach any conclusion in taxonomic changes because of insufficient sampling and less resolution of the molecular phylogeny. For *Amblyanthopsis*, *Amblyanthus*, *Fittingia*, *Sadiria*, *Systellantha* and *Tetrardisia* we used only one sample each in this study, and their phylogenetic relationships with *Ardisia* should be tested using more samples. For *Ardisia*, more samples, especially the type of genus viz. *Ar. tinifolia* Sw. and other Mesoamerican species described by Swartz (1788: 3[48]) are required for further discussion on the generic circumscription. Additional samples across the subfamily, especially for the related genera *Antistrophe*, *Conandrium*, *Loheria* and *Tapeinosperma*, as well as effort to increase more molecular data are needed to obtain better phylogenetic resolution for inter- and intrageneric delimitation by not only

improving the support values, but also providing more informative characters (e.g. Nabhan & Sarkar 2012).

SUBGENERIC DELIMITATION

All of the subgenera analyzed only partially agree with the morphological circumscription of subgenera by Mez (1902), supporting the hypothesis that the current subgeneric groupings may not be monophyletic (e.g., see Stone 1989 [p. 264], 1990b [p. 235]). As inferred by the current phylogenetic tree using nrITS data (Figure 1.2), the circumscription of subgenera obviously needs to be revised by excluding some members from their current group and reassigning them either into existing ones or creating new groups to accommodate them.

For the subgenus *Stylardisia* as currently circumscribed is characterized by having terminal inflorescences on lateral branches and the style protruding from the corolla prior to anthesis. However, the subgenus was not monophyletic because the clade consisting of *Ardisia montana* King & Gamble – *Ar*. *colorata* Roxb. was highly supported (PP = 1, BS = 100%) but four species, *Ar*. *gasingoides* Julius & Utteridge, *Ar*. *oocarpa* Stapf, *Ar*. sp. vel aff. *oocarpa* and *Ar*. *suffruticosa* Ridl., were not included in it (Figure 1.2). The former three were sister to taxa in subgenus *Acrardisia*. These non-*Stylardisia* taxa will reassign into subgenus *Acrardisia* in any future taxonomic treatments, as well as critically examine characters used for the circumscription of subgenus *Acrardisia*. In a case of *Ardisia suffruticosa*, it was thought that misinterpretation of morphological characters has resulted in mistaken placement to subgenus *Stylardisia*, for example, this species has axillary inflorescences at the uppermost sections of the lateral branches giving a ‘terminal inflorescence’ and was consequently placed by Stone (1989) in subgenus *Stylardisia*. Based on the axillary inflorescences, together with other characters, *Ardisia suffruticosa* might be

placed in subgenus *Akosmos* because the species nested within the subgenus and formed a strongly supported clade (PP = 1 / BS = 94%) with *Ar. quinquegona* Blume. The non-monophyletic relationship among the members of subgenus *Stylardisia* clearly indicates that not all species assigned to this subgenus may belong to this group, and each taxon should be critically reevaluated; the key diagnostic character for the 'core', well-supported members of *Stylardisia* is the style exserted from the corolla bud immediately prior to anthesis, but this is problematic to observe in young flowering material or specimens with fruit only.

For subgenus *Akosmos*, its circumscription has been queried by Stone (1990) who speculated that this subgenus is heterogeneous and may not represent a natural group, implying members of the group are not monophyletic. Recent study with less samples included in the phylogenetic analysis (Julius et al., 2019 [accepted]) seems to agree with Stone's (1990) perception of the group because *Ardisia quinquegona* and *Ar. sieboldii* Miq. (both subgenus *Akosmos* fide Mez 1902, p.105), were recovered as paraphyletic in the tree. However, the result may merely be due to low sampling and, therefore, additional sequences viz. *Ardisia conspersa* E. Walker and *Ar. fordii* Hemsl. for this group was obtained from GenBank and analyzed together with *Ar. quinquegona* and *Ar. sieboldii*. The inferred tree shows all members of subgenus *Akosmos* together with *Ar. suffruticosa* formed a weakly supported clade (PP = 0.85 / BS = <50%) in the present study.

The subgenus *Tinus* which was suggested to be combined with subgenus *Tinopsis* by Stone (1990: 235) but the treatment which extremely ambiguous as to why, stating just that 'other evidence [not given] appears to weaken the difference between' the two subgenera turn out to be closely related to taxa of subgenus *Pyrgus*, excluding *Ardisia serrata* (Cav.) Pers., and genus *Hymenandra* as weakly supported clade (PP = 0.76 / BS = 55%) in Clade I. Subgenus *Pyrgus* has terminal inflorescences subtended by several, crowded subverticillate leaves or 1–2 leaves immediately below the inflorescence peduncle (Table 1.0b). By studying

recent collections with detailed field observations from members of both subgenera, all taxa found in this clade have a similar arrangement of lateral inflorescence bearing branches in-between subverticillate leaves on the main stem. Moreover, the recent morphological observation had revealed that the subverticillate leaves along the main stem which characterizes the members of this clade, remains throughout the plant's life except for *Ar. elliptica* Thunb. (where the subverticillate leaf arrangement becomes more dispersed as the plant matures). Although the leaf arrangement on the main stem has not been used as one of the diagnostic characters for groupings as circumscribed by Mez (1902), this feature does characterize this clade. On the other hands, *Ardisia serrata* which has been classified into subgenus *Pyrgus* but not included in this clade, has lateral branches continuously grow (sympodially) flowering (personal observations in the field), and the subverticillate leaves subtending the inflorescences are not present in the other members of subgenus *Pyrgus*, which have only one or two leaves below the inflorescence and without continuous sympodial growth after flowering. Subgenus *Pyrgus* is thus best split based to two groups based on these characters as well as the phylogenetic relationship, but further morphological studies need to be carried out to define additional morphological characters which will be useful for the circumscription of the two groups.

For subgenera *Pimelandra* and *Bladhia*, their monophyletic status has never been questioned based on morphological characters, and both groups are easy to recognize: subgenus *Pimelandra* by the axillary inflorescences along the lateral branches, and subgenus *Bladhia* mainly by its sharply serrulate-denticulate leaf margin without nodules at the sinuses (Julius & Utteridge 2012). Some members from these subgenera, however, appear not to be to correctly assigned. For example, *Ar. congesta* Ridl. has axillary inflorescences on lateral branches similar to members of subgenus *Pimelandra*, therefore it is understandable for Ridley (1920) to assign it to subgenus *Pimelandra*. However, the phylogenetic relationship

placed the species outside the clade comprising most species of subgenus *Pimelandra* (including *Ardisia fuliginosa* Blume – *Ar. korthalsiana* Scheff. The isolation of *Ar. congesta* from subgenus *Pimelandra* is also supported by several morphological characters: (1) small stature with height c. 30 cm (vs. > 1.5 m in height), (2) the lateral branches arranged in between subverticillate leaves (vs. lateral branches subtended by normal leaves), and (3) the corolla and anther connectives are elongated and twisted (vs. both corolla and anther connectives are short with obtuse tips). With these features, present study is currently unable to place *Ar. congesta* into an existing subgenus and a new group may be needed for this species, as the type of *Pimelandra* (*Ar. pachysandra*) is clustered together with the other members of the subgenus. The elongated, twisted corolla and anther connective morphology places *Ar. congesta* as morphologically similar to the genus *Antistrophe* and *Hymenandra wallichii* A. DC. However, this relationship could not be tested as representative species of those taxa were not available for this phylogenetic analysis.

Subgenus *Bladhia* has been recently revised based mainly on the following characters: 1) the leaf arrangement and leaf margin, 2) inflorescence type and position, 3) calyx-lobes shape and 4) the scales shape (Yang & Dwyer 1989). Members of the subgenus have been classified into two sections *Bladhia* and *Odontophylla* Yang, with these further subdivided into five subsections, *Bladhia* and *Faberi* Yang of section *Bladhia*; and *Fimbriata* Yang, *Gigantifolia* Yang and *Odontophylla* Yang of section *Odontophylla*). Representative species of both sections were grouped in the phylogenetic relationship according to the section proposed by Yang & Dwyer (1989) but placed in different lineages. *Ardisia japonica* (Thumb.) Blume, *Ar. maclurei* Merr., *Ar. purpureovillosa* C.Y. Wu & C. Chen ex C.M. Hu and *Ar. pusilla* A. DC., members of section *Bladhia* positioned in Clade I, whereas *Ar. affinis* Hemsl., *Ar. albomaculata* Pit., *Ar. balansana* Y.P. Yang, *Ard. demissa* Miq., *Ard. gigantifolia* Stapf, *Ar. ordinata* E. Walker, *Ar. pubinevula* E. Walker, *Ar. scalarinervis* E.

Walker, *Ar. silvestris* Pit. var. *appressa* C.M. Hu & J.E. Vidal and *Ar. theifolia* King & Gamble, members of section *Odontophylla* formed a highly supported clade (PP = 1 / BS = 100%) in Clade II. The subgenus is clearly not monophyletic although the two sections were reciprocally monophyletic. The lineage consisting members of section *Odontophylla* may require a new name and circumscription since the type species of subgenus *Bladhia*, *Ar. japonica* was placed in the other clade. Yang & Dwyer (1989) speculated that subgenus *Bladhia* is closely related to subgenus *Tinus* based on the inflorescence position, but result do not support this.

For members of subgenus *Crispardisia*, all analyzed taxa formed a weakly supported clade (PP = 0.66 / BS < 50%) in Clade I. Majority of the members were recovered in the clade consisting *Ar. alyxiifolia* Tsiang ex C. Chen – *Ar.* cf. *cadieri* Guilaumin (PP = 1 / BS = 98%), while another clade comprising *Ar. crenata* Sims – *Ar.* sp. vel aff. *vidalii* C.M. Hu + *Amblyanthopsis bhotanica* (C.B. Clarke) Mez (PP = 1 / BS = 94%).

PHYLOGENETIC RELATIONSHIP AMONG NODULATED GENERA

According to molecular phylogeny, the nodulated genera *Amblyanthopsis*, *Amblyanthus* and *Ardisia* subgenus *Crispardisia* are all placed in Clade I with weak support (PP = 1 / BS = 58%). Their phylogenetic position is, however, not monophyletic as inferred from the phylogenetic tree (Figure 1.2). The genus *Amblyanthus*, is obviously distantly related from the other two and positioned as sister taxon to *Sadiria longistyla*. Moreover, the taxon *Amblyanthus* sp. vel aff. *multiflorus* examined here characterized by the inflorescences axillary on lateral branches which differ from the other two (inflorescences terminal on uppermost part of lateral branches in *Amblyanthopsis* and *Ardisia* subgenus *Crispardisia*).

Based on inflorescence position, it shows that the *Amblyanthopsis* has an affinity with *Ardisia crenata* alike.

Although paraphyletic position of nodulated genera is morphologically supported in the phylogenetic tree but their phylogenetic inference needs further test for resolution better to re-evaluate their relationship. To do so, phylogenetic analyses using concatenated data sets of cpDNA and nrITS will be carried out on following chapter not only to reconfirm their phylogenetic status but the sought information will be used to infer the evolution of leaf nodulation within Myrsinoideae

1.4 CONCLUSION

Ardisia is not monophyletic based on the nrITS phylogeny presented here, and the members form two major clades. The proposed subgenera of *Ardisia* by Mez (1902) are only partially reflected, suggesting the need for a thorough re-evaluation of their traditional circumscription. *Hymenandra* was clearly shown to be non-monophyletic within *Ardisia*. Although nrITS data alone could not provide fully resolved relationships among the analyzed taxa, this study highlighted that either *Ardisia* could be expanded to become an extremely large and heterogeneous group, or it could be split up to smaller groups. Whilst some groups are well-supported as distinct clade and may deserve to be recognized as distinct taxon for example *Afrardisia*, we cannot finalize the taxonomic circumscription of them until a fully resolved phylogenetic tree is obtained. Additional markers and increased taxon sampling are needed, especially to include the type species of the genus *Ardisia* from the Caribbean, as well as additional species to represent Neotropical diversity, the subgenera *Tinopsis* (mainly Bornean) and *Scherantha* (from the Philippines), as well as other related genera including *Antistrophe*, *Conandrium*, *Loheria* and *Tapeinosperma*. I am preparing a revision of the delimitation and circumscription for the Malesian subgenera of *Ardisia*, as well as the placement of species within them, and I believe that this study will give us preliminary idea to improve systematic framework to better understand the diversity and infra-generic groups of this remarkable genus. Preparation for phylogenetic paper on Bornean *Hymenandra* and taxonomic revision of *Tetrardisia* are also ongoing.

1.5 REFERENCES

Alvarez I. and Wendel J.F. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. **29** (3):417–434.

Angiosperm Phylogeny Group (APG). 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Bot. J. Linn. Soc. **161**: 105–121.

Baldwin B., Sanderson J.M., Porter M.F., Wojciechowski C.S., Campbell M.J. and Donoghue M. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Mo. Bot. Gard. **82**: 247–277.

Bone R.E., Strijk J.S., Fritsch P.W., Buerki S., Strasberg D., Thébaud C. and Hodkinson T.R. 2012. Phylogenetic inference of *Badula* (Primulaceae), a rare and threatened genus endemic to the Mascarene Archipelago. Bot. J. Linn. Soc. **169** (2): 284–296.

De Candolle, A.P. 1844. Prodromus systematis naturalis regni vegetabilis, 8th ed. pp. 75–140. Sumptibus Sociorum Treuttel et Würtz, Paris.

Doyle J.J. and Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11–15.

Edgar R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32** (5): 1792–1797.

Frodin D.G. 2004. History and concepts of big plant genera. Taxon **53** (3): 753–776.

Furtado C.X. 1959. Some new or noteworthy species in Malaysia. Gard. Bull. Singapore **17**: 279–311.

Giri G.S., Das S.K. and Chowdary H.J. 2002. A new species of *Amblyanthus* A. DC., family Myrsinaceae from Arunachal Pradesh, India. J. Bombay Nat. Hist. Soc. **99** (3): 471–473.

Gruenstaeudl M., Urtubey E., Jansen R.K., Samuel R., Barfuss M.H., Stuessy T. 2009. Phylogeny of Barnadesioideae (Asteraceae) inferred from DNA sequence data and morphology. Mol. Phylogenet. Evol. **51** (3): 572–587.

Hao G., Yuan Y.-M., Hu C.M., Ge X.-J. and Zhao N.-X. 2004. Molecular phylogeny of *Lysimachia* (Myrsinaceae) based on chloroplast *trn*L-F and nuclear ribosomal ITS sequences. Mol. Phylogenet. Evol. **31** (1): 323–339.

Hu C.M. 2002. New species of *Ardisia* (Myrsinaceae) from Malesia. Blumea **47**: 493–512.

Hu C.M. and Vidal J.E. 2004. Myrsinaceae. In: Morat, Ph. (ed.) Flore du Cambodge du Laos et de Viêtnam 32. 288 pp. Muséum National D'Histoire Naturelle, Paris.

Hu, C. M. and Deng, Y. F. 2012. *Sadiria aberrans*, a new combination in Chinese Myrsinaceae. Ann. Bot. Fennici **49**: 395–396.

Julius A. and Utteridge T.M.A. 2012. Revision of *Ardisia* subgenus *Bladhia* in Peninsular Malaysia; studies in Malaysian Myrsinaceae I. Kew Bull. **67**: 379–388.

Julius A., Gutiérrez-Ortega, J.S., Sabran S., Tagane S., Naiki A., Darnaedi D., Aung M.M., Dang S.V., Nogoc N.V., Binhk, T.H., Watano Y., Utteridge T.M.A. and Kajita T. 2019 [accepted]. Phylogenetic Relationship of Tropical Asian *Ardisia* and Relatives (Primulaceae) Shows Non-monophyly of Recognized Genera and Subgenera. J. Jap. Bot.

Ku C. and Hu J.M. 2014. Phylogenetic and cophylogenetic analyses of the leaf-nodule symbiosis in *Ardisia* subgenus *Crispardisia* (Myrsinaceae): evidence from nuclear and chloroplast markers and bacterial *rrn* operons. Int. J. Plant Sci. **17** (1): 92–109.

Kumar S., Stecher G. and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molec. Biol. Evol. **33** (7):1870–1874.

Larsen K. and Hu C.M. 1994. Reduction of *Tetrardisia* to *Ardisia*. Nord. J. Bot. **15**(2): 161–162.

Larsen K. and Hu C.M. 1996. Myrsinaceae. Fl. Thailand **6** (2): 81–178.

Martins L., Oberprieler C. and Hellwig F.H. 2003. A phylogenetic analysis of Primulaceae s.l. based on internal transcribed spacer (ITS) DNA sequence data. Pl. Syst. Evol. **237**: 75–85.

Mez C. 1902. Myrsinaceae. Pflanzenr. [Engler] **9**: 1–437.

Mez C. 1922. *Fittingia* novum Myrsinacearum genus. Bot. Arch. **1**: 105–106.

Nabhan A.R. and Sarkar I.N. 2011. The impact of taxon sampling on phylogenetic inference: a review of two decades of controversy. Brief. Bioinformatics **13** (1): 122–134.

Posada D. 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. **25**: 1253–1256.

Ridley H.R. 1920. On a collection of plants from Peninsular Siam. J. Fed. Malay States Mus. **10**: 63–126.

Ronquist F., Teslenko M., Van Der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M.A., Huelsenbeck J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**: 539–542.

Shaw J., Lickey E.B., Schilling E.E. and Small R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Amer. J. Bot. **94** (3): 257–288.

Silvestro D. and Michalak I. 2012. raxmlGUI: a graphical front-end for RAxML. Org. Divers Evol. **12**: 335.

Sleumer H. 1988a. The genera *Discocalyx* Mez, *Fittingia* Mez, *Loheria* Merr., and *Tapeinosperma* Hook. *f*. (Myrsinaceae) in New Guinea. Blumea **33**: 81–107.

Sleumer H. 1988b. The genus *Conandrium* Mez (Myrsinaceae). Blumea **33**: 109–113.

Sleumer H. 1988c. A revision of the genus *Ardisia* Sw. (Myrsinaceae) in New Guinea. Blumea **33**: 115–140.

Ståhl B. and Anderberg A.A. 2004. Myrsinaceae. *In*: Kubitzki K. (ed.) The families and genera of vascular plants VI. pp. 266–281. Springer, Berlin.

Stamatakis A. 2008. RAxML-VI-HPC: a tool for phylogenetic analysis and post- analysis of large phylogenies. Bioinformatics **30**: 1312–1313.

Stevens P.F. 2001 onwards. Angiosperm Phylogeny Website. Version 14, July 2017. http://www.mobot.org/MOBOT/research/APweb/.

Stone B.C. 1982. New and noteworthy Malaysian Myrsinaceae, I. Malaysian Forester **45**: 101–121.

Stone B.C. 1988. New and noteworthy Malesian Myrsinaceae II. *Emblemantha*, a new genus from Sumatra. Proc. Acad. Nat. Sci. Philadelphia **140** (2): 275–280.

Stone B.C. 1989. Myrsinaceae. *In*: Ng, F.S.P. (ed.) Tree Flora of Malaya 4. pp. 264–284. Longman Malaysia.

Stone B.C. 1990a. Studies in Malesian Myrsinaceae, V. Additional new species of *Ardisia* Sw. Proc. Acad. Nat. Sci. Philadelphia **142**: 21–58.

Stone B.C. 1990b. Myrsinaceae as an example of plant diversity in Malesia with special reference to the species in Borneo. *In*: Kheong Y.S. and Win L.S. (eds.), Proceedings of the International Conference on Tropical Biodiversity 'In Harmony with Nature', 12–16 June 1990. pp. 230–237. Malayan Nature Society, Kuala Lumpur.

Stone B.C. 1991. The genus *Loheria* Merr. (Myrsinaceae). Micronesica **24** (1): 65–80.

Stone B.C. 1992a. A revision of the genus *Tetrardisia* Mez (Myrsinaceae). Malayan Nat. J. **46**: 1–11.

Stone B.C. 1992b. *Systellantha,* a new genus of Myrsinaceae from Borneo. Malayan Nat. J. **46**: 13–24.

Stone B.C. 1992c. New and Noteworthy Malesian Myrsinaceae, VI. Revision of the Genus *Hymenandra* A. DC. Gard. Bull. Sing. **43**: 1–17.

Swartz O.P. 1788. *Ardisia*. Prodromus [Swartz] **3**: 48.

Taton A. 1979. Contribution a l'etude du genre *Ardisia* SW. (Myrsinaceae) en Afrique tropicale. Bull. Jard. Bot. Nat. Belgie **49** (1/2): 81–120.

Wang L.-Y., Wang Z.-H., Chen L., Zhao F. and Li R.-J. 2018. *Sadiria longistyla* (Myrsinoideae, Primulaceae), a new species from Yunnan, China. Phytotaxa **345**: 293–297.

White T.J., Bruns T.D., Lee S.H. and Taylor J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis M.A., Gefland D.H., Sninsky J.J. and White T.J. (eds.), PCR protocols: A guide to methods and application. pp. 315–322. Academic Press, San Diego.

Yang Y.-P. and Dwyer J.D. 1989. Taxonomy of subgenus *Bladhia* of *Ardisia* (Myrsinaceae). Taiwania **34** (2): 192 – 297.

Table 1.0a. List of main characters for generic delimitation following the classification outline by Mez (1902, 1922) and Stone (1992). Included only in the list are the genera closely related to *Ardisia* mainly found in Malesia region towards adjacent area and candidates from African (in bold face). Asterisk indicates the available genera subjected to phylogenetic analysis.

		Characters							
		Leaf nodule	**Flowers**						**Exocarp of fruits**
Tribe	**Genus**	presence/absence	merosity	sexuality	aestivation	anther connation	calyx	stigma	
Ardisieae A. DC. (many ovules in pluriseriate)	*Ardisia* Sw.*	absence (except for subgenus *Crispardisia)*	5-merous	Bisexual, monoecious	Right	Free	Deeply lobed to the base	Inconspicuously	Thin
	Conandrium Mez	Absence	5-merous	Bisexual, monoecious	Right	Connate laterally by the thecal margins	Deeply lobed to the base	Inconspicuously	Thin
	Hymenandra A. DC. *	Absence	5-merous	Bisexual, monoecious	Right	Connate laterally by the thecal margins	Deeply lobed to the base	Inconspicuously	Thin
Myrsineae Pax (fewer ovules in uniseriate)	***Afrardisia*** Mez*	Absence	5-merous	Bisexual, monoecious	Right	Free	Deeply lobed to the base	Inconspicuously	Thin
	Amblyanthus A. DC. *	Presence	5-merous	Bisexual, monoecious	Right	Connate laterally by the thecal margins	Deeply lobed to the base	Discoid	Thin
	Amblyanthopsis Mez*	Presence	5-merous	Bisexual, monoecious	Right	Free	Deeply lobed to the base	Capitate	Thin
	Antistrophe A. DC.	Absence	5-merous	Bisexual, monoecious	Left	Free	Deeply lobed to the base	Inconspicuously	Thin
	Badula Juss. *	Absence	5-merous	Bisexual, monoecious	Right	Free	Deeply lobed to the base	Punctiform	Thin
	Discocalyx Mez *	Absence	4(–5)-merous	Unisexual (rarely bisexual), dioecious	Right	Free	Cup-shaped	Discoid, capitate-ovoid or subglobose	Thin
	Fittingia Mez*	Absence	(4–)5-merous	Unisexual, dioecious	Right	Free	Deeply lobed to the base	Discoid, subcapitate, peltate	Thick, spongy, becoming flattened or wing-like by pressure during preparation for herbarium specimens
	Loheria Mez	Absence	4(–5)-merous	Unisexual, dioecious	Right	Free	Deeply lobed to the base	Capitate	Thin

Table 1.0a. *Continued.*

	Characters							
	Leaf nodule	**Flowers**						**Exocarp of fruits**
Genus	presence/absence	merosity	sexuality	aestivation	anther connation	calyx	stigma	
Oncostemum A. Juss. *	Absence	(4–)5-merous	Bisexual, monoecious	Right	Free	Deeply lobed to the base	Discoid	Thin
Sadiria Mez*	Absence	5-merous	Bisexual, monoecious	Right	Free	Corollas united above middle	Punctiform	Thin
Systellantha B.C. Stone*	Absence	4-merous	Unisexual, monoecious	Right	Free	Deeply lobed to the base	Inconspicuously	Thin
Tapeinosperma Hook. *f.* *	Absence	5-merous	Bisexual, monoecious	Right	Free	Cup-shaped	Truncate, inconspicuously discoid or subcapitate	Thin
Tetrardisia Mez*	Absence	4-merous	Bisexual, monoecious	Right	Free	Deeply lobed to the base	Inconspicuously	Thin

Table 1.0b. List of main characters for subgeneric delimitation following the classification outline by Mez (1902). Included only in the list are the subgenera for tropical Asian *Ardisia*.

	Characters				
Subgenera	**Habit**	**Leaf margin**	**Inflorescence position**	**Style**	**Calyx**
Acrardisia Mez	Shrub, rarely attained to a big tree with 15m high, lateral branches plagiotropic	Entire	Terminal on lateral branches	Included before anthesis	Lobes spreading during the anthesis
Akosmos Mez	Shrub, lateral branches plagiotropic	Entire	Inflorescence combined terminal and axillary, or if axillary, then in axil of last leaf; inflorescence subtended by normal or reduced leaves	Included before anthesis	Lobes spreading during the anthesis
Crispardisia Mez	Shrub, lateral branches normally pointing upwards	Leaves crenulate, the sinuses each with a nodule locally overlapped by the margin	Terminal on lateral branches, sometimes lateral on main branches	Included before anthesis	Lobes spreading during the anthesis
Bladhia (Thunb.) Mez	Low subshrub or suffrutescent and monocaul, with procumbent lower stem (or referring as creeping rhizome)	Sharply serrulate-denticulate without nodule on the sinuses	Lateral on main branches	Included before anthesis	Lobes spreading during the anthesis
Pimelandra (A. DC.) Mez	Shrub to small tree, lateral branches plagiotropic	Entire, rarely serrulate-denticulate	Inflorescences strictly axillary, compact or condensed, subtended by normal leaves or from the axils of fallen leaves on older shoots	Normally included before anthesis, sometimes exserted before anthesis	Lobes spreading during the anthesis
Pyrgus (Lour.) Mez	Shrub to subshrub, lateral branches pointing upwards	Entire, rarely serrulate-denticulate	Inflorescence terminal on lateral branches, with only 1- to 2-pair of leaves present; rarely distally crowded pseudo-verticillate leaves just below it	Included before anthesis	Lobes distinctly imbricate during the anthesis
Tinopsis Mez	Shrub, lateral branches plagiotropic	Entire	Inflorescence terminal on normal shoot	Included before anthesis	Lobes distinctly imbricate during the anthesis
Tinus (Burm.) Mez	Shrub, lateral branches plagiotropic	Entire	Inflorescence lateral, in axil or a normal or usually a reduced leaf	Included before anthesis	Lobes distinctly imbricate during the anthesis
Stylardisia Mez	Shrub to small tree, lateral branches plagiotropic	Entire	Inflorescences strictly terminal on lateral branches	Excerted before anthesis	Lobes imbricate during the anthesis

Table 1.1. List of taxa with vouchers information are given. The *Ardisia* samples organized according to subgenera and listed alphabetically. The numbering labels with asterisk indicating a given number for the leaf tissue extracted from RBGK whereas no asterisk referring to the given tube's number for the samples extracted in Chiba University and one accession from GenBank. The outgroup indicates by '#'.

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID	GenBank Accession no.
1	*Afrardisia* Mez	*Af. staudtii* Mez	Koumbemba *et al*. 187, CON, K	45208*	LC440217
2		*Afrardisia* sp.	Mpandzou ALM 292, CAM, K	547	LC440139
3		*Afrardisia* sp. 1	Mpandzou *et al*. 984, CON, K	45183*	LC440135
4		*Afrardisia* sp. 8	Tchiengue et al. 3605, CAM, K	45181*	LC440136
5	*Amblyanthopsis* Mez	*Amblyanthopsis bhotanica* (C.B. Clarke) Mez	KEA883, MYN, NYBG	517	LC440221
6	*Amblyanthus* Mez	*Amblyanthus* sp. vel aff. *multiflorus* Mez	MY881, MYN, TNS	654	LC440222
	Ardisia Sw.				
7	subgen. *Acrardisia* Mez	*Ar.* cf. *amboinensis* Scheff.	SAJ1433, PNG, BISH	184	LC440202
8		*Ar*. cf. *amboinensis* Scheff.	SAJ1055, PNG, BISH	166	LC440218
9		*Ar.* cf. *squarrosa* Mez	SAJ0249, PNG, BISH	189	LC440210
10	subgen. *Akosmos* Mez	*Ar. conspersa* E. Walker	–	–	MF926190
11		*Ar. fordii* Hemsl.	–	–	MF926197
12		*Ar*. *quinquegona* Blume	2015/04-J, JPN, TBRC	203	LC440210
13		*Ar. quinquegona* Blume	T4692, THA, FU	272	LC440149
14		*Ar*. *sieboldii* Miq.	2015/02-J, JPN, TBRC	201	LC440150
15		*Ar*. *sieboldii* Miq.	2015/01-J, JPN, TBRC	202	LC440168
16	subgen. *Bladhia* (Thunb.) Mez	*Ar*. *albomaculata* Pit.	V3270, VNM, FU	429a	LC440169
17		*Ar. balansana* Y.P. Yang	–	–	MF926209
18		*Ar. balansana* Y.P. Yang	–	–	MF682156
19		*Ar*. *demissa* Miq.	T4066, THA, FU	268	LC440132
20		*Ar*. *demissa* Miq.	FRI72048, PM, KEP	45436*	LC440131
21		*Ar*. *gigantifolia* Stapf	V3154, VNM, FU	362	LC440140
22		*Ar*. *japonica* Blume	Chen 2007298, CHI, SCBG	–	JN645201
23		*Ar. maclurei* Merr.	–	–	MF926202

Table 1.1. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID	GenBank Accession no.
24		*Ar. maclurei* Merr.	–	–	MF682149
25		*Ar. ordinata* E. Walker	–	–	MF926207
26		*Ar. ordinata* E. Walker	–	–	MF682154
27		*Ar. pubinevula* E. Walker	–	–	MF926217
28		*Ar. pubinevula* E. Walker	–	–	MF682164
29		*Ar. purpureovillosa* C.Y. Wu & C. Chen ex C.M. Hu	–	–	MF926218
30		*Ar. purpureovillosa* C.Y. Wu & C. Chen ex C.M. Hu	–	–	MF682165
31		*Ar. pusilla* A.DC.	FU201507-8, JPN, FU	219	LC440148
32		*Ar. pusilla* A.DC.	–	–	MF926219
33		*Ar. silvestris* Pit. var. *appressa* C.M. Hu &J.E. Vidal	–	–	MF926222
34		*Ar. scalarinervis* E. Walker	–	–	MF926221
35		*Ar. theifolia* King & Gamble	FRI71872, PM, KEP	45422*	LC440220
36	subg. *Crispardisia* Mez	*Ar. affinis* Hemsl.	–	–	MF926181
37		*Ar. affinis* Hemsl.	–	–	MF682128
38		*Ar. alyxiifolia* Tsiang ex C. Chen	–	–	MF926182
39		*Ar. alyxiifolia* Tsiang ex C. Chen	–	–	MF682129
40		*Ar. brevicaulis* Diels	–	–	MF926186
41		*Ar. brevicaulis* Diels	–	–	MF682133
42		*Ar. caudata* Hemsl.	–	–	MF926188
43		*Ar. caudata* Hemsl.	–	–	MF682135
44		*Ar. corymbifera* Mez	T907, THA, FU	250	LC440122
45		*Ar. corymbifera* Mez	–	–	MF926191
46		*Ar. crenata* Sims subsp. *crenata*	FRI73564, PM, KEP	45373*	LC440124
47		*Ar. crenata* Sims subsp. *crenata*	SNP37015, SBH, SNP	455a	LC440125
48		*Ar. crenata* Sims subsp. *obtusifolia* Chatan & W. Promprom	T4630, THA, FU	271	LC440123
49		*Ar. crispa* (Thunb.) A. DC. var. *crispa*	FU201507-1, JPN, FU	212	LC440183

Table 1.1. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID	GenBank Accession no.
50		*Ar. crispa* (Thunb.) A. DC. var. *crispa*	–	–	MF926192
51		*Ar. crispa* (Thunb.) A. DC. var. *amplifolia* E. Walker	–	–	MF926231
52		*Ar. crispa* (Thunb.) A. DC. var. *amplifolia* E. Walker	–	–	MF682178
53		*Ar. ensifolia* E. Walker	–	–	MF926195
54		*Ar. ensifolia* E. Walker	–	–	MF682142
55		*Ar. hanceana* Mez	–	–	JN645188
56		*Ar. hanceana* Mez	–	–	MF926199
57		*Ar. hanceana* Mez	–	–	MF682146
58		*Ar. lindleyana* D. Dietrich	–	–	JN645191
59		*Ar. maculosa* Mez	T4760, THA, FU	273	LC440137
60		*Ar. maculosa* Mez	T3059, THA, FU	263	LC440143
61		*Ar. maculosa* Mez	–	–	MF926203
62		*Ar. maculosa* Mez	–	–	MF682150
63		*Ar. mamillata* Hance	–	–	MF926204
64		*Ar. polysticta* Miq.	V1937, VNM, FU	303	LC440146
65		*Ar. polysticta* Miq.	V3054, VNM, FU	361	LC440127
66		*Ar. polysticta* Miq.	SNP37006, SBH, SNP	448a	LC440114
67		*Ar. polysticta* Miq.	–	–	MF926212
68		*Ar. polysticta* Miq.	–	–	MF682159
69		*Ar. primulifolia* Gardner & Champ.	–	–	MF926186
70		*Ar. primulifolia* Gardner & Champ.	–	–	MF682133
71		*Ar. pseudocrispa* Pit	–	–	MF926215
72		*Ar. pseudocrispa* Pit	–	–	MF682162
73		*Ar. omissa* C.M. Hu	–	–	MF926206
74		*Ar. omissa* C.M. Hu	–	–	MF682153
75		*Ar. rosea* King & Gamble	FRI64029, PM, KEP	45425* (rep_2)	LC440154
76		*Ar. rosea* King & Gamble	FRI75002, PM, KEP	45439*	LC440156
77		*Ar. rosea* King & Gamble	FRI64029, PM, KEP	96 (rep_1)	LC440158
78		*Ar. rosea* King & Gamble	FRI64030, PM, KEP	45426*	LC440155
79		*Ar. rosea* King & Gamble	FRI71778, PM, KEP	45450*	LC440157
80		*Ar. rosea* King & Gamble	FRI45374, PM, KEP	45374*	LC440162

Table 1.1. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID	GenBank Accession no.
81		*Ar. verbascifolia* Mez	–	–	MF926227
82		*Ar. verbascifolia* Mez	–	–	MF682174
83		*Ar. verbascifolia* Mez	–	–	JN638408
84		*Ar. villosa* Roxb. var. *villosa*	T3508, THA, FU	265	LC440164
85		*Ar. villosa* Roxb. var. *villosa*	–	–	MF926228
86		*Ar. villosa* Roxb. var. *oblanceolata* E. Walker	–	–	MF926229
87		*Ar. villosa* Roxb. var. *oblanceolata* E. Walker	–	–	MF682176
88		*Ar. virens* Kurz	IS274, SUM, FU	399	LC440138
89		*Ar. virens* Kurz	FRI54105, PM, KEP	45420*	LC440177
90		*Ar. virens* Kurz	T3060, THA, FU	264	LC440178
91		*Ar.*cf. *annamensis* Pit.	V2550, VNM, FU	355	LC440129
92		*Ar.* cf. *cadieri* Guillaumin	V1905, VNM, FU	302	LC440113
93		*Ar.* cf. *ridleyi* King & Gamble	FRI73504, PM, KEP	463	LC440130
94		*Ar.*cf. *villosa* var. *ambovestita* E. Walker	V3761, VNM, FU	435a	LC440126
95		*Ar.*cf. *tsangii* E. Walker	V2415, VNM, FU	353	LC440128
96		*Ar.* sp. vel. aff. *vidalii* C.M. Hu	V1955, VNM, FU	304	LC440175
97		*Ar.* sp. vel aff. *villosa* Roxb.	T2177, THA, FU	256	LC440176
98	subgen. *Pimelandra* (A.DC.) Mez	*Ar. congesta* Ridl.	MY210, MYN, FU	607	LC440120
99		*Ar. congesta* Ridl.	MY570, MYN, FU	608	LC440121
100		*Ar. ferox* Furtado	FRI73534, PM, KEP	478	LC440191
101		*Ar. ferox* Furtado	FRI73526, PM, KEP	481	LC440192
102		*Ar. fuliginosa* Blume	J878, JAVA, FU	280	LC440152
103		*Ar. korthalsiana* Scheff.	SAN156115, SBH, SAN	45441*	LC440142
104		*Ar. korthalsiana* Scheff.	Marsio *et al*. 18, SUM, K	45174*	LC440197
105		*Ar. retinervia* Ridl.	FRI72063, PM, KEP	45437*	LC440213
106		*Ar. retinervia* Ridl.	FRI72084, PM, KEP	45438*	LC440214
107		*Ar.* cf. *korthalsiana* Scheff.	FRI57775, PM, KEP	45443*	LC440180
108		*Ar.* cf. *pachysandra* Mez	SAN153345, SBH, SAN	45317*	LC440182
109	subgen. *Pyrgus* (Lour.) Mez	*Ar. murtonii* H.R. Fletcher	C3394, C, FU	416	LC440144
110		*Ar. porifera* E. Walker	–	–	MF926213
111		*Ar. porifera* E. Walker	–	–	MF682160

Table 1.1. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID	GenBank Accession no.
112		*Ar. rigida* Kurz	T579, THA, FU	247	LC440153
113		*Ar. vaughanii* Ridl.	FRI72412, PM, KEP	45418*	LC440223
114		*Ar. serrata* Pers.	SAN153642, SBH, SAN	45318*	LC440166
115		*Ar. serrata* Pers.	SAN155852, SBH, SAN	45325*	LC440167
116		*Ar.* sp. vel aff. *breviramea* Merr.	SAN155854, SBH, SAN	45326*	LC440112
117		*Ar.* sp. vel aff. *breviramea* Merr.	SWK1910, SWK, FU	603	LC440179
118		*Ar.* sp. vel aff. *poilanei* Pit.	MS78, SBG, K	195	LC440181
119		*Ar.* sp. vel aff. *poilanei* Pit.	MS77, SBG, K	196	LC440203
120	subgen. *Stylardisia* Mez	*Ar. colorata* Roxb.	FRI77315, PM, KEP	45383*	LC440116
121		*Ar. colorata* Roxb.	SAN155969, SBH, SAN	45430*	LC440117
122		*Ar. colorata* Roxb.	FRI48303, PM, KEP	45432*	LC440118
123		*Ar. colorata* Roxb.	FRI71741, PM, KEP	45449*	LC440119
124		*Ar. gasingoides* Julius & Utteridge	FRI74697, PM, KEP	45419*	LC440194
125		*Ar. lamponga* Miq.	SNP20180, SBH, SNP	45312*	LC440199
126		*Ar. lamponga* Miq.	SNP20181, SBH, SNP	45313*	LC440200
127		*Ar. lamponga* Miq.	SAN156444, SBH, SAN	45378*	LC440201
128		*Ar. miqueliana* Scheff.	FRI77779, PM, KEP	45372*	LC440204
129		*Ar. montana* King & Gamble	FRI73650, PM, KEP	45390*	LC440205
130		*Ar. montana* King & Gamble	FRI64024, PM, KEP	45382*	LC440206
131		*Ar. paralleloneura* K. Larsen & C.M. Hu	FRI65236, PM, KEP	45386*	LC440145
132		*Ar. sanguinolenta* Blume	T1909, THA, FU	254	LC440165
133		*Ar. suffruticosa* Ridl.	FRI75651, PM, KEP	45417*	LC440172
134		*Ar. oocarpa* Stapf	SAN156423, SBH, SAN	45334*	LC440151
135		*Ar. oocarpa* Stapf	SAN155966, SBH, SAN	45329*	LC440159
136		*Ar.* cf. *livida* Mez	S104008, SWK, SAN	530	LC440171
137		*Ar.* sp. vel aff. *livida* Mez	SAN156132, SBH, SAN	45333*	LC440160
138		*Ar.* sp. vel aff. *livida* Mez	SAN153993, SBH, SAN	45335*	LC440161
139		*Ar.* sp. vel aff. *pterocaulis* Miq.	SAN156484, SBH, SAN	45377*	LC440163
140	subgen. *Tinus* (Burm.) Mez	*Ar. attenuata* Wall. & A. DC.	FRI72352, PM, KEP	45376*	LC440174
141		*Ar. attenuata* Wall. & A. DC.	–	–	MF926184
142		*Ar. chevalieri* Pit.	V1567, VNM, FU	296	LC440115
143		*Ar. elliptica* Thunb.	FRI54116, PM, KEP	45367*	LC440133
144		*Ar. elliptica* Thunb.	MS72, SING, K	191	LC440134

Table 1.1. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID	GenBank Accession no.
145		*Ar*. *elliptica* Thunb.	–	–	JN645200
146		*Ar*. *elliptica* Thunb.	–	–	FJ980441
147		*Ar*. *hulletii* Mez	FRI77353, PM, KEP	658	LC440141
148		*Ar*. *polycephala* Wall. & A. DC.	MY10, MYN, FU	606	LC440211
149		*Ar*. *pubicalyx* Miq. var. *collinsiae* (H.R. Fletcher) C.M. Hu	C903, C, FU	261	LC440147
150		*Ar*. *pubicalyx* Miq. var. *collinsiae* (H.R. Fletcher) C.M. Hu	–	–	MF926216
151		*Ar*. *smaragdinoides* Yahara & Tagane	C758, C, FU	412	LC440170
152		*Ar*. *solanacea* Roxb.	FRI70341, PM, KEP	45387*	LC440216
153		*Ar. waitakii* C.M. Hu	–	–	MF926230
154		*Ar. waitakii* C.M. Hu	–	–	MF682177
155		*Ar.* sp. vel aff. *multipunctata* Fletcher	MY721, MYN, FU	610	LC440173
156	*Badula* A. DC.	*B*. *balfouriana* Mez	–	–	HE590600
157		*B. barthesia*A. DC.	–	–	HE590601
158		*B. borbonica* A. DC.	–	–	HF548930
159		*B. grammisticta* (Cordem.) Coode	–	–	HE548934
160		*B. nitida* (Coode) Coode	–	–	HE590642
161	*Discocalyx* Mez	*D*. *schlechteri* K. Schum.	SAJ0003, PNG, BISH	159	LC440185
162		*Discocalyx* sp.	Baker 826, PNG, K	45203*	LC440186
163		*Discocalyx* sp.	SAJ0255, PNG, BISH	161	LC440184
164	*Fittingia* Mez	*F*. *conferta* (S. Moore) Sleumer	SAJ1370, PNG, BISH	164	LC440193
165	*Hymenandra* A. DC.	*H*. *beamanii* B.C. Stone	SAN157135, SBH, SAN	498	LC440196
166		*H*. *rosea* B.C. Stone	SAN 155243, SBH, SAN	45323a	LC440195
167	*Oncostemum* A. Juss.	*O. acuminatum* Mez	–	–	HE590659
168		*O. elephantipes* H. Perrier	–	–	HE590667
169		*O. forsythii* Mez	–	–	HE590675
170		*O. gracile* Mez	–	–	HE590671
171		*O. nervosum* Baker	–	–	HE590677
172		*Oncostemum* sp.	–	–	HF548947
173		*Oncostemum* sp.	–	–	HF548945
174	*Sadiria* Mez	*Sa*. *longistyla* Ze H. Wang & H. Peng,	WZH201705_001, CHI, KUN	–	LT964874

Table 1.1. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID	GenBank Accession no.
175	*Systellantha* B.C. Stone	*Sy. brookeae* B.C. Stone	SAN157322, SBH, SAN	497	LC440215
176	*Tetrardisia* Mez	*T. corneri* Furtado	FRI73543, PM, KEP	470	LC440219
177	#*Embelia* Burm.*f.*	*E. amentacea* C.B. Clarke	FRI57778, PM, KEP	45444*	LC440188
178		*E. pergamacea* A. DC.	FRI64012, PM, KEP	45421*	LC440187
179		*E. ribes* Burm.*f.*	FRI73606, PM, KEP	45448*	LC440189
180	#*Labisia* Lindl.	*L. pumila* (Blume) Mez	MS79, SING, K	197	LC440212
181		*L. pumila* (Blume) Mez	IS265, SUM, FU	398	LC440190
182		*L. pumila* (Blume) Mez	S104008, SWK, SAN	531	LC440198
183	#*Myrsine* L.	*M. cacuminum* (Mez) Pipoly	SAJ1255, PNG, BISH	179	LC440208
184		*M. rhombata* (P. Royen) Pipoly	SAJ1261, PNG, BISH	180	LC440209
185		*M. stolonifera* (Koidz.) Walker	V4870, VNM, FU	604	LC440207
186	#*Pleiomeris* A. DC	*P. canariensis* A. DC.	–	–	KJ189026
187		*P. canariensis* A. DC.	–	–	KJ189027

[a]Origin: C=Cambodia, CAM = Cameroon, CHI= China, CON = Congo, CUL = Cultivated, KAL = Kalimantan, MYN = Myanmar, PM = Peninsular Malaysia, PNG = Papua New Guinea, SBG = Singapore Botanic Garden, SBH = Sabah, SING= Singapore, SUM = Sumatra, SWK = Sarawak, THA = Thailand, VNM = Vietnam

[b]Herbarium: BISH = Bishop Museum, E = RBGE, FU = Kyushu University, K = RBGK, KEP = The herbarium of Forest Research Institute Malaysia, KUN= Kunming Institute of Botany, Chinese Academy of Sciences, SAN = Sandakan Herbarium, SNP = Sabah National Park Herbarium, TBRC = Tropical Biosphere Research Center, University of the Ryukyus, TNS = National Science Museum, Tsukuba.

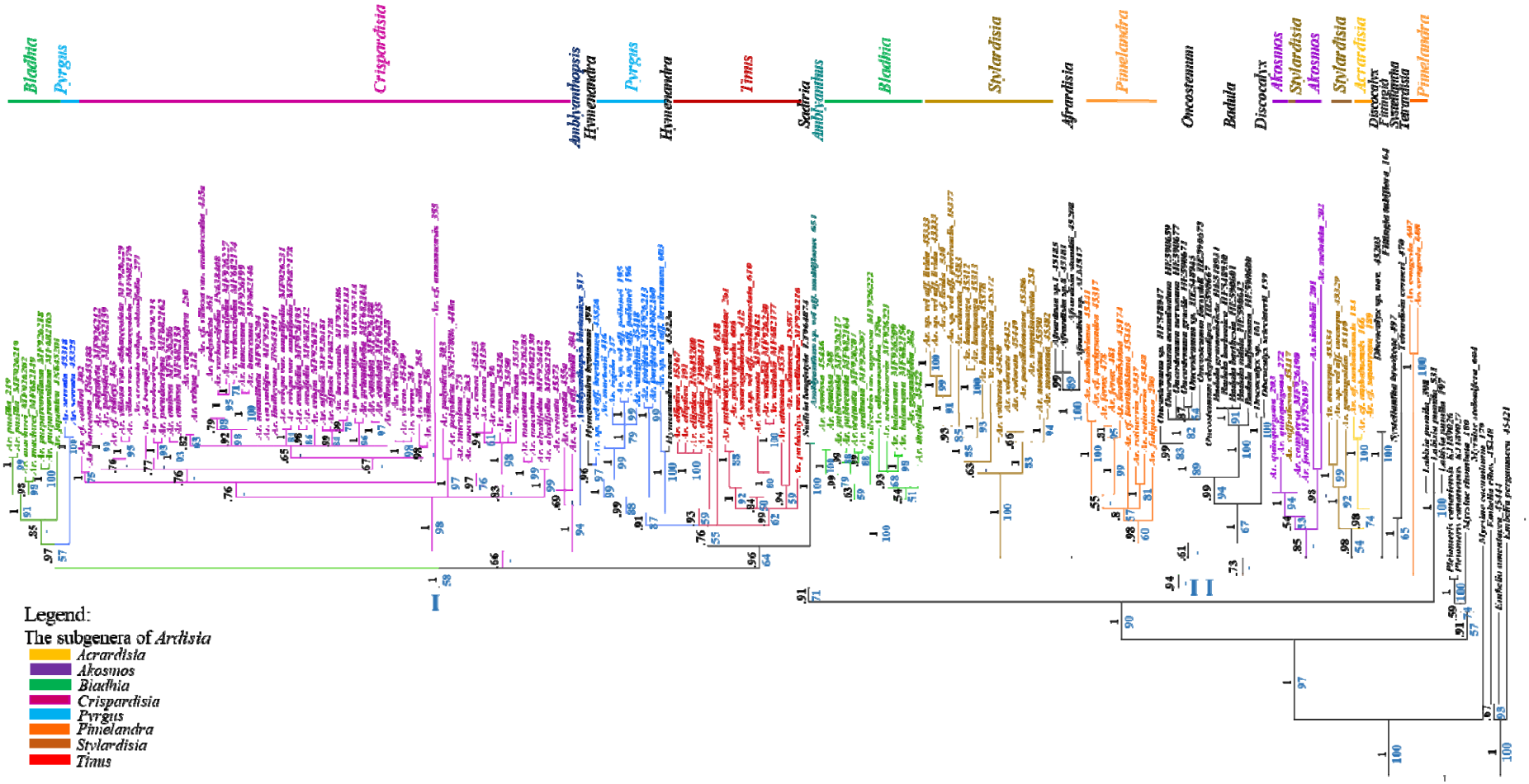

Figure 1.2. Bayesian based phylogenetic tree obtained from the analysis of nrITS sequences for *Ardisia* and relatives. Values above the branches are Bayesian posterior probabilities and values below (>50%) are ML bootstrap supports. Bootstrap supports <50% are labelled with '-'. The two major clades labelled as I and II.

CHAPTER 2

Phylogenetic inferences of leaf nodulation origin in Primulaceae-Myrsinoideae

ABSTRACT

Bacterial leaf nodule symbiosis has been hypothesized to have evolved only once in Primulaceae subfamily Myrsinoideae, but this has only been partly confirmed by previous studies which only examined species from *Ardisia* subgenus *Crispardisia*. However, leaf nodules were also found in two other genera in the subfamily, *Amblyanthopsis* and *Amblyanthus*, and this study tested the evolution of leaf nodulation within Myrsinoideae by including all three nodulating genera. Phylogenetic reconstruction of host-plant relationships from 60 samples using a combination of chloroplast *rpL*32-*trn*L and nrITS markers showed that *Ardisia* subgenus *Crispardisia* and *Amblyanthopsis* are closely related and formed a clade together, while *Amblyanthus* is sister to a clade containing some members of *Ardisia* and *Hymenandra*. The housekeeping genes *atp*D, *gyr*B, *lep*A and *rec*A amplified from the leaf nodulating endosymbionts suggested they form a clade within *Burkholderia*. This indicates only a single genus *Burkholderia* is able to make symbiotic associations with nodulated genera within Myrsinoideae. The reconstructed phylogenetic tree of the endosymbionts also suggested that the leaf nodulating symbionts for the former two nodulated genera were closely related and clustered together, while the leaf nodulating endosymbionts of *Amblyanthus* positioned as a sister to the clade consisting of leaf nodulating endosymbionts of Rubiaceae. The current results suggest that the leaf nodule

symbiotic association between nodulated genera and their endosymbionts, *Burkholderia,* was formed twice independently throughout the evolutionary history of Primulaceae. PCR detection confirmed the presence of bacterial symbionts only in nodule parts and the seeds from nodulated taxa and none from non-nodulated taxa. Therefore, the present study corroborated previous observations in which symbiotic bacteria are restricted only to the nodulated taxa.

2.0 INTRODUCTION

Bacterial leaf nodule symbiosis or leaf nodulation, characterized by the presence of specialized nodules along the leaf margin in which endosymbionts are housed, is a unique plant-bacterial association in angiosperms. This symbiotic association is unique because of the intimate interaction between plant and bacteria in which the endosymbionts are vertically transmitted between plant generations via seeds, but also because it is known from only approximately 500 species of Rubiaceae and Primulaceae subfamily Myrsinoideae (Miller 1990– the latter as Myrsinaceae prior to the Angiosperm Phylogeny Group classification). Nodulated species are reported mostly from Rubiaceae, primarily in three distantly related genera *Pavetta* L. (Ixoroideae-Pavetteae; c. 350 from 400 species with nodules; De Block et al. 2015), *Psychotria* L. (Rubioideae-Psychotrieae; c. 80/1400 species; Anderson 2002) and *Sericanthe* Robbr. (Ixoroideae-Coffeeae; c. 12/17 species; Lemaire et al. 2011c). In contrast to Rubiaceae, leaf nodulation is rare in Myrsinoideae and has been reported in less than 100 species in the following groups: the c. 70 species of *Ardisia* subgenus *Crispardisia* (see Mez 1902, Sleumer 1988, Stone 1989, Larsen & Hu 1996, Chen & Pipoly 1996, Hu & Vidal 2004), the four species of *Amblyanthus* A. DC., and the single species in the monotypic genus *Amblyanthopsis* Mez (Miller 1990).

Previous studies on bacterial leaf nodule symbiosis for Myrsinoideae, however, have focused on *Ardisia* subgenus *Crispardisia* (Lemaire et al. 2011a, Ku & Hu 2014), without thoroughly testing species from other subgenera, and especially not from sister groups to subgenus *Crispardisia*. *Ardisia* is the largest genus within Primulaceae comprising from c. 450 to over 1000 species (Stevens 2001, Frodin 2004). The genus is found predominantly in the understory of moist tropical and subtropical rain forests and material for the genus is relatively easy to obtain. The presence of bacterial symbionts, identified as *Burkholderia*

Yabuuchi et al. (Yabuuchi et al. 1992), has been studied in the leaf nodulated taxa of subgenus *Crispardisia* using samples only from east Asia (China, Taiwan, Japan: cultivated material in Lemaire et al. (2011b), field collected material in Ku & Hu (2014)). The subgenus, however, is widely distributed from Japan through China (reaching Nepal) and south into Malesia reaching the islands of Sumatra, Borneo and the Philippine archipelago. The other two nodulated genera are so far recorded only from Assam, India and study material has been difficult to obtain and the presence of bacteria, and if present they are members of the genus *Burkholderia*, in the leaf nodules of *Amblyanthopsis* and *Amblyanthus* is still uncertain (Lemaire et al. 2011b).

Previous phylogenetic analyses showed both the nodulated taxa of *Ardisia* and the bacterial symbionts as being monophyletic groups (Lemaire et al. 2011a, Ku & Hu 2014). These studies revealed that the leaf nodulation has evolved only once in *Ardisia* and suggesting a single origin of leaf nodulation throughout the history of symbiotic association between *Ardisia* and its bacterial symbionts (Lemaire et al. 2011a, Ku & Hu 2014). To further clarify evolution of leaf nodulation within the Primulaceae, this study includes representative species of *Amblyanthopsis* and *Amblyanthus* recently found and collected from Myanmar, as well as additional samples of subgenus *Crispardisia* from Indo-China and Malesia, specifically Cambodia, Myanmar, Vietnam, Thailand and Malaysia (Sabah and Peninsular Malaysia). In addition, taxa from other genera in the Myrsinoideae and species from additional *Ardisia* subgenera were examined to verify if endosymbionts are restricted to the three groups as currently reported. Phylogenetic trees are produced to infer relationships among the nodulated hosts and their symbionts to investigate the evolution of leaf nodulation in Myrsinoideae; in addition, we identify the bacterial symbionts of *Amblyanthopsis* and *Amblyanthus*.

2.1 MATERIALS AND METHODS

TAXON SAMPLING

PHYLOGENETIC ANALYSIS

Plant species

For this study, 23 nodulated samples of *Ardisia* subgenus *Crispardisia* and a representative each for nodulated genera *Amblyanthopsis* and *Amblyanthus* were included in the analysis (Table 2.1a). Another 40 representatives of non-nodulated taxa from *Ardisia* and its closely related genera were analyzed together to investigate phylogenetic relationship among the nodulated genera to clarify evolution of leaf nodulation in Myrsinoideae. The materials were originated from the living collection of Royal Botanic Garden, Edinburgh (RBGE) or newly collected from the wild. Reference specimens of the materials are deposited in the herbaria/institutes BISH, K, KEP, FU, NYBG, SAN, SNP, TBRC and TNS. Other relevant data such as voucher information and the samples identification are provided in Table 2.1a. Samples identification was done in Royal Botanic Gardens, Kew (RBGK) and Royal Botanic Garden, Edinburgh (RBGE), and undertaken with the use of relevant literatures (for examples: Furtado 1969, Giri et al. 2002, Hu 2002, Larsen & Hu 1996, Sleumer 1988, Stone 1989, 1990). Types at K and E, and images of types from Global Plants JSTOR (http://plants.jstor.org/) and the BioPortal of Naturalis Biodiversity Center (http://bioportal.naturalis.nl/) were also consulted.

Bacterial symbionts

The samples used in the phylogenetic tree reconstruction based on housekeeping genes *gyr*B and *rec*A for bacterial symbionts of leaf nodulated genera Myrsinoideae are listed in the Table 2.1b. Sixteenth accessions of leaf nodulating endosymbionts were newly generated in

this study, representing *Amblyanthopsis* (a single accession), *Amblyanthus* (two accessions) and *Ardisia* (13 accessions), supplemented by 67 accessions of non-nodulating bacterial sequences of *Burkholderia*, 2 and 122 accessions of leaf nodulating endosymbionts of Myrsinoideae and Rubiaceae, respectively, which were generated previously from the study by Lemaire et al. (2011b). Two accessions of *Cupriavidus* Makkar & Casida were chosen as the outgroup (Platero et al. 2016).

LEAF NODULATING ENDOSYMBIONT IDENTIFICATION

The occurrence and identification of leaf nodulating endosymbionts of Myrsinoideae was carried out using both nodulated and non-nodulated taxa with 25 and 6 samples, respectively (see Table 2.1c). To show host specificity, in which a host harbors only a single bacterium, and to ensure only the true endosymbiont was amplified, 14 out of 25 nodulated taxa were available with at least two replicates per sample from the same individual or more than one individual per taxa (see Table 2.1d). Both vegetative and reproductive parts from untreated, dried herbarium specimens were used to investigate the presence of bacterial symbionts (see Figure 2.1a). For vegetative parts, bacterial symbionts were detected using both non- and sterilized lamina/twig. For reproductive parts, sterilized floral parts of three selected taxa and seed of *Ardisia crenata* were examined. To ensure the floral parts were intact without contamination from the environment, flower buds were used in this study. The surface-sterilization process was done by first cleansing the specimen surface with sterile water until the dirt was removed and then wiping with 70% ethanol.

DNA EXTRACTION, AMPLIFICATION, PURIFICATION AND SEQUENCING

For leaf samples, total genomic DNA was extracted from either silica dried leaves or

herbarium specimens, while bacterial DNA was obtained from excised leaf nodules or seeds using the method described in Doyle & Doyle (1987). The amplification of DNAs for leaf samples was conducted for internal transcribed spacer region of the nuclear ribosomal DNA (nrITS) and an intergenic noncoding region of chloroplast DNA (*rpL*32-*trn*L), while bacterial DNAs were amplified using housekeeping genes *atp*D, *gyr*B, *lep*A and *rec*A PCR primer sets (see Table 2.1e for the primers detail). PCR were performed using either TaKaRa Ex Taq (Takara Bio, Shiga, Japan) or KAPA HiFi DNA polymerase (KAPA Biosystems, Wilmington, USA) protocols. For TaKaRa Ex Taq protocol, PCR were performed at 12.5 μL volumes containing 0.4 μM of each of the primers, 10X Ex Taq buffer, 2.5mM MgCl2, 250 μM of each dNTP, 5U Ex Taq DNA Polymerase and 1 μL of template DNA. For KAPA HiFi protocol, PCR were performed at 10 μL volumes containing 2.5 μL of KAPA HiFi mixture reaction, 0.4 μM of each of the primers and 1 μL of template DNA. The amplification profile using Ex Taq for nrITS of host plants was 35 cycles of 95ºC for 30 s, 56ºC for 30 s, 72ºC for 3 min, preceded by initial denaturation at 95ºC for 5 min and followed by the final extension at 72ºC for 7 min. For cpDNA region of host plants and housekeeping genes of symbiont bacteria, PCR were performed using KAPA HiFi protocol and their amplification profile was 35 cycles of 98ºC for 20 s, 60ºC for 15 s, 72ºC for 15 s, preceded by initial denaturation at 95ºC for 3 min and followed by the final extension at 72ºC for 3 min. PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA). Cycle sequencing-reactions were prepared with BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) by modifying the manufacturer's protocol, and analyzed on an ABI 3500 DNA sequencer (Applied Biosystems) and partly by Eurofins Genomics (Tokyo, Japan).

DNA ALIGNMENT AND PHYLOGENETIC ANALYSES

DNA sequence chromatograms were edited using the DNA Baser Sequence Assembler

v.4.36.0 (http://www.dnabaser.com) software. The alignment of sequences was done using the MUSCLE algorithm (Edgar 2004) in MEGA7 software (Kumar et al. 2016). Finally, the sequences were manually trimmed to form a matrix data set with uniform length. Phylogenetic trees were obtained using Bayesian inference (BI) and maximum likelihood (ML) methods. BI analyses were run on Mr. Bayes 3.2.5 (Ronquist et al. 2012) after determining the appropriate model of evolution with the jModelTest v.2.1.1 (Posada 2008) under the Akaike information criterion. Model test selected for the different datasets the following models of evolution: nrITS = SYM+I+G, *rpL*32-*trn*L = GTR+G, *atp*D = GTR+G, *gyr*B = HKY+G and *rec*A = GTR+G. In the combined analysis, mixed-model approach was used. The concatenated datasets were partitioned and the same models were assigned to separate partitions as selected for single analyses. Four iterations consisting of five million Monte Carlo Markov Chains were run discarding 25% of the generations as burn-in. The ML phylogenetic analysis was performed in RaxML v.8.0.26 (Stamatakis 2008) with raxmlGUI (Silvestro & Michalak 2012) using 10000 rapid bootstraps under the GTR gamma model. To verify whether there was a significant amount of conflict between nrITS and the cpDNA regions, the test of Farris et al. (1995) was performed using the partition-homogeneity test as implemented in PAUP*4.0a (Swofford 2002). The test was performed with 10000 replicates. Each replicate consisted of a heuristic search of random taxon addition and TBR branch swapping.

2.2 RESULTS

IDENTIFICATION AND DISTRIBUTION OF LEAF NODULATING ENDOSYMBIONT

This study shows that the genes of bacterial symbionts were amplified only from nodulated taxa and none from non-nodulated members as shown in Table 2.2a. The PCR detection of bacterial symbionts was tested on leaf nodules as well as non-nodulated areas of lamina and twigs from nodulated and non-nodulated taxa (see Figure 2.2a). Where endosymbionts were present in vegetative parts, PCR detection revealed bacterial symbionts only in the nodulated parts and absent in the non-nodulated areas of laminas and twigs. PCR detection of young floral parts showed that bacterial symbionts do not occur in pedicels, bracts, calyx, corolla or stamens (see Figure 2.2a). Among the four housekeeping genes, amplification using *gyr*B is more successfully, followed by *lep*A and *atp*D compared to *rec*A. Using the GenBank BLASTn search, DNA sequences retrieved for all three nodulated genera of Myrsinoideae were found to show 98% to 100% similarity to *Burkholderia* accessions.

The bacterial symbionts were successfully amplified from the first and second replicates for almost all taxa examined here, except, especially, the second duplicate of *Amblyanthus* (Table 2.2b). DNA sequences of the first and second replicates are 99% to 100% identical. Likewise, a high level of similarity of symbiont DNA sequences was obtained each from the nodules [B26a (nodule)] and seed [B26b (seeds)] of *Ardisia crenata*. The symbiont sequences from *Amblyanthus* and *Ardisia* samples were distinct and recovered in different clades in phylogenies using all three markers (Figure 2.2b) and indicate that each host harbors their own specific *Burkholderia* lineage supporting previous observations (e.g. Miller 1990, Lemaire et al. 2011a, Ku & Hu 2014).

HOST PLANT PHYLOGENY

The partition homogeneity test (*p*-value = 0.01) indicates that the nrITS and *rpL*32-*trn*L trees have significantly different topologies but major branching patterns are congruent and therefore the data can be combined for phylogenetic analysis. The alignment of the concatenated dataset yielded 1622 bp in length (654 bp for nrITS and 968 bp for the *rpL32-trnL* regions). The nrITS provided more parsimony informative characters (217 bp) than *rpL32-trn*L (129 bp). The topology of the Bayesian tree is highly congruent to the maximum likelihood tree and differs only in support values; therefore, only the Bayesian tree is presented (Figure 2.2c). Among the three nodulated groups, *Ardisia* subgenus *Crispardisia* and *Amblyanthopsis* form a weakly supported clade (PP = 0.93 / BS = 56%), while *Amblyanthus* is placed at different position in the tree as a sister to some members of non-nodulated *Ardisia* (subgenera *Tinus* and *Pyrgus*) and *Hymenandra* (PP = 0.93 / BS = 56%).

ENDOSYMBIONT PHYLOGENY

Five phylogenetic trees of endosymbionts were generated. The individual phylograms using housekeeping genes of *atp*D, *gyr*B and *lep*A were presented in Figure 2.2b. All these three markers showed that the symbiont sequence recovered from *Amblyanthus* forms a well-supported clade with the symbiont from *Pavetta* (Rubiaceae) and that these are sister to all other *Burkholderia* sequences from *Amblyanthopsis* and *Ardisia* subgenus *Crispardisia*, as well as soil bacteria (*Burkholderia glathei* in the *lep*A phylogeny). Another two enlarge trees of endosymbionts were generated by including more samples viz. concatenated tree of *rec*A and *gyr*B with 171 accessions and an independent tree of *gyr*B with 206 accessions. The

independent tree of *gyr*B was generated to include accessions of non-nodulated endosymbionts of *Psychotria*, gut symbionts of insects and, soil and plant associated *Burkholderia*.

The enlarge *gyr*B tree comprised 422 bp in length and 256 sites were variable, 226 sites of them were phylogenetically informative. While the concatenated alignment for *rec*A and *gyr*B datasets comprised 980 bp in length and 534 sites were variable, 442 sites of them were phylogenetically informative. Bayesian and maximum likelihood analyses for each dataset yielded similar tree topology. Thus, only the Bayesian tree is presented for each dataset as shown in Figure 2.2d (*gyr*B + *rec*A) and Figure 2.2e (*gyr*B).

The leaf nodulating endosymbionts of Myrsinoideae are obviously not monophyletic and split into two main groups. The first group comprises endosymbionts of *Amblyanthus* (the two samples form a well-supported branch indicated by A1, PP = 1 / BP = 100%) and is positioned as sister to all endosymbionts of Rubiaceae (Clade A2, PP = 1 / BS <50%) in Figure 2.2d but as an immediate sister to leaf nodulating endosymbiont of *Psychotria kikwitensis* in Figure 2.2e. This relationship corroborates that recovered in each of three gene trees of leaf nodulating endosymbionts (Figure 2.2b). A strongly supported Clade (indicated by B) was formed by the endosymbionts of *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* (PP =1 / BS = 100%) and sister to soil associated *Burkholderia glathei* in Figure 2.2d. Similarly, the endosymbionts of *Amblyanthopsis* formed a monophyletic clade with *Ardisia* subgenus *Crispardisia* (PP = 1 / BS = 77%) in Figure 2.2e, and positioned as immediate sister to soil associated *Burkholderia udeis*.

Ku & Hu (2014) identified *Burkholderia sordidicola* as sister to the endosymbionts of *Ardisia* subgenus *Crispardisia* using 16S rDNA marker, but in this study, we have used the sequences *rec*A and *gyr*B. Problematically, when we used those regions of *rec*A and *gyr*B available in GenBank for *Burkholderia sordidicola*, the species was nested within 'the

Burkholderia cepacia complex (BCC) opportunistic pathogen clade' (Mahenthiralingam et al. 2008), and so we have not included that species in the reconstruction of *rec*A + *gyr*B tree due to uncertainty over its identification as given on GenBank.

2.3 DISCUSSION

IDENTIFICATION AND DISTRIBUTION OF LEAF NODULATING SYMBIONTS

The current results are, to date, the first to demonstrate that the two nodulated genera *Amblyanthopsis* and *Amblyanthus* harbour their own symbiotic bacteria. Similarly, this study reveals for the first time the presence of symbiotic bacteria in nodulated *Ardisia* from Indo-China and Malesia. The association between *Burkholderia* and two nodulated genera *Amblyanthus* and *Amblyanthopsis* is another case of astounding adaptability of *Burkholderia* taxa to plants.

Previous studies have shown no symbiotic bacteria in the leaves of several non-nodulated *Ardisia* (Miehe 1911, Ku & Hu 2014), but these taxa were distantly related to the nodulated groups. Therefore, this study used several non-nodulated taxa from the same group with *Amblyanthopsis, Amblyanthus* and *Ardisia* subgenus *Crispardisia* in Clade I (see Figure 2.2c) to determine the presence of bacteria. Results show bacteria do not occur in *Ardisia serrata*, *Ar*. sp. vel aff. *breviramea*, *Hymenandra beamanii* and *H*. *rosea* (as shown in Table 2.2a, Figure 2.2a), indicating bacterial symbionts are restricted to nodulated taxa only. This is in contrast to the presence of leaf associated *Burkholderia* endophytes in non-nodulated species of *Psychotria* (Lemaire et al. 2012a).

The PCR detection results show that the amplified DNAs from the leaf nodules using bacterial gene primer sets are from the true symbionts for each nodulated taxa examined here (Figure 2.2a). Symbiotic bacteria DNAs were not amplified from non-nodulated taxa, and was also absent in the non-nodulated areas of lamina and twig of nodulated taxa. If the amplified bacterial DNAs from the nodule structures are either contaminant or phyllosphere

bacteria, then it would also be amplified from non-nodulated taxa and the non-nodulated areas of lamina and twig.

This study is the second to observe symbiotic bacteria in reproductive parts after Ku & Hu (2014). The PCR detection of bacterial symbionts from lamina and floral parts of nodulated taxa *Ardisia polysticta* and *Ar.* sp. vel aff. *clemensii* using gyrB is consistent with the observation by Ku & Hu (2014) in which no symbiotic bacteria are detected in the calyx and corolla, and additionally, this study demonstrates for the first time that there are no symbiotic bacteria in the pedicels and bracts (Figure 2.1a). In contrast to the previous study, no symbiotic bacteria are observed in the stamens from the two nodulated species examined here (*Ardisia polysticta* and *Ar*. cf. *virens*). Although a weak DNA band on the PCR products was observed by Ku & Hu (2014, Figure 4], they were uncertain if the amplified DNA in the stamens was associated with symbiotic bacteria exposed to bacterial mucilage during floral development (Miller 1990). In addition, Ku & Hu (2014) did not mention if they used mature flowers at anthesis (which tend to exposed by pollinators) or flower buds to detect the presence of symbiotic bacteria in the reproductive parts, and it is possible that PCR products obtained from the stamen were due to bacteria transferred by pollinators. Further observations are needed to verify this with both open, mature flowers and unopened flower buds to determine the presence of symbiotic bacteria in the stamen. This observation will be useful to verify if there is paternal contribution of symbionts in Myrsinoideae (Lemaire et al. 2011a, Ku & Hu 2014), but this is not strongly supported by our results, and a purely maternal (ovules and seeds) mechanism of symbiont vertical transfer is more likely.

DNA sequences of symbionts obtained from the nodules and seeds of *Ardisia crenata* were highly identical, as were those obtained from nodules using different individuals of *Amblyanthus* (Figure 2.2b (B) and (C)), congruent with previous studies revealing that each host is infected by a single bacterium species (Yabuuchi et al. 1992). Being distantly related

from the other two nodulated genera, and consistently recovered, phylogenetic position of the *Amblyanthus* endosymbionts also suggests that endosymbiotic bacteria in *Amblyanthus* are heritable symbionts (i.e. vertically transferred), similar to the mechanisms in nodulated taxa of *Ardisia*.

RELATIONSHIP, EVOLUTION AND ORIGIN OF LEAF NODULATION IN MYRSINOIDEAE

This study is the first attempt to investigate the phylogenetic relationships among nodulated and non-nodulated genera using a combination of nrITS and chloroplast DNA markers. The host plant phylogenies showed a close relationship between nodulated genera *Amblyanthopsis* and *Ardisia* subgenus *Crispardisia* in Clade A1, whilst *Amblyanthus* was sister to some non-nodulated genera of *Ardisia* (including species from subgenera *Pyrgus* and *Tinus*) and *Hymenandra* (Figure 2.2c, see Table 2.1a for subgenera of the taxa examined) in Clade A2. Similar results were previously obtained using nrITS alone in which *Ardisia* subgenus *Crispardisia* shows affinity with *Amblyanthopsis*, whereas *Amblyanthus* with above mentioned non-nodulated genera (see Chapter 1).

Based on morphological observation, the leaves and lateral inflorescence bearing branches arrangement are the main synapomorphic characteristics to define the taxa grouped in Clade I. The leaves are arranged in pseudowhorls along the main stem and the lateral inflorescence bearing branches are normally crowded terminally between the pseudowhorls. However, the pseudowhorls leaves become more disperse with age and the lateral inflorescence bearing branches are subtended by a simple leaf that distinguished *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* from *Amblyanthus*. *Amblyanthus*, as well as

members of *Ardisia* (subgenera *Tinus* and *Pyrgus*) and *Hymenandra*, have leaves in pseudowhorls on the main stem which remain apparent at all stages of the life cycle. *Amblyanthus* can be further distinguished by having axillary inflorescences on lateral branches while *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* can be identified by terminal, or sometimes lateral inflorescences either on lateral branches or on the main stem.

The non-monophyletic relationship of nodulated genera suggests two possible hypotheses regarding evolution of leaf nodulation in Myrsinoideae (Figure 2.2c). First, it might have evolved only once on branch A, inferring that leaf nodulation was lost in the *serrata-pusilla* and *attenuata-breviramea* lineages. An alternative hypothesis is that leaf nodulation evolved independently on branches A1 and A2 with the symbiotic association occurring only in the *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* clade and *Amblyanthus*. The second hypothesis seems more likely because it is an obligate symbiotic association in which both partners depend on each other to survive (Miller 1990). The bacteria-free plants produced by heat-treatment or antibiotics do not develop normally, are of slow growth, and usually produce abnormal or deformed leaves, and eventually die (Miller 1990). Moreover, no endosymbiotic bacteria were detected from several non-nodulated taxa examined in this study (Table 2.2a). Additionally, the bacterial phylogeny gives support for this hypothesis in which *Amblyanthus*-nodulating endosymbionts are distantly related to the other nodulating endosymbionts of Myrsinoideae (Figure 2.2d). Based on these reasons, it can be hypothesized that the leaf nodulation has evolved at least twice in Myrsinoideae and with separate single common ancestors for the *Ardisia* subgenus *Crispardisia* + *Amblyanthopsis* clade and *Amblyanthus*, respectively.

THE ORIGIN OF BACTERIAL LEAF NODULE SYMBIOSIS BETWEEN *BURKHOLDERIA* AND MYRSINOIDEAE

The phylogenies of leaf nodulating endosymbionts of Myrsinoideae reveal that the bacterial leaf nodule symbiosis has two different origins which evolved independently as indicated by the non-monophyletic relationship of *Ardisia* subgenus *Crispardisia* + *Amblyanthopsis*- and *Amblyanthus*-nodulating endosymbionts (Figure 2.2d). This scenario probably explained by Lemaire et al. (2011b) who speculated frequent infections of free-living bacteria occurred from the environment to nodulated Rubiaceae and Myrsinoideae taxa. Based on current observations, it can be hypothesized that the ancestor of the nodulating endosymbionts of *Amblyanthus* is distantly related from the ancestor of the nodulating endosymbionts of *Ardisia* subgenus *Crispardisia* + *Amblyanthopsis*.

More interestingly, the *Amblyanthus*-nodulating endosymbiont is recovered as a sister to all leaf nodulating endosymbionts of Rubiaceae in Figure 2.2d, and positioned as immediate sister to *Burkholderia kikwitensis* in Figure 2.2e. The affinity between leaf nodulating endosymbionts of *Amblyanthus* and *Psychotria kikwitensis* from Congo (Africa) possibly can be explained by 1) a common free-living ancestor from environment that evolved the ability to form bacterial leaf nodule symbiosis with both *Amblyanthus* and *Psychotria kikwitensis* or 2) two closely related free-living ancestors from the environment independently evolved the ability to form symbiosis with *Amblyanthus* and Rubiaceae. However, additional samples with suitable candidate taxa especially from soil associated bacteria are needed for future study to verify the current opinion. The inclusion of soil associated bacteria in the future study is suggested because the leaf nodulation was hypothesized triggered by aridification in 3–9 Mya that might cause soil associated bacteria to form symbiosis with plant to survive from the drought season (Lemaire et al. 2011b).

Moreover, the leaf nodulating endosymbionts of Myrsinoideae and Rubiaceae were shown to have affinities with soil associated bacteria in Pinto-Carbó et al. (2018).

2.4 CONCLUSION

In conclusion, this study is the first attempt to investigate the evolution of leaf nodulation in Myrsinoideae at family level. It can be hypothesized that the leaf nodulation of Myrsinoideae have two origins: first, in the *Amblyanthopsis-Crispardisia* clade and secondly in the genus *Amblyanthus*. Hence, the bacterial leaf nodule symbiotic association between nodulated genera and their endosymbionts, *Burkholderia,* was formed twice independently throughout the evolutionary history of Primulaceae. Additionally, investigation of symbiotic bacteria associated with nodulated genera *Amblyanthopsis* and *Amblyanthus* were also first effort done in this study. More interestingly, the current finding shows unexpected result, in which the leaf nodulating endosymbionts of *Amblyanthus* more closely related to the leaf nodulating endosymbionts of Rubiaceae from different family compared to the other nodulated genera of Myrsinoideae.

2.5 REFERENCES

Anderson L. 2002. Relationships and generic circumscriptions in the *Psychotria* complex (Rubiaceae, Psychotrieae). Syst. Geogr. Plants **72**: 167–202.

Chen J. and Pipoly J.J. 1996. Myrsinaceae. *In*: Wu, Raven P.H. (eds.) Fl China. Science, Beijing. **15**: 1–38.

De Block P., Razafimandimbison S.G., Janssens S., Ochoterena H., Robbrecht E. and Bremer B. 2015. Molecular phylogenetics and generic assessment in the tribe Pavetteae (Rubiaceae). Taxon **64** (1): 79–95. Available from doi: 10.12705/641.19

Doyle J.J. and Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11–15.

Edgar R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32** (5): 1792–1797. doi: 10.1093/nar/gkh340

elnour a-A. 2017. The Impact of Genomics Evolution and Genomic Island in *Burkholderia pseudomallei*. MOJ Proteomics Bioinform. **6** (4): 00199.

Farris J.S., Källersjö M., Kluge A.G. and Bult C. 1995. Testing significance of incongruence. Cladistics **10**: 315– 319. doi: 10.1111/j.1096-0031. 1994.tb00181.x

Frodin D.G. 2004. History and concepts of big plant genera. Taxon **53** (3): 753–776.

Furtado C.X. 1959. Some new or noteworthy species in Malaysia. Gard. Bull. Singapore **17**: 279–311.

Giri G.S., Das S.K. and Chowdary H.J. 2002. A new species of *Amblyanthus* A. DC., family Myrsinaceae from Arunachal Pradesh, India. J. Bombay Nat. Hist. Soc. **99** (3): 471–473.

Hu C.M. 2002. New species of *Ardisia* (Myrsinaceae) from Malesia. Blumea **47**: 493–512.

Hu C.M. and Vidal J.E. Myrsinaceae. 2004. *In*: Morat, Ph. (ed.), Flore du Cambodge du Laos et de Viêtnam 32. 288 pp. Muséum National D'Histoire Naturelle, Paris.

Ku C. and Hu J.M. 2014. Phylogenetic and cophylogenetic analyses of the leaf-nodule symbiosis in Ardisia subgenus Crispardisia (Myrsinaceae): evidence from nuclear and chloroplast markers and bacterial *rrn* operons. Int. J. Plant Sci. **17** (1): 92–109. Available from doi: 10.1086/673306.

Kumar S., Stecher G. and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molec. Biol. Evol. **33** (7): 1870–1874. Available from doi: 10.1093/molbev/msw054

Larsen K. and Hu C.M. 1996. Myrsinaceae. Fl. Thailand **6** (2): 81–178.

Lemaire B., Smets E. and Dessein S. 2011a. Bacterial leaf symbiosis in *Ardisia* (Myrsinoideae, Primulaceae): molecular evidence for host specificity. Res. Microbiol. **162**: 528–534. Available from doi: 10.1371/journal.pone.0024430

Lemaire B., Vandamme P., Merckx V., Smets E. and Dessein S. 2011b. Bacterial leaf symbiosis in angiosperms: host specificity without co-speciation. PLoS ONE **6** (9): e24430. Available from doi:10.1371/journal.pone.0024430

Lemaire B., Oevelen S.V., De Block P., Verstraete B., Smets E., Prinsen E. and Dessein S. 2011c. Identification of the bacterial endosymbionts in leaf nodules of *Pavetta* (Rubiaceae). Int. J. Syst. Evol. Microbiol. **62**: 201–209. Available from doi:10.1111/j.1574-6941.2012.01356.x

Lemaire B., Lachenaud O., Persson C., Smets E. and Dessein S. 2012a. Screening for leaf-associated endophytes in the genus *Psychotria* (Rubiaceae). FEMS Microbiol. Ecol. **81**: 364–372.

Lemaire B., Janssens S., Smets E. and Dessein S. 2012b. Endosymbionts transmission mode in bacterial leaf nodulation as revealed by a population genetic study of *Psychotria leptophylla*. Appl. Environ. Microbiol. **78** (1): 284– 287. Available from doi: 10.1128/AEM.06130-11

Mahenthiralingam E., Baldwin A. and Dowson C. 2008. *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology. J. Appl. Microbiol. **104** (6): 1539–1551. Available from doi: 10.1111/j.1365-2672.2007.03706. x.

Mez C. Myrsinaceae. 1902. *In*: Engler A. (ed.), Das Pflanzenreich, Heft 9 (IV. Fam. 236). pp. 1–437. W. Engelmann, Leipzig.

Miehe H. 1911. Die Bakterienknoten an den Blatträndern der *Ardisia crispa* A. DC. Abhandl Mat-Phys Kl Kön Sächs Ges Wiss Leipzig. **32**: 399–431.

Miller I.M. 1990. Bacterial leaf nodule symbiosis. Adv. Bot. Res. **17**: 163–234.

Pinto-Carbó M., Gademann K., Eberl L. and Carlier A. 2018. Leaf nodule symbiosis: function and transmission of obligate bacterial endophytes. Current Opinion in Plant Biology **44**: 2 3–31.

Platero R., James E.K., Rios C., Iriarte A., Sandes L., Zabaleta M., Battistoni F. and Fabiano E. 2016. Novel *Cupriavidus* strains isolated from root nodules of native Uruguayan Mimosa species. Appl. Environ. Microbiol. **82**: 3150–3164. doi: 10.1128/AEM.04142-15

Ronquist F., Teslenko M., Van Der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M.A. and Huelsenbeck J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**: 539–542.

Shaw J., Lickey E.B., Schilling E.E. and Small R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am. J. Bot. **94** (3): 275–288.

Silvestro D. and Michalak I. 2012. raxmlGUI: a graphical front-end for RAxML. Org. Divers. Evol. **12**: 335. Available from doi: 10.1007/s13127-011-0056-0

Sleumer H. 1988. A revision of the genus *Ardisia* Sw. (Myrsinaceae) in New Guinea. Blumea **33**: 115–140.

Spilker T., Baldwin A., Bumford A., Dowson C.G., Mahenthiralingam E. and LiPuma J.J. 2009. Expanded multilocus sequence typing for *Burkholderia* species. J. Clin. Microbiol. **47**: 2607–2610.

Stamatakis A. 2008. RAxML-VI-HPC: a tool for phylogenetic analysis and post- analysis of large phylogenies. Bioinformatics **30**: 1312–1313.

Stone B.C. 1989. Myrsinaceae *In*: Ng F.S.P. (ed.) Tree Flora of Malaya 4. pp. 264–284. Longman, Malaysia.

Stone B.C. 1990. Studies in Malesian Myrsinaceae, V. Additional new species of *Ardisia* Sw. Proc Acad. Nat. Sci. Philadelphia **142**: 21–58.

Stevens P.F. 2001 onwards. Angiosperm Phylogeny Website. Version 14, July 2017. http://www.mobot.org/MOBOT/research/APweb/.

Swofford D.L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods) version 4. Sinauer Associates, Sunderland, Massachusetts.

White T.J., Bruns T.D., Lee S.H. and Taylor J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis M.A., Gefland D.H., Sninsky J.J. and White T.J. (eds.) PCR protocols: A guide to methods and application. pp. 315–322. Academic Press, San Diego.

Yabuuchi E., Kosako Y., Oyaizu H., Yano I., Hotta H., Hashimoto Y., Ezaki T. and Arakawa M. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36**: 1251–1275. doi:10.1111/j.1348-0421.1992.tb02129.x

Table 2.1a. List of samples used in the host phylogenetic analysis. The numbering ID with asterisk indicating a given number for the leaf tissue extracted from RBGK whereas no asterisk referring to the given tube's number for the samples extracted in Chiba University. The outgroup indicates by '#'.

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID
1	*Amblyanthopsis*	*Amblyanthopsis bhotanica*	KEA883, MYN, NYBG	517
2	*Amblyanthus*	*Amblyanthus* sp. vel aff. *multiflorus*	MY881, MYN, TNS	654
	Ardisia			
3	subgen. *Acrardisia*	*Ar*. cf. *amboinensis*	SAJ1433, PNG, BISH	184
4		*Ar*. cf. *amboinensis*	SAJ1055, PNG, BISH	166
5	subgen. *Akosmos*	*Ar. quinquegona*	2015/04-J, JPN, TBRC	203
6	subgen. *Bladhia*	*Ar*. *albomaculata*	V3270, VNM, FU	429a
7		*Ar. pusilla*	FU201507-8, JPN, FU	219
8		*Ar. demissa*	FRI72048, PM, KEP	45436*
9		*Ar. demissa*	T3059, THA, FU	268
10	subgen. *Crispardisia*	*Ar*. cf. *cadieri*	V1905, VNM, FU	302
11		*Ar. corymbifera*	T907, THA, FU	250
12		*Ar*. *crenata*	FRI73583, PM, KEP	482
13		*Ar*. *crenata*	SNP37015, SBH, SNP	455a
14		*Ar. crenata* subsp. *obtusifolia*	T4630, THA, FU	271
15		*Ar. crenata* subsp. *obtusifolia*	T4407, THA, FU	653
16		*Ar*. *crispa*	FU201507-1, JPN, FU	212
17		*Ar. maculosa*	T4760, THA, FU	273
18		*Ar. maculosa*	T3059, THA, FU	263
19		*Ar. polysticta*	V1937, VNM, FU	303
20		*Ar. polysticta*	V3054, VNM, FU	361
21		*Ar. polysticta*	2015/03-J, JPN, TBRC	200
22		*Ar. polysticta*	SNP37006, SBH, SNP	448a
23		*Ar. rosea*	FRI64029, PM, KEP	45425*
24		*Ar. rosea*	FRI64030, PM, KEP	45426*
25		*Ar. virens*	IS274, SUM, FU	399

Table 2.1a. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID
26		*Ar. virens*	FRI54105, PM, KEP	45420*
27		*Ar. virens*	T3060, THA, FU	264
28		*Ar.* cf. *ridleyi*	KEP73504, PM, KEP	463
29		*Ar.* cf. *villosa* var. *ambovestita*	V2415, VNM, FU	435a
30		*Ar.* sp. vel aff. *clemensii*	V2741, VNM, FU	356
31		*Ar.* sp. vel aff. *vidalii*	V1955, VNM, FU	304
32		*Ar.* sp. vel aff. *villosa*	T2177, THA, FU	256
33	subgen. *Pimelandra*	*Ar. congesta*	MY210, MYN, FU	607
34		*Ar. congesta*	MY570, MYN, FU	608
35		*Ar. ferox*	KEP73534, PM, KEP	478
36		*Ar. ferox*	KEP73526, PM, KEP	481
37	subgen. *Pyrgus*	*Ar. murtonii*	C3394, C, FU	416
38		*Ar. rigida*	T579, THA, FU	247
39		*Ar. vaughanii*	FRI72412, PM, KEP	45418*
40		*Ar. serrata*	SAN153642, SBH, SAN	45318*
41		*Ar. serrata*	SAN155852, SBH, SAN	45325*
42		*Ar.* sp. vel aff. *breviramea*	SAN155854, SBH, SAN	45326*
43		*Ar.* sp. vel aff. *breviramea*	SWK1910, SWK, FU	603
44		*Ar.* sp. vel aff. *poilanei*	MS77, SBG, K	196
45	subgen. *Stylardisia*	*Ar. sanguinolenta*	T1909, THA, FU	254
46		*Ar.* sp. vel aff. *livida*	SAN156132, SBH, SAN	45333*
47		*Ar.* sp. vel. aff. *pterocaulis*	SAN156484, SBH, SAN	45377*
48	subgen. Tinus	*Ar. attenuata*	FRI72352, PM, KEP	45376*
49		*Ar. chevalieri*	V1567, VNM, FU	296
50		*Ar. elliptica*	FRI54116, PM, KEP	45367*
51		*Ar. hulletii*	FRI77353, PM, KEP	658
52		*Ar. smaragdinoides*	C1694, C, FU	412
53		*Ar. solanacea*	FRI70341, PM, KEP	45387*

Table 2.1a. *Continued.*

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID
54	*Fittingia*	*F. conferta*	SAJ1370, PNG, BISH	164
55		*Fittingia* sp.	SAJ0442, PNG, BISH	188
56	*Hymenandra*	*H beamanii*	SAN153136, SBH, SAN	499
57		*H. rosea*	SAN155964, SBH, SAN	45328*
58	*Systellantha*	*S. fruticosa*	SWK870, SWK, FU	390
59	*Tetrardisia*	*T. corneri*	FRI76024, PM, KEP	45370*
60		*T. corneri*	FRI73501, PM, KEP	460
61	*#Embelia*	*E. amentacea*	FRI57778, PM, KEP	45444*
62		*E. pergamacea*	FRI64012, PM, KEP	45421*
63	*#Labisia*	*L. pumila*	MS79, SBG, K	197
64	*#Myrsine*	*M. stolonifera*	V4870, VNM, FU	604
65		*M. cacuminum*	SAJ1255, PNG, BISH	179

[a]Origin: C=Cambodia, JPN=Japan, MYN=Myanmar, PM=Peninsular Malaysia, PNG=Papua New Guinea, SBG=Singapore Botanic Garden, SBH=Sabah, SUM=Sumatra, SWK= Sarawak, THA=Thailand, VNM=Vietnam

[b]Herbarium: BISH= Bishop Museum, K= RBGK, KEP= The herbarium of Forest Research Institute Malaysia, FU= Kyushu University, NYBG=New York Botanic Garden, SAN= Sandakan Herbarium, SNP= Sabah National Park Herbarium, TBRC= Tropical Biosphere Research Center, University of the Ryukyus, TNS= National Science Museum, Tsukuba.

Table 2.1b. List of bacterial strains used in Lemaire et al. (2011b, 2012), Pinto-Carbó (2018), Platero et al. (2016) and newly amplified leaf nodulating *Ardisia* endosymbionts for the independent and combined DNA analyses. The '#' indicates outgroup, *Can*.= candidatus, Endo= endosymbionts and GS= gut symbiont.

Taxa	Strain/voucher	Origin	Accession numbers	
			***rec*A**	***gyr*B**
Burkholderia ambifaria	LMG 19182		HQ849130	HQ849186
Burkholderia anthina	LMG 20980		HQ849132	HQ849187
Burkholderia arationis	LMG293324	–	–	LT158635
Burkholderia arvi	LMG29317	–	–	LT158628
Burkholderia bryophila	LMG 23644		HQ849133	HQ849188
Burkholderia caledonica	LMG 19076		HQ849134	HQ849189
Burkholderia caribensis	LMG 18531		HQ849135	HQ849190
Burkholderia catudaia	LMG29318	–	–	LT158629
Burkholderia cepacia	LMG 1222		JF295011	HQ849191
Burkholderia choica	LMG22940	–	–	HE985163
Burkholderia dolosa	LMG 18943		HQ849136	HQ849192
Burkholderia ferrariae	LMG 23612		HQ849137	HQ849193
Burkholderia fungorum	LMG 16225		HQ849138	HQ849194
Burkholderia gladioli	LMG 11626		HQ849139	HQ849195
Burkholderia gladioli	LMG 2216		HQ849140	HQ849196
Burkholderia glathei	LMG 14190		HQ849141	HQ849197
Burkholderia mimosarum	LMG 23256		HQ849146	HQ849202
Burkholderia mineralivorans	PML116	–	–	KY711344
Burkholderia nodosa	LMG 23741		HQ849147	HQ849204
Burkholderia oklahomensis	LMG 23618		HQ849148	HQ849205
Burkholderia pedi	LMG29323	–	–	LT158634
Burkholderia peredens	LMG29314	–	–	LT158625
Burkholderia phenoliruptrix	LMG 22037		HQ849150	HQ849207
Burkholderia phymatum	LMG 21445		HQ849151	HQ849208
Burkholderia plantarii	LMG 16020		HQ849154	HQ849209
Burkholderia plantarii	LMG 16020		HQ849154	HQ849209
Burkholderia pterochthonis	LMG29326	–	–	LT158637
Burkholderia pyrrocinia	LMG 14191		AF143794	EU024236
Burkholderia pyrrocinia	LMG 14191		HQ849155	HQ849211
Burkholderia sacchari	LMG 19450		HQ849156	HQ849212
Burkholderia silvatlantica	LMG 23149		HQ849157	HQ849213
Burkholderia sordidicola	LMG22029	–	–	NZ_FCOC02000002
Burkholderia stabilis	LMG 14294		HQ849159	JF295010
Burkholderia telluris	LMG22936	–	–	HE985161
Burkholderia terrestris	LMG22936	–	–	HE985170
Burkholderia terricola	LMG 20581		HQ849160	HQ849215
Burkholderia tropica	LMG 22274		HQ849161	HQ849216

Table 2.1b. *Continued*.

Taxa	Strain/voucher	Origin	Accession numbers	
			***rec*A**	***gyr*B**
Burkholderia tuberum	LMG 21444		HQ849162	HQ849217
Burkholderia udeis	LMG27135	–	–	HE985179
Burkholderia vietnamiensis	LMG 10929		AF143793	EU024229
Burkholderia vietnamiensis	LMG 10929		HQ849163	HQ849218
Burkholderia xenovorans	LMG 21463		HQ849164	HQ849219
Burkholderia sp.	Y-123	–	–	CP003087
Can. Burkholderia alatipes	BR-Dessein et al. 2547	Cameroon	JN054098	JN053967
Can. Burkholderia amboniana	UPS-Luke 8344	Kenya	JN054100	JN053969
Can. Burkholderia andongensis	BR-Lemaire et al. 259	South Africa	JF916912	JF916907
Can. Burkholderia andongensis	BR-Lemaire et al. 293	South Africa	JF916914	JF916909
Can. Burkholderia andongensis	BR-Lemaire et al. 271	South Africa	JF916913	JF916908
Can. Burkholderia andongensis	BR-Dessein et al. 1097	Zambia	JF916915	JF916905
Can. Burkholderia anthocleistifolia	BR-Dessein et al. 1875	Gabon	JN054101	JN053970
Can. Burkholderia anthocleistifolia	BR-Dessein et al. 1917	Gabon	JN054102	JN053971
Can. Burkholderia bidentata	BR-Lachenaud et al. 593	Cameroon	JN054103	JN053972
Can. Burkholderia bifaria	BR-Lachenaud et al. 707A	Cameroon	JN054107	JN053976
Can. Burkholderia bifaria	BR-Lachenaud et al. 707	Cameroon	JN054106	JN053975
Can. Burkholderia bifaria	BR-Dessein et al. 2862A	Cameroon	JN054104	JN053973
Can. Burkholderia bifaria	BR-Dessein et al. 2862D	Cameroon	JN054105	JN053974
Can. Burkholderia bimbiensis	BR-2804	–	–	JN643607
Can. Burkholderia brachyantha	BR-Lachenaud et al. 876B	Cameroon	JN054110	JN053979
Can. Burkholderia brachyantha	BR-Dessein et al. 2731	Cameroon	JN054109	JN053978
Can. Burkholderia brachyanthoides	BR-2009044596	Unknown	JN054108	JN053977
Can. Burkholderia brevipaniculata	BR-Dessein et al. 2916	Cameroon	JN054111	JN053980
Can. Burkholderia calva	BR-Lachenaud et al. 748A	Cameroon	JN054112	JN053981
Can. Burkholderia calva	BR-Lachenaud et al. 748B	Cameroon	JN054113	JN053982
Can. Burkholderia camerunensis	BR-Dessein et al. 1390	Cameroon	JN054115	JN053984
Can. Burkholderia camerunensis	BR-Dessein et al. 3165A	Cameroon	JN054198	JN053986
Can. Burkholderia camerunensis	BR-Dessein et al. 3165B	Cameroon	JN054199	JN053987
Can. Burkholderia camerunensis	BR-Dessein et al. 1465	Unknown	JN054114	JN053983
Can. Burkholderia catophylla	BR-Lemaire et al. 180	South Africa	JN054118	JN053991
Can. Burkholderia catophylla	BR-Lemaire et al. 219	South Africa	JN054120	JN053992
Can. Burkholderia catophylla	BR-Lemaire et al. 179	South Africa	JN054117	JN053990
Can. Burkholderia cooperi	BR-Lemaire et al. 75	South Africa	JN054121	JN053993
Can. Burkholderia cooperi	BR-Lemaire et al. 247	South Africa	JN054122	JN053994
Can. Burkholderia darwiniana	BR-Dessein et al. 2720A	Cameroon	JN054124	JN053996
Can. Burkholderia darwiniana	BR-Dessein et al. 2682	Cameroon	JN054123	JN053995
Can. Burkholderia edentula	BR-Lemaire et al. 60	South Africa	JN054125	JN053997
Can. Burkholderia edentula	BR-Lemaire et al. 135	South Africa	JN054127	JN053999
Can. Burkholderia edentula	BR-Lemaire et al. 70C	South Africa	JN054126	JN053998

Table 2.1b. *Continued*.

Taxa	Strain/voucher	Origin	Accession numbers	
			***rec*A**	***gyr*B**
Can. Burkholderia expanssissima	BR-Groeninckx et al. 4	Madagascar	JN054128	JN054000
Can. Burkholderia eylesii	BR-Lemaire et al. 87	South Africa	JN054129	JN054001
Can. Burkholderia fleuryana	BR-Dessein et al. 2578	Cameroon	JN054130	JN054003
Can. Burkholderia fleuryana	BR-Dessein et al. 2675	Cameroon	JN054131	JN054004
Can. Burkholderia gardeniifolia	BR-Lemaire et al. 276	South Africa	JN054132	JN054005
Can. Burkholderia gardeniifolia	BR-Lemaire et al. 136	South Africa	JN054133	JN054007
Can. Burkholderia hispidae	BR-Lachenaud et al. 732	Cameroon	HQ849179	HQ849232
Can. Burkholderia hispidae	BR-Dessein et al. 3176	Cameroon	HQ849178	HQ849231
Can. Burkholderia holtzii	UPS-Luke 8342	Kenya	JN054134	JN054008
Can. Burkholderia humilis	BR-Dessein et al. 1581	Cameroon	JN054135	JN054009
Can. Burkholderia humilis	BR-Dessein et al. 1497	Cameroon	JN054136	JN054010
Can. Burkholderia humilis	BR-Lachenaud et al. 820	Cameroon	JN054138	JN054012
Can. Burkholderia humilis	BR-Dessein et al. 3175	Cameroon	JN054137	JN054011
Can. Burkholderia inandensis	BR-Lemaire et al. 244	South Africa	JN054139	JN054013
Can. Burkholderia ingentifolia	BR-2546	–	–	JN643615
Can. Burkholderia kikwitensis	BR-2004145187	Zambia	JN054140	JN054014
Can. Burkholderia kimuenzae	BR-Stoffelen et al. 7	D.R. Congo	JN054142	JN054016
Can. Burkholderia kimuenzae	BR-Stoffelen et al. 7	D.R. Congo	JN054142	JN054016
Can. Burkholderia kirkii	BR-De Block et al. 372	Kenya	JN054182	JN054053
Can. Burkholderia kirkii	BR-19536779	Unknown	HQ849165	HQ849220
Can. Burkholderia kirkii	BR-2001051392	Unknown	HQ849169	HQ849224
Can. Burkholderia kirkii	BR-2000194661	D.R. Congo	HQ849166	HQ849221
Can. Burkholderia kirkii	BR-2002120315	Unknown	HQ849168	HQ849223
Can. Burkholderia kirkii	BR-1998182519	Kenya	HQ849170	HQ849225
Can. Burkholderia kirkii	BR-200103624	Unknown	HQ849171	HQ849226
Can. Burkholderia kirkii	BR-2002152647	Unknown	HQ849167	HQ849222
Can. Burkholderia konguensis	BR-Lachenaud et al. 932	Cameroon	JN054153	JN054027
Can. Burkholderia konguensis	BR-Dessein et al. 1705	Gabon	JN054150	JN054024
Can. Burkholderia konguensis	BR-Dessein et al. 2306	Gabon	JN054151	JN054025
Can. Burkholderia konguensis	BR-Dessein et al. 1434	Cameroon	JN054149	JN054023
Can. Burkholderia konguensis	BR-Lachenaud et al. 636E	Cameroon	JN054152	JN054026
Can. Burkholderia kotzei	BR-Lemaire et al. 126	South Africa	JN054154	JN054028
Can. Burkholderia lanceolata	BR-Lemaire et al. 40	South Africa	JN054155	JN054029
Can. Burkholderia lanceolata	BR-Lemaire et al. 41	South Africa	JN054156	JN054030
Can. Burkholderia laxithyrsa	BR-1951	–	–	JN643618
Can. Burkholderia leptophylla	BR-Dessein et al. 2570B	Cameroon	JN054160	JN054034
Can. Burkholderia leptophylla	BR-Lachenaud et al. 591	Cameroon	JN054163	JN054163
Can. Burkholderia leptophylla	BR-Dessein et al. 3111A	Cameroon	JN054157	JN054031
Can. Burkholderia leptophylla	BR-Dessein et al. 3159	Cameroon	JN054159	JN054033
Can. Burkholderia letouzeyi	BR-Lachenaud et al. 931	Cameroon	JN054167	JN054040

Table 2.1b. *Continued*.

Taxa	Strain/voucher	Origin	Accession numbers	
			***rec*A**	***gyr*B**
Can. Burkholderia letouzeyi	BR-Dessein et al. 1731	Gabon	JN054165	JN054038
Can. Burkholderia letouzeyi	BR-Dessein et al. 2140	Gabon	JN054166	JN054039
Can. Burkholderia lokohensis	BR-Tosh et al. 238	Madagascar	JN054168	JN054041
Can. Burkholderia mamillata	BR-10005023	Unknown	JN054169	JF416290
Can. Burkholderia mamillata	BR-10005024	Unknown	JN054170	JF416291
Can. Burkholderia mannii	BR-Dessein et al. 2053	Gabon	JN054174	JN054046
Can. Burkholderia mannii	BR-Dessein et al. 2053B	Gabon	JN054175	JN054045
Can. Burkholderia mannii	BR-Dessein et al. 1793	Gabon	JN054172	JN054043
Can. Burkholderia nigropunctata	BR-Dessein et al. 1849	D.R. Congo	HQ849174	HQ849228
Can. Burkholderia pendulothyrsa	BR-Lachenaud et al. 647B	Cameroon	JN054180	JN054051
Can. Burkholderia petitii	BR-Lachenaud et al. 658	Cameroon	JF916917	JF916910
Can. Burkholderia petitii	BR-Dessein et al. 1592	Cameroon	JF916916	JF916911
Can. Burkholderia psychotriodes	BR-3093	–	–	JN643622
Can. Burkholderia psychotriodes	BR-2639	–	–	JN643623
Can. Burkholderia pumila	BR-2004143571	Zambia	JN054181	JN054052
Can. Burkholderia recurva	BR-Dessein et al. 2550A	Cameroon	JN054183	JN054054
Can. Burkholderia recurva	BR-Dessein et al. 2575	Cameroon	JN054184	JN054055
Can. Burkholderia rhizomatosa	BR-Dessein et al. 2551B	Cameroon	JN054192	JN054063
Can. Burkholderia rhizomatosa	BR-Dessein et al. 2240	Gabon	JN054190	JN054060
Can. Burkholderia rhizomatosa	BR-Dessein et al. 2368	Gabon	JN054186	JN054061
Can. Burkholderia rhizomatosa	BR-Dessein et al. 2432	Gabon	JN054191	JN054062
Can. Burkholderia rhizomatosa	BR-Dessein et al. 2239	Gabon	JN054178	JN054049
Can. Burkholderia rhizomatosa	BR-Dessein et al. 1772	Gabon	JN054185	JN054056
Can. Burkholderia rhizomatosa	BR-Dessein et al. 2674	Cameroon	JN054193	JN054064
Can. Burkholderia rhizomatosa	BR-Dessein et al. 1785	Gabon	JN054187	JN054057
Can. Burkholderia rhizomatosa	BR-Dessein et al. 2223	Gabon	JN054189	JN054059
Can. Burkholderia rigidae	BR-Lachenaud et al. 694	Cameroon	HQ849176	HQ849229
Can. Burkholderia rigidae	BR-Lachenaud et al. 877	Cameroon	HQ849177	HQ849230
Can. Burkholderia rubripilis	BR-Dessein et al. 3174	Cameroon	JN054197	JN054069
Can. Burkholderia rubripilis	BR-Dessein et al. 1973	Gabon	JN054195	JN054067
Can. Burkholderia rubripilis	BR-Dessein et al. 2077	Gabon	JN054196	JN054068
Can. Burkholderia rubripilis	BR-Dessein et al. 2295	Gabon	JN054194	JN054066
Can. Burkholderia schumanniana	BR-Lemaire et al. 1	South Africa	HQ849184	HQ849237
Can. Burkholderia schumanniana	BR-Lemaire et al. 99	South Africa	HQ849185	HQ849238
Can. Burkholderia schumanniana	BR-Dessein et al. 1137	Zambia	HQ849181	HQ849234
Can. Burkholderia schumanniana	BR-2004143066	Zambia	HQ849183	HQ849236
Can. Burkholderia schumanniana	BR-Dessein et al. 1099	Zambia	HQ849180	HQ849233
Can. Burkholderia schumanniana	BR-2001944257	D.R. Congo	HQ849182	HQ849235
Can. Burkholderia subobliqua	BR-2891	–	–	JN643627
Can. Burkholderia subpunctata	BR-Lachenaud et al. 815	Cameroon	JN054211	JN054082
Can. Burkholderia taedoumgii	BR-2873	–	–	JN643605

Table 2.1b. *Continued.*

Taxa	Strain/voucher	Origin	Accession numbers	
			***rec*A**	***gyr*B**
Can. Burkholderia trichardtensis	BR-Lemaire et al. 282	South Africa	JN054213	JN054084
Can. Burkholderia trichardtensis	BR-Lemaire et al. 295	South Africa	JN054214	JN054085
Can. Burkholderia trichardtensis	BR-Lemaire et al. 280	South Africa	JN054212	JN054083
Can. Burkholderia verschuerenii	BR-Lachenaud et al. 655A	Cameroon	JN054221	JN054096
Can. Burkholderia verschuerenii	BR-without voucher	Unknown	JN054218	JN054093
Can. Burkholderia verschuerenii	BR-Dessein et al. 1760	Gabon	JN054219	JN054094
Can. Burkholderia verschuerenii	BR-750204	Cameroon	JN054220	JN054095
Can. Burkholderia sp.	BR-2004114076	Unknown	JN054204	JN054073
Can. Burkholderia sp.	BR-20041440	Unknown	JN054202	JN054071
Can. Burkholderia sp.	BR-Dessein et al. 2719	Cameroon	JN054206	JN054076
Can. Burkholderia sp.	BR-2006012338	Unknown	JN054203	JN054072
Can. Burkholderia sp.	BR-Dessein et al. 1974	Gabon	JN054205	JN054074
Can. Burkholderia sp.	BR-Lachenaud et al. 616	Cameroon	JN054207	JN054077
Can. Burkholderia sp.	BR-Lachenaud et al. 919	Cameroon	JN054208	JN054078
GS of Dricanosephalus agilis	Kue594	–	–	LT221843
GS of Dricanosephalus agilis	Kue595	–	–	LT221836
GS of Dricanosephalus albipes	Kue464	–	–	LT221816
GS of Dricanosephalus lateralis	YM3	–	–	LT221775
GS of Dricanosephalus lateralis	YM3	–	–	LT221777
GS of Dricanosephalus medius	Kue321	–	–	LT221800
GS of Dricanosephalus medius	Kue576	–	–	LT221820
GS of Riptortus pedestris	RPE67	–	–	AP014576
GS of Riptortus pedestris	RPE64	–	–	AP013058
Endo. of Amblyanthopsis bhotanica	KEA883	Myanmar	–	–
Endo. of Amblyanthus sp. vel aff. *multiflorus*	16Aa/MY881	Myanmar	–	–
Endo. of Amblyanthus sp. vel aff. *multiflorus*	16Ac/MY881	Myanmar	–	–
Endo. of Ardisia crenata subsp. *crenata*	3/T4049	Thailand	–	–
Endo. of Ardisia crenata subsp. *crenata*	22/SNP37015	Malaysia	–	–
Endo. of Ardisia crenata subsp. *obtusifolia*	2/T4630	Thailand	–	–
Endo. of Ardisia maculosa	7c/T3059	Thailand	–	–
Endo. of Ardisia maculosa	10/T4760	Thailand	–	–
Endo. of Ardisia cf. *cadieri*	32/V1905	Vietnam	–	–
Endo. of Ardisia cf. *polysticta*	29/V1907	Vietnam	–	–
Endo. of Ardisia cf. *ridleyi*	25/FRI73503	Malaysia	–	–
Endo. of Ardisia cf. *ridleyi*	25c/FRI73503	Malaysia	–	–
Endo. of Ardisia cf. *tsangii*	28/V2415	Vietnam	–	–
Endo. of Ardisia cf. *vidalii*	11/V1955	Vietnam	–	–
Endo. of Ardisia sp. vel aff. *clemensii*	12/V2741	Vietnam	–	–
Endo. of Ardisia sp.	1/V728	Vietnam	–	–
#*Cupriavidus* sp.	UYMM02A		KT198755	KT198753
#*Cupriavidus* sp.	UYMS13B		KT198756	KT198754

Table 2.1c. List of nodulated and non-nodulated taxa examined for the presence bacterial symbionts. Asterisk indicated that the presence of bacterial symbionts was examined on the floral parts.

No.	Samples	Origin, voucher, herbaria	ID
Nodulated taxa			
1	*Amblyanthopsis bhotanica*	MYN, KEA883, FU	27
2	*Amblyanthus* sp. vel. aff. *multiflorus*	MYN, MY881, TNS	16
3	*Amblyanthus* sp. vel. aff. *multiflorus*	MYN, MY806, TNS	17
4	*Ar*. *corymbifera*	THA, T907, FU	4
5	*Ar. crenata* subsp. *crenata*	SBH, SNP37015, SNP	22
6	*Ar. crenata* subsp. *crenata*	THA, T4049, FU	3
7	*Ar. crenata* subsp. *crenata*	CB, (an escape plant)	26
8	*Ar*. *crenata* subsp. *obtusifolia*	THA, T4630, FU	2
9	*Ar*. *maculosa*	THA, T3059, FU	7*
10	*Ar*. *maculosa*	THA, T4760, FU	10
11	*Ar*. *polysticta*	VNM, V3054, FU	15*
12	*Ar*. *polysticta*	SBH, SNP37006, SNP	23
13	*Ar. villosa*	PM, FRI73504, KEP	24
14	*Ar*. *virens*	THA, T3060, FU	6
15	*Ar*. *virens*	SUM, IS274, K	31
16	*Ar*. cf. *cadieri*	VNM, V1905, FU	32
17	*Ar*. cf. *hanceana*	VNM, V1937, FU	30
18	*Ar*. cf. *polysticta*	VNM, V1907, FU	29
19	*Ar.* cf. *ridleyi*	PM, FRI73503, PM	25
20	*Ar.* cf. *tsangii*	VNM, V2415, FU	28
21	*Ar.* sp. vel aff. *clemensii*	VNM, V2741, FU	12*
22	*Ar.* sp. vel aff. *vidalii*	VNM, V1955, FU	11
23	*Ar*. sp. vel aff. *villosa*	THA, T2177, FU	8
24	*Ardisia* sp.	VNM, V728, FU	1
25	*Ardisia* sp.	C, C6954, FU	9
Non-nodulated taxa			
26	*Ar*. *serrata*	SBH, SAN155852, SAN	529
27	*Ar*. sp. vel aff. *breviramea*	SBH, SAN155854, SAN	644
28	*Hymenandra beamanii*	SBH, SAN153136, SAN	499
29	*Hymenandra beamanii*	SBH, SAN157135, SAN	498
30	*Hymenandra rosea*	SBH, SAN155243, SAN	602
31	*Hymenandra rosea*	SBH, SAN157318, SAN	500

Table 2.1d. Fourteen samples of nodulated taxa listed in Table 2.1c were selected for host specificity examination.

No.	Samples	Veg./Rep. Parts	ID	
			rep. 1	rep. 2
1	*Amblyanthus* sp. vel aff. *multiflorus*	nodule	16Aa	16Ac
2	*Amblyanthus* sp. vel aff. *multiflorus*	nodule	17B	-
3	*Ar*. *corymbifera*	nodule	4	4c
4	*Ar. crenata* subsp. *crenata*	nodule	3	3c
5	*Ar. crenata* subsp. *crenata*	nodule	22	22c
6	*Ar. crenata* subsp. *crenata*	nodule, seeds	26a	26b
7	*Ar*. *crenata* subsp. *obtusifolia*	nodule	2	2c
8	*Ar*. *maculosa*	nodule	7	7c
9	*Ar*. *polysticta*	nodule	15	15c
10	*Ar*. *virens*	nodule	6	6c
11	*Ar.* cf. *ridleyi*	nodule	25	25c
12	*Ar.* sp. vel aff. *clemensii*	nodule	12	12c
13	*Ar.* sp. vel aff. *vidalii*	nodule	11	11c
14	*Ardisia* sp.	nodule	9	9c

Table 2.1e. Oligonucleotide primers for amplification and sequencing.

Region	Primer	Primer sequence (5'-3')	Reference
ITS	ITS4-F	TCCTCCGCTTATTGATATGC	White et al. (1990)
	ITS5-R	GAAGTAAAAGTCGTAACAAGG	
*rpL*32-*trn*L$^{(UAG)}$	*rpL*32	CAGTTCCAAAAAAACGTACTTC	Shaw et al. (2007)
	*trn*L	CTGCTTCCTAAGAGCAGCGT	
*rec*A	*recA*-F (forward)	AGGACGATTCATGGAAGAWAGC	Spilker et al. (2009)
	recA-R (reverse)	GACGCACYGAYGMRTAGAACTT	
*gyr*B	*gyrB*-F (forward)	ACCGGTCTGCAYCACCTCGT	Spilker et al. (2009)
	gyrB-R (reverse)	YTCGTTGWARCTGTCGTTCCACTGC	
*lep*A	*lepA*-F (forward)	CTSATCATCGAYTCSTGGTTCG	Spilker et al. (2009)
	lepA-R (reverse)	CGRTATTCCTTGAACTCGTARTCC	
*atp*D	*atpD*-F (forward)	ATGAGTACTRCTGCTTTGGTAGAAGG	Spilker et al. (2009)
	*atp*D-R (reverse)	CGTGAAACGGTAGATGTTGTCG	

Table 2.2a. Amplification of bacterial symbionts from total DNAs of leaf tissues using housekeeping genes *atp*D, *gyr*B, *lep*A and *rec*A. Bacterial symbionts DNAs were amplified only from nodulated taxa but none from non-nodulated taxa. The 'X' means amplification unsuccessful from nodulated taxa, while 'O' means no bacterial symbionts were detected from non-nodulated taxa.

		DNA sequences				
			Markers			
No.	**Samples**	**ID**	***rec*A**	***gyr*B**	***lep*A**	***atp*D**
Nodulated taxa (leaf nodule)						
1	*Amblyanthopsis bhotanica*	27	OK	OK	OK	OK
2	*Amblyanthus* sp. vel aff. *multiflorus*	16	OK	OK	OK	OK
3	*Amblyanthus* sp. vel aff. *multiflorus*	17	X	OK	X	OK
4	*Ar. corymbifera*	4	OK	OK	OK	OK
5	*Ar. crenata* subsp. *crenata*	22	OK	OK	OK	X
6	*Ar. crenata* subsp. *crenata*	26	X	OK	OK	OK
7	*Ar. crenata* subsp. *crenata*	3	OK	OK	OK	OK
8	*Ar. crenata* subsp. *obtusifolia*	2	OK	OK	OK	OK
9	*Ar. maculosa*	7	OK	OK	OK	OK
10	*Ar. maculosa*	10	OK	OK	OK	OK
11	*Ar. polysticta*	15	X	OK	OK	OK
12	*Ar. polysticta*	23	X	OK	OK	OK
13	*Ar. polysticta*	30	X	OK	OK	OK
14	*Ar. villosa*	24	X	OK	OK	OK
15	*Ar. virens*	6	X	OK	OK	OK
16	*Ar. virens*	31	X	OK	OK	OK
17	*Ar.* cf. *cadieri*	32	OK	OK	OK	OK
18	*Ar.* cf. *polysticta*	29	OK	OK	OK	X
19	*Ar.* cf. *ridleyi*	25	OK	OK	OK	OK
20	*Ar.* cf. *tsangii*	28	OK	OK	OK	OK
21	*Ar.* sp. vel aff. *clemensii*	12	OK	OK	OK	OK
22	*Ar.* sp. vel aff. *vidalii*	11	OK	OK	OK	OK
23	*Ar.* sp. vel aff. *villosa*	8	X	OK	OK	X
24	*Ardisia* sp.	1	OK	OK	OK	X
25	*Ardisia* sp.	9	X	OK	OK	OK
Non-nodulated taxa (non-nodulated leaf)						
26	*Ar. serrata*	529	O	O	O	O
27	*Ar.* sp. vel aff. *breviramea*	644	O	O	O	O
28	*Hymenandra beamanii*	499	O	O	O	O
29	*Hymenandra beamanii*	498	O	O	O	O
30	*Hymenandra rosea*	602	O	O	O	O
31	*Hymenandra rosea*	500	O	O	O	O

Table 2.2b. Bacterial symbiont amplification from 14 selected nodulated samples. The 'X' shows bacterial symbiont DNA was not successfully sequenced.

			DNA sequences					
			Markers					
			***gyr*B**		***atp*D**		***lep*A**	
No.	**Samples**	**ID**	**rep. 1**	**rep. 2**	**rep. 1**	**rep. 2**	**rep. 1**	**rep. 2**
1	*Amblyanthus* sp. vel aff. *multiflorus*	16Aa	OK	OK	OK	OK	X	OK
2	*Amblyanthus* sp. vel aff. *multiflorus*	17B	OK	X	X	OK	X	X
3	*Ar*. c*orymbifera*	4	OK	OK	OK	X	OK	X
4	*Ar. crenata* subsp. *crenata*	3	X	OK	OK	X	OK	X
5	*Ar. crenata* subsp. *crenata*	22	OK	OK	OK	X	OK	X
6	*Ar. crenata* subsp. *crenata*	26a	OK	OK	OK	OK	OK	OK
7	*Ar. crenata* subsp. *obtusifolia*	2	OK	OK	OK	X	OK	X
8	*Ar. maculosa*	7	OK	OK	OK	OK	OK	X
9	*Ar. polysticta*	15	OK	OK	OK	OK	OK	X
10	*Ar. virens*	6	OK	OK	OK	OK	OK	OK
11	*Ar.* cf. *ridleyi*	25	OK	OK	OK	OK	OK	OK
12	*Ar.* sp. vel aff. c*lemensii*	12	OK	OK	OK	OK	OK	OK
13	*Ar.* sp. vel aff. *vidalii*	11	OK	OK	OK	OK	OK	OK
14	*Ardisia* sp.	9	OK	OK	OK	OK	OK	OK

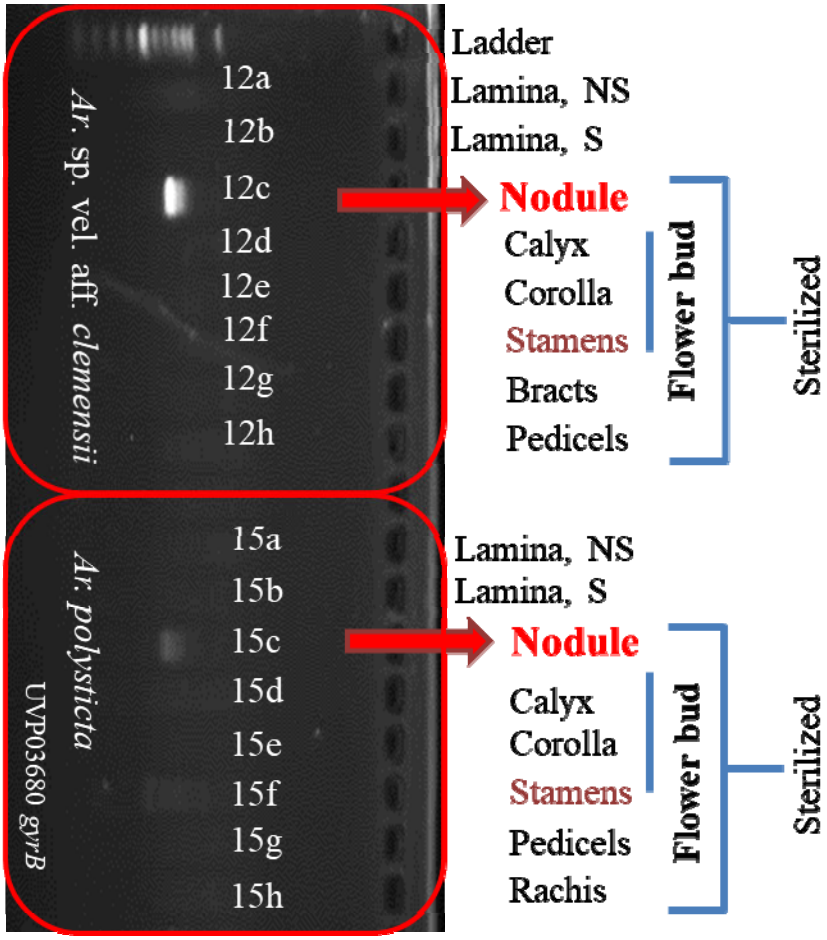

Figure 2.1a. PCR detection of bacterial symbionts from lamina and floral parts of *Ardisia polysticta* and *Ar.* sp. vel aff. *clemensii* using *gyr*B. The bacterial symbionts detected only on nodule parts but absent from the rest. (NS= non-sterilized, S=sterilized).

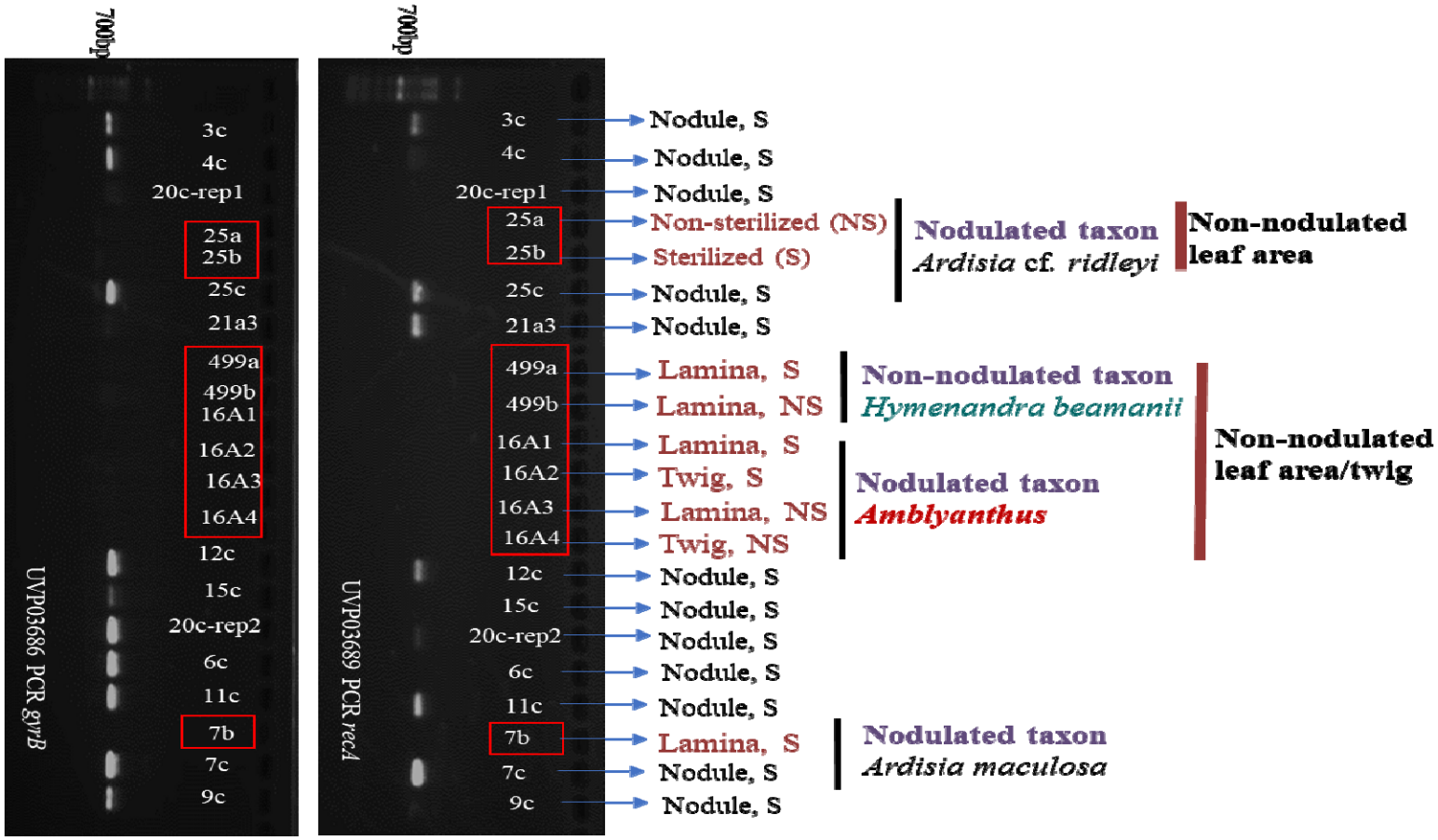

Figure 2.2a. **PCR detection of bacterial symbionts from nodulated and non-nodulated taxa using *gyr*B and *rec*A.** None of bacterial symbionts were amplified from non-nodulated leaf area/twig using both non-nodulated (e.g. *Hymenandra beamanii*) and nodulated taxa (e.g. *Amblyanthus*, *Ardisia maculosa*, *Ar*. cf. *ridleyi*). (NS= non-sterilized, S=sterilized).

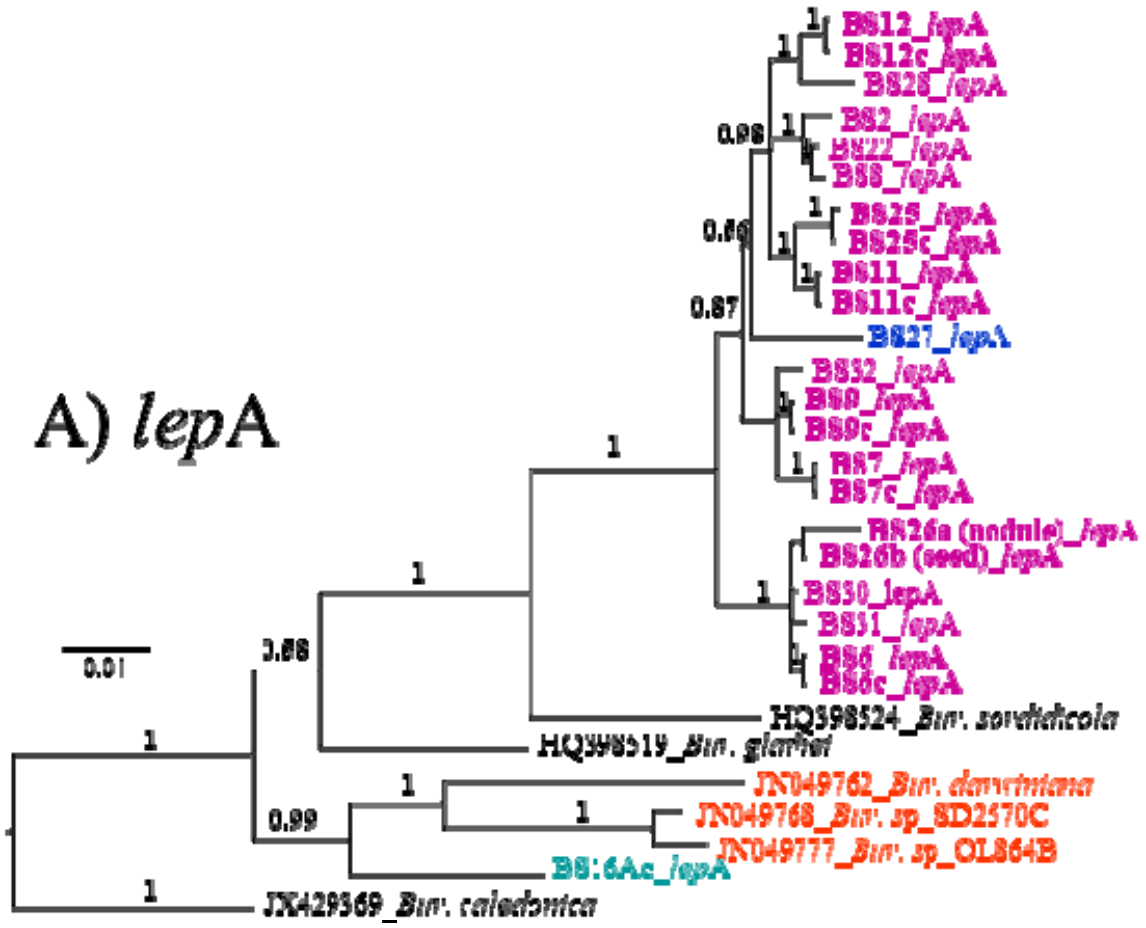

Figure 2.2b. Individual phylograms for the three endosymbiont markers *lep*A (A), *atp*D (B) and *gyr*B (C). Taxa in boldface have two sample replicates. Endosymbionts for *Ardisia* subgenus *Crispardisia*=pink, *Amblyanthopsis*= blue, *Amblyanthus*= green and *Pavetta*= orange. BS= bacterial symbiont and the number after it is its ID.

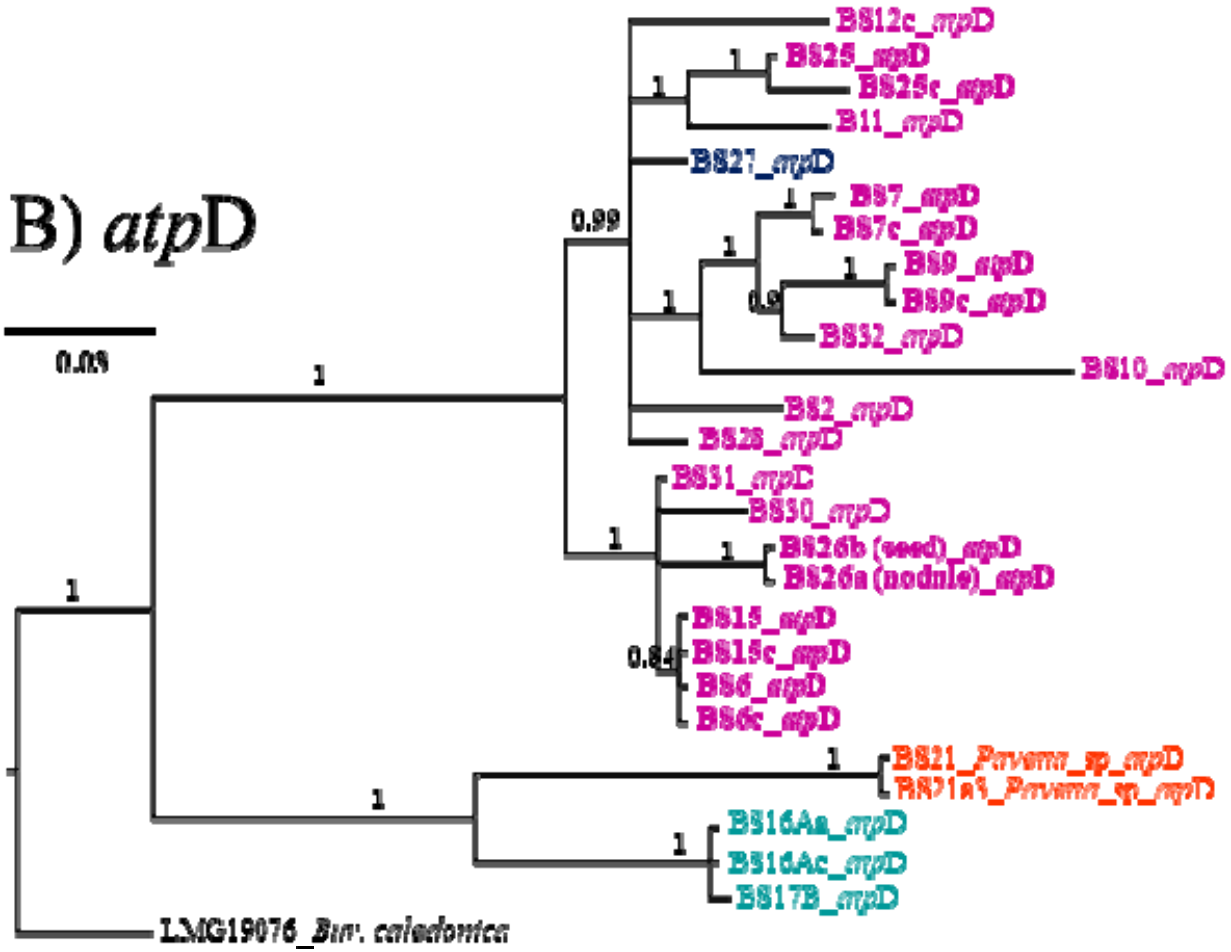

Figure 2.2b. *Continued*.

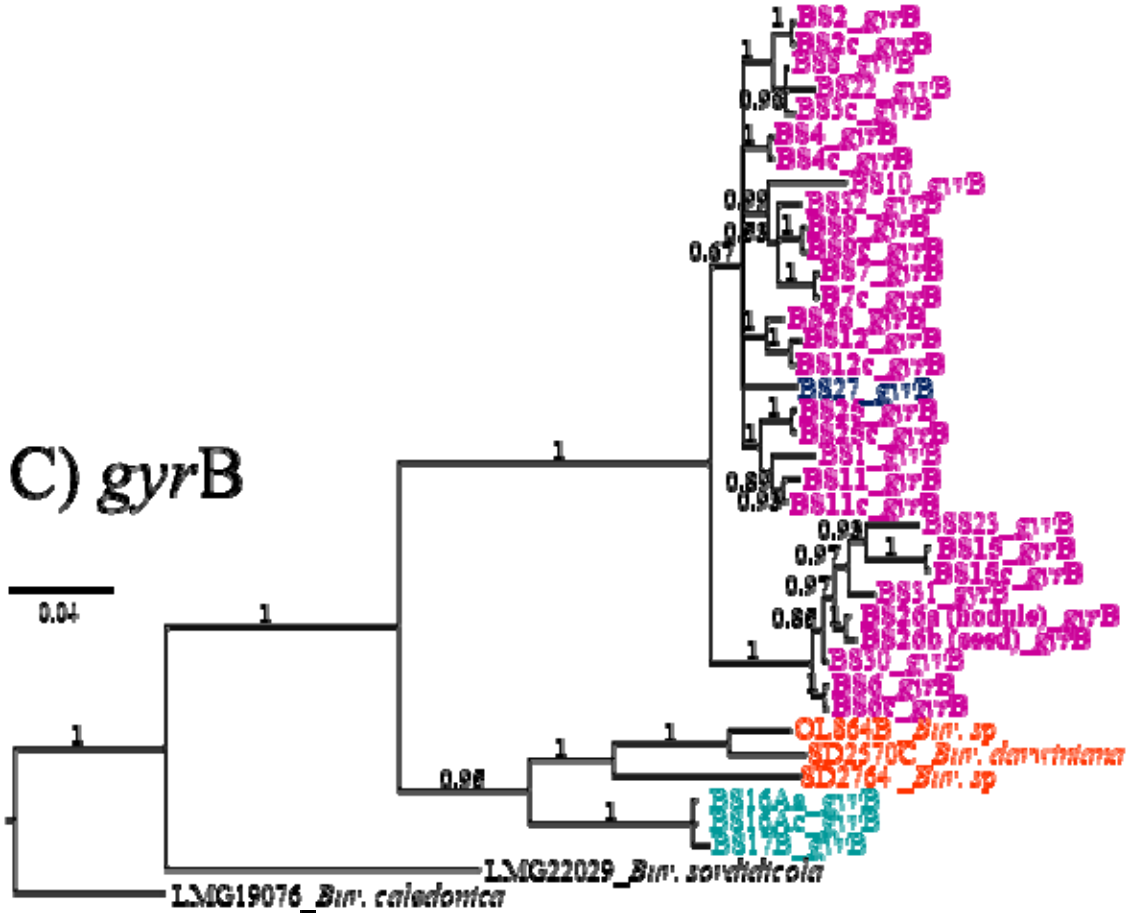

Figure 2.2b. *Continued.*

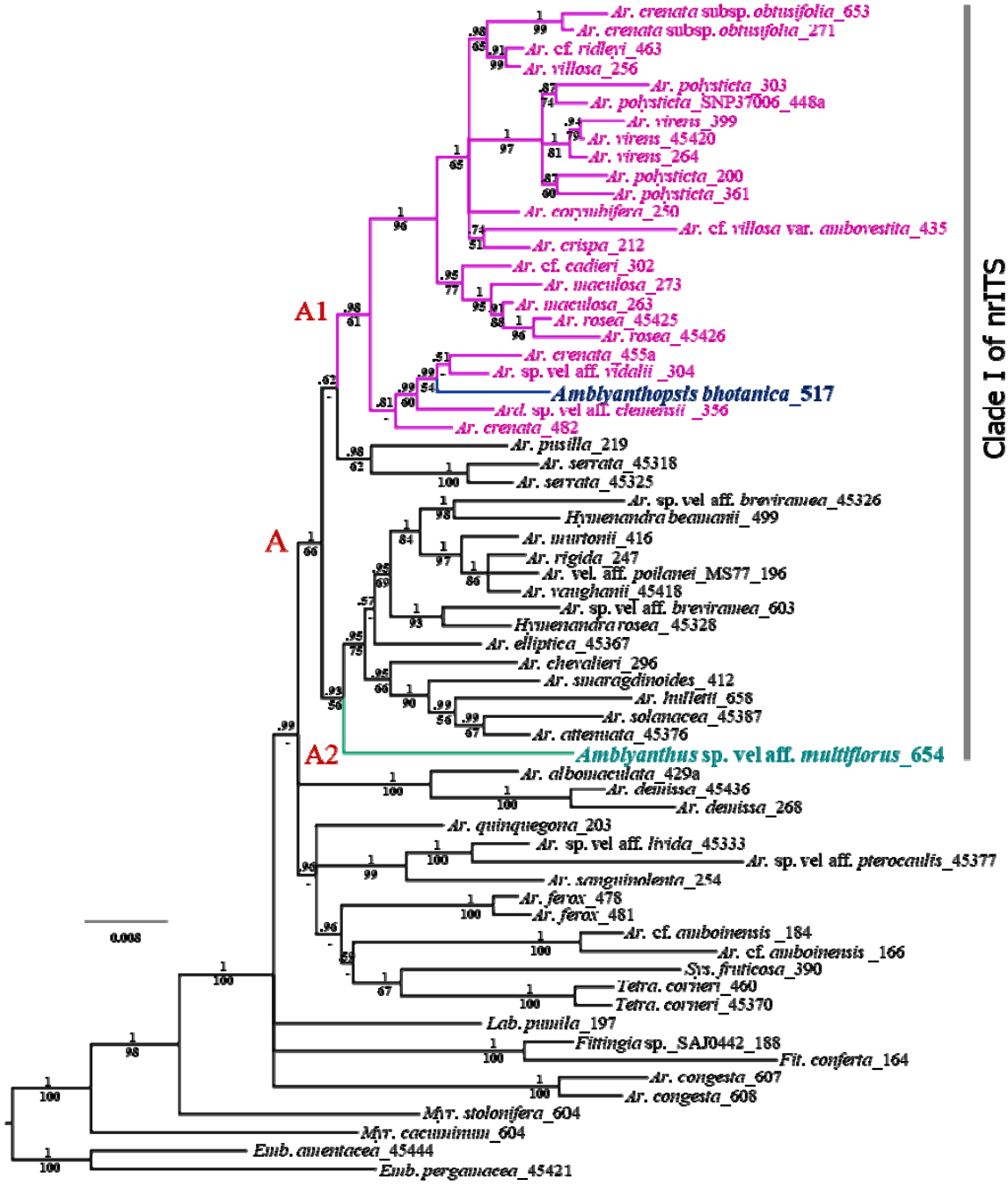

Figure 2.2c. Phylogenetic position of leaf nodulated genera in Myrsinoideae. Phylogeny inferred from Bayesian analysis using concatenated alignment of cpDNA and nrITS data sets. Posterior probabilities of BI (> 0.5) and bootstrap supports of ML analyses (> 50%) were provided above and below the branches, respectively. Leaf nodulation possibly originated twice, once in a common ancestor of *Crispardisia-Amblyanthopsis* lineage (indicated by A1) and independently in *Amblyanthus* (indicated by A2). The color represents different nodulated genera and their position in the tree. The *Ar.*= *Ardisia*, *Emb.*= *Embelia*, *Fit.*= *Fittingia*, *Lab.*= *Labisia*, *Myr.*= *Myrsine* and *Tetra.*= *Tetrardisia*.

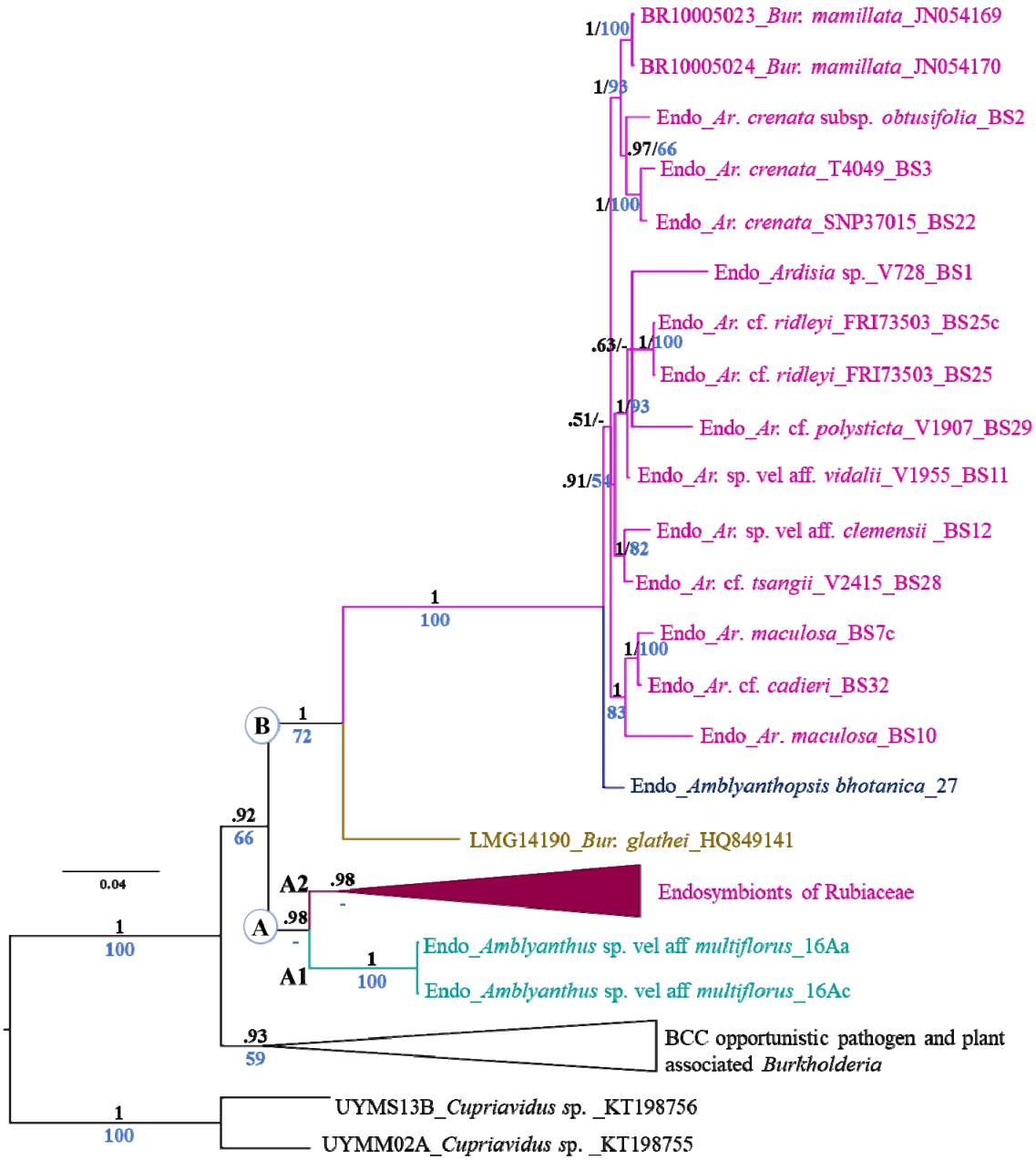

Figure 2.2d. Endosymbiont phylogenetic tree based on *rec*A and *gyr*B with branches collapsed at nodes endosymbionts of Rubiaceae and, BCC opportunistic pathogen and plant associated *Burkholderia*. The posterior probabilities of BI (> 0.5) and bootstrap supports of ML analyses (> 50%) are in black and blue, respectively. The endosymbionts of Myrsinoideae are placed in separate positions: *Amblyanthus* in the Clade A, whereas *Amblyanthopsis* and *Ardisia* subgenus *Crispardisia* in Clade B. The *Ar.*= *Ardisia*, BCC = *Burkholderia cepacia* complex, *Bur.*= *Burkholderia* and Endo= Endosymbiont.

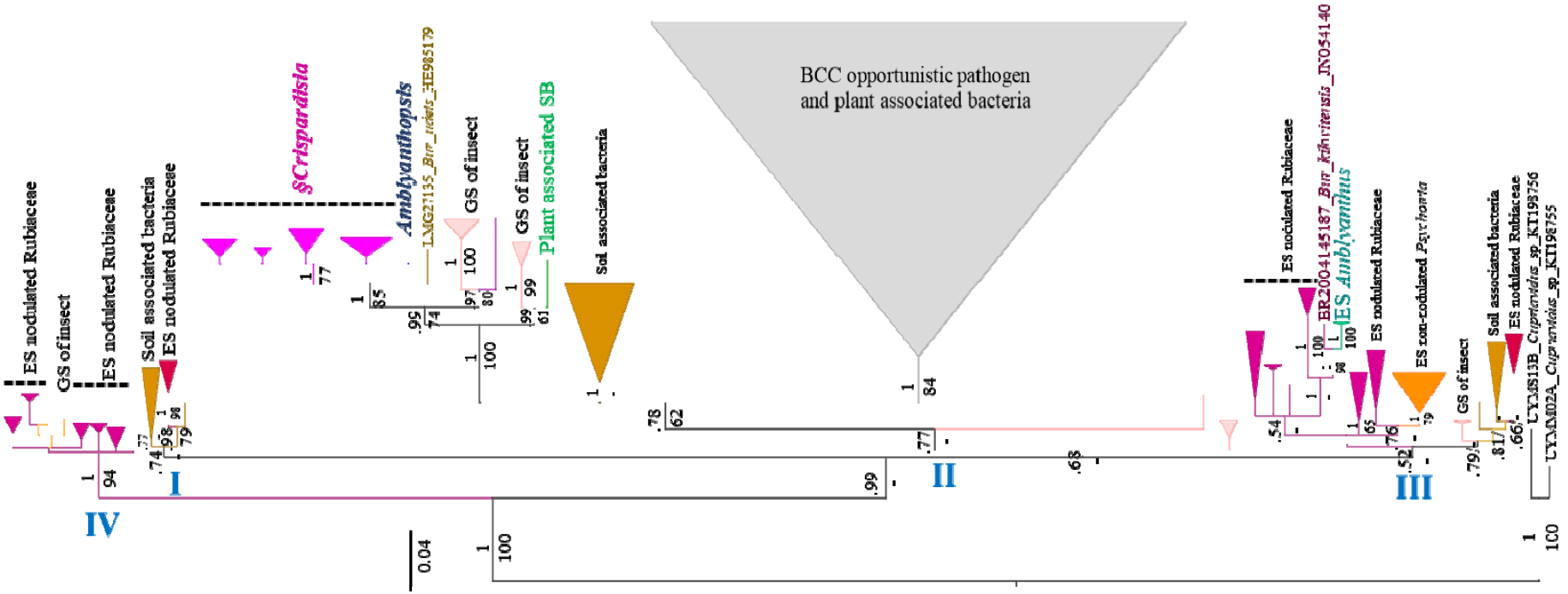

Figure 2.2e. Endosymbiont phylogenetic tree based on *gyr*B. Posterior probabilities of BI (> 0.5) and bootstrap supports of ML analyses (> 50%) were provided above and below the branches, respectively. The endosymbionts of Myrsinoideae are placed in separate positions: *Amblyanthus* in Clade III, whereas *Amblyanthopsis* and *Ardisia* subgenus *Crispardisia* in Clade II. The BCC = *Burkholderia cepacia* complex, ES= Endosymbiont and GS= gut symbiont.

CHAPTER 3

Cophylogenetic study between leaf nodulated Myrsinoideae and their endosymbionts

ABSTRACT

Cophylogenetic studies have been investigated in various host-symbiont associations and provided different results: from strict congruence to lacking correspondence between the host and symbiont trees and sometimes mixed where the symbionts phylogeny is partially mirrored by the host phylogeny. Few cophylogenetic studies have been carried out in Myrsinoideae and the most comprehensive ones studied *Ardisia* subgenus *Crispardisia* but only on several Chinese taxa. In this study, the cophylogenetic pattern in the symbiotic association between leaf nodulated Myrsinoideae-hosts and their endosymbionts were investigated to test further the strict co-speciation hypothesis which has previously been used to describe the evolutionary relationships between these two partners. The leaf nodulated host-endosymbiont association was studied using representative species from the nodulated genera *Amblyanthopsis* and *Ardisia* subgenus *Crispardisia*. Phylogenetic trees were generated for leaf nodulated hosts and their endosymbionts using Bayesian inference and maximum likelihood analyses, and their congruence was assessed using these trees, and the cophylogenetic relationship assessed using topology- or event-based approaches with the software JANE 4.0 and TreeMap v3.0β. The phylogenetic trees of leaf nodulated host and their endosymbionts were incongruent except for few taxa. The partially topological incongruences between the two partners suggest an intermittent interaction between the hosts and their endosymbionts involving vertical and horizontal transmission of bacterial symbiont

in leaf nodulation of Myrsinoideae. Reconciliation reconstruction detected both co-speciation and the duplication and host switch events in the association between leaf nodulated Myrsinoideae-hosts and their endosymbionts. Although vertical transmission of bacterial symbiont implied strict-co-speciation between host–symbiont bacteria relationships, this study suggests that co-speciation may not be kept always in the leaf nodulation of *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* clade. Duplication and host switch may be more common events than it has reported previously.

3.0 INTRODUCTION

Cophylogenetic studies involve the comparison of two or more phylogenetic trees to reveal patterns of co-cladogenesis or co-divergence (e.g. Charleston & Perkins 2006, Clayton et al. 2003). These analyses are most commonly used to infer the relationship between hosts-parasites systems and different patterns were inferred, from strict co-divergence in which parasite phylogeny mirrors host phylogeny, to complete lack of correspondence between trees (e.g. Page 2003). Sometimes, such methods are used to assess the relationships in other associations, such us between evolving pairs of genes/species (e.g. Szöllősi et al. 2014) and endemism/areas parasites (e.g. Peterson et al. 2010). Examining cophylogenetic between hosts and parasites will be able to reveal on the evolutionary processes that rule the interaction between these partners (Charleston & Perkins 2006). In an obligate and heritable symbiotic association, the general assumption is that the phylogenetic tree of parasite should mirror that of the host phylogenetic tree, and thus both phylogenies are congruent to each other. On the other hand, in an association without long-term co-speciation, an incongruent topology between the parasite's phylogeny and the host would be expected.

Few cophylogenetic studies have been done for bacterial leaf nodule symbiosis in angiosperms (e.g. Lemaire et al. 2011a, 2011b, 2012, Ku & Hu 2014); more cophylogenetic studies, however, have been undertaken in Rubiaceae compared to Myrsinoideae. Lemaire et al. (2011b, 2012), in their recent reconciliation reconstructions at generic and population level of the family Rubiaceae, showed some topological incongruency between phylogenetic trees of hosts and their endosymbionts on related host. Both co-speciation and non-co-speciation events were detected in their reconciliation analyses. The observation of these non-co-speciation events were considered to be a result of frequent horizontal transmission of bacterial symbionts from soil, inferred from the close relationship between leaf nodulated

endosymbionts and soil bacteria as indicated in their 16S rDNA tree. Theoretically, these results suggest the association between the two partners are without long-term strict-co-speciation as speculated in previous study (Lemaire et al. 2011b). Their observations also suggest a mixed mode bacterial-symbiont transmission, involving both vertical inheritance and horizontal transfers, is possible.

In Myrsinoideae, nodulation is known to occur in three nodulated genera: *Amblyanthopsis, Amblyanthus* and *Ardisia*, only restricted to subgenus *Crispardisia* in the latter. Previous cophylogenetic studies have only studied *Ardisia* subgenus *Crispardisia* (Lemaire et al. 2011a, Ku & Hu 2014). Two hypotheses were postulated to describe the evolutionary relationship between the leaf nodulated hosts in subg. *Crispardisia* and their symbiotic bacteria. Lemaire et al. (2011b), in their study using 16S rDNA with extensive sampling of leaf nodulating endosymbionts and free living *Burkholderia*, showed the occurrence of multiple horizontal transfers of bacteria from the environment to leaf nodulated plants, thereby rejecting the hypothesis of long-term co-speciation between the leaf nodulated host and their symbiotic bacteria. However, only a few representatives of *Crispardisia*-nodulating endosymbionts were included in their study and their sampling may not have been sufficient to reflect the co-evolutionary history of bacterial leaf nodule symbiosis in *Ardisia*. In a subsequent study, Ku & Hu (2014) increased the representatives of *Crispardisia*-nodulating endosymbionts and their reconciliation, assessed using phylogenetic reconstruction based on the *rrn* operon marker, indicated co-speciation events played a more prevalent role than duplication and host switch events (horizontal transfer), and therefore supported the hypothesis of strict co-speciation between the symbiotic bacteria with their nodulated host of subgenus *Crispardisia*. Given different results from different cophylogenetic studies, it is suspected that co-speciation is not the main coevolutionary process in the relationship between leaf nodulating endosymbionts of *Ardisia* subgenus

Crispardisia and their host. Moreover, the emphasis only on Chinese specimens of *Ardisia* subgenus *Crispardisia*, means the cophylogenetic patterns at family level are still unclear.

Therefore, this study investigates the cophylogenetic pattern in the symbiotic association between leaf nodulated Myrsinoideae hosts and their endosymbionts, and will give a broader understanding of evolutionary relationships between these two partners by using representative species from nodulated genera *Amblyanthopsis* and *Ardisia* subgenus *Crispardisia*.

3.1 MATERIALS AND METHODS

TAXON SAMPLING

For host and endosymbiont phylogenetic trees comparison, both host and endosymbiont phylogenies reconstructed using 15 nodulated samples (see Tables 3.1a and 3.1b). *Amblyanthus* was chosen as outgroup.

DNA EXTRACTION, AMPLIFICATION, PURIFICATION AND SEQUENCING

For leaf samples, total genomic DNA was extracted from either silica dried leaves or herbarium specimens, while bacterial DNA was obtained from excised leaf nodules or seeds using the method described in Doyle & Doyle (1987). The amplification of DNAs for leaf samples were conducted nrITS and an intergenic non-coding region of chloroplast DNA *rpL*32-*trn*L and *trn*S-*trn*G, while bacterial DNAs were amplified using housekeeping genes *gyr*B, *lep*A and *rec*A (see Table 3.1c for primers details). PCR were performed using either TaKaRa Ex Taq (Takara Bio, Shiga, Japan) or KAPA HiFi DNA polymerase (KAPA Biosystems, Wilmington, USA) protocols. For TaKaRa Ex Taq protocol, PCR were performed at 12.5 µL volumes containing 0.4 µM of each of the primers, 10X Ex Taq buffer (Takara), 2.5mM MgCl2, 250 µM of each dNTP, 5U Ex Taq DNA Polymerase (Takara) and 1 µL of template DNA. For KAPA HiFi protocol, PCR were performed at 10 µL volumes containing 2.5 µL of KAPA HiFi mixture reaction, 0.4 µM of each of the primers and 1 µL of template DNA. The amplification profile using Ex Taq for nrITS was 35 cycles of 95ºC for 30 s, 56ºC for 30 s, 72ºC for 3 min, preceded by initial denaturation at 95ºC for 5 min and followed by the final extension at 72ºC for 7 min. For cpDNA regions and housekeeping

genes, PCR were performed using KAPA HiFi protocol and therefore amplification profile were 35 cycles of 98ºC for 20 s, 60ºC for 15 s, 72ºC for 15 s, preceded by initial denaturation at 95ºC for 3 min and followed by the final extension at 72ºC for 3 min. PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA). Cycle sequencing-reactions were prepared with BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) by modifying the manufacturer's protocol, and performed on an ABI 3500 DNA sequencer (Applied Biosystems) and partly by Eurofins Genomics (Tokyo, Japan).

DNA ALIGNMENT AND PHYLOGENETIC ANALYSES

DNA sequence chromatograms were edited using the DNA Baser Sequence Assembler v.4.36.0 (http://www.dnabaser.com) software. The alignment of sequences was done using the MUSCLE algorithm (Edgar 2004) in MEGA7 software (Kumar et al. 2016). Finally, the sequences were manually trimmed to form a matrix data set with uniform length. Phylogenetic trees were obtained using Bayesian inference (BI) and maximum likelihood (ML) methods. BI analyses were run on Mr. Bayes 3.2.5 (Ronquist et al. 2012) after determining the appropriate model of evolution with the jModelTest v.2.1.1 (Posada 2008) under the Akaike information criterion. Model test selected for the different datasets the following models of evolution: nrITS = SYM+I+G, *rpL*32-*trn*L = GTR+G, *trn*S-*trn*G = GTR+G, *gyr*B = HKY+G and *lep*A = GTR+I. In the combined analysis, mixed-model approach was used. The concatenated datasets were partitioned and the same models were assigned to separate partitions as selected for single analyses. Four iterations consisting of five million Monte Carlo Markov Chains were run discarding 25% of the generations as burn-in. For ML analysis, RaxML v.8.0.26 (Stamatakis 2008) as implemented in raxmlGUI (Silvestro & Michalak 2012) was done on ML search using 10000 rapid bootstraps under the GTR gamma model. To verify whether there was a significant amount of conflict between

nrITS and the two cpDNA regions, the test of Farris et al. (1995) was performed using the partition- homogeneity test as implemented in PAUP*4.0a (2002). This test compares the sums of the lengths of the most parsimonious trees of the data analyses, to the distribution of the sums of lengths of the most parsimonious trees from random partition of the characters. The test was performed with 10000 replicates. Each replicate consisted of a heuristic search of random taxon addition and TBR branch swapping. Tree branches were collapsed to create polytomies if the maximum branch length was equal to zero.

COPHYLOGENETIC ANALYSIS

Cophylogenetic analyses can be constructed using two different methods: distant-based method e.g. ParaFit (Legendre 2002) and tree- or event-based methods e.g. TreeMap v3.0β (Charleston & Robertson 2002) and JANE 4.0 (Conow et al. 2010). Both methods have been summarized and reviewed in de Vienna et al. (2013). Distance-based methods determine if parasites and their hosts are associated randomly by comparing genetic distances from homologous gene regions for the associated partners without testing the presence of co-evolutionary events (Legendre 2002). While tree- or event-based methods compare only the branching structure of parasites and hosts phylogenetic trees to determine if tree topologies are more similar than would be expected by chance (e.g. Light et al. 2008). This method applies heuristics to find solutions that minimize the overall cost of evolutionary historical reconstruction given a cost regime for different events including co-speciation, duplication and host switching, lineage sorting and failure to diverge. However, the event-based methods is that they need fully resolved phylogenies.

In this study, event-based method implemented in JANE 4.0 for reconciliation reconstruction between leaf nodulating endosymbionts of Myrsinoideae and their hosts, was chosen. The new version JANE 4.0, has a graphical tree builder for constructing

host/symbionts trees, their tip associations, and other annotations. Hence, tanglegram, in which each of endosymbiont tip linked to a host tip between selected nodulated Myrsinoideae-hosts and their endosymbionts were built using JANE 4.0 based on the ML host and endosymbiont trees as input. However, the tanglegram was visualized using TreeMap v3.0β and plotted onto the hosts-endosymbionts trees (Figure 3.2a). In addition, JANE 4.0 is always able to provide correct solutions that are often optimal to find a reconciliation of minimal total cost as compared to another event-cost method (e.g. Martinez-Aquino et al. 2016, Mendlová et al. 2012).

In JANE 4.0, the cost regime for evolutionary events including (a) co-speciation/co-divergence, in which both partners, host and parasite speciate simultaneously, (b) duplication, in which a parasite speciates and both of the new species remain in the same host, (c) duplication and host switch where a parasite speciates and one of the new species switches onto a different host, (d) loss or lineage sorting where a host speciates and the parasite remains only on one of the new host species), and (e) failure to diverge, in which a host speciates and the parasite remains on both new host species (see simplified diagram in Figure 3.1). The cost assigned to each type of evolutionary event is related to the possibility of its occurrence. In this study, the reconciliation analysis was performed with 100 generations, population sizes of 100, a maximum of 99999 stored solutions in each run, and a default cost setting matrix of 0 for co-speciation/co-divergence, 1 for duplication, 2 for duplication and host switch, 1 for loss or lineage sorting, and 1 for failure to diverge.

3.2 RESULTS

HOST PHYLOGENY

The host phylogenetic tree was reconstructed using DNA sequences generated from previous chapters together with newly generated DNA sequences of *trn*S-*trn*G (16 accessions) in this study. The aligned concatenated dataset of nrITS, *rpL*32-*trn*L and *trn*S-*trn*G yielded 2258 bp in length and 174 sites were variable, 57 sites of which were potentially parsimony informative. The topology for both Bayesian inference and maximum likelihood trees is congruent, therefore, only maximum likelihood tree is presented as shown in Figure 3.2a (host).

ENDOSYMBIONTS PHYLOGENY

The endosymbiont phylogenetic tree was reconstructed using DNA sequences generated from previous chapter together with newly generated DNA sequences of *lep*A (16 accessions) in this study. The aligned concatenated dataset of *gyr*B and *lep*A yielded 1427 bp in length and 359 sites were variable, 98 sites of which were potentially parsimony informative. The topology for both Bayesian inference and maximum likelihood trees is congruent, therefore, only maximum likelihood tree is presented as shown in Figure 3.2a (endosymbionts).

TOPOLOGY BASED ANALYSIS

The tanglegram of the cophylogenetic relationship between Myrsinoideae-nodulated hosts and their endosymbionts was generated and the association between these two partners were plotted onto the hosts-endosymbionts trees in Figure 3.2a. Reconciliation reconstruction of event-cost based analysis using JANE 4.0 with the default cost setting detected 5 co-

speciation events, 9 duplication and host switching events, 1 lost or lineage sorting event and 0 for each duplication or failure to diverge events with minimum total cost 19 (Figure 3.2b). Based on the result, the host-endosymbiont topologies are congruent in some parts, but duplication and host switch among/within clades in a clade of *Ardisia* subgenus *Crispardisia* are also detected. Thus, the global congruence between hosts and endosymbionts was not significant (*p*-value = 0.16).

3.3 DISCUSSION

The hosts-endosymbionts trees reconciliation analysis shows a low-level co-speciation between Myrsinoideae-nodulated host and their endosymbionts. This is quite surprising for plant host-symbiont system as in nodulated genera of Myrsinoideae where the association between the two partners has been described as an obligate and intimate. Theoretically, this obligate and intimate relationship will lead to strict-co-speciation where the symbionts speciate or evolve simultaneously with their host. Unfortunately, this is not often the case for bacterial leaf nodule symbiosis in Myrsinoideae as currently inferred.

The topological incongruences between *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* -nodulated hosts and their endosymbionts are not only conflicting with previous result obtained by Ku & Hu (2014), in which their host-symbiont phylogenetic trees are highly congruence but also rejecting the history of long term strict-co-speciation as hypothesized in leaf nodulation of subgenus *Crispardisia*.

The reason for the differences probably due to inclusion of more taxa with few samples represented by multiple candidates viz. *Ardisia maculosa*, *Ar. virens* and *Ar. polysticta* in the present study compared to previous one (Ku & Hu 2014). The horizontal transmission of bacterial symbiont not only detected between taxa from different clade but also occurs within a species or between closely related species in the same clade by increasing the sample per taxon, for example in the *polysticta–virens* clade (Figure 3.2a). Similar observation has been detected in the cophylogenetic study of *Psychotria*, in which host-endosymbiont trees are incongruence except for few terminal taxa (Lemaire et al. 2011b).

It should be noted that there are potential confounding effects such as incomplete lineage sorting or recent host switching events when constructed co-speciation analyses

among lineages that have diverged recently (Hughes et al. 2007). The divergence time for leaf nodulated of *Ardisia* is estimated 5 Mya and is considered relatively recent origin of leaf nodulation in *Ardisia* subgenus *Crispardisia* (Lemaire et al. 2011b).

3.4 CONCLUSION

Although vertical transmission of symbiont bacteria implied strict-co-speciation between host–symbiont bacteria relationships, this study suggests that co-speciation may not be kept always in the leaf nodulation of *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* clade. Duplication and host switches may be more common events than it has reported previously.

3.5 REFERENCES

Charleston M.A. and Perkins S.L. 2006. Traversing the tangle: Algorithms and applications for cophylogenetic studies. J. Biomed. Inform. 3**9** (1): 62–72.

Charleston M.A. and Robertson D.L. 2002. Preferential host switching by primate lentiviruses can account for phylogenetic similarity with the primate phylogeny. Syst. Biol. **51** (3): 528–535.

Clayton D.H., Al-Tamimi S. and Johnson K.P. 2003. The ecological basis of coevolutionary history. *In*: Page R.D.M. (ed.). Tangled Trees: Phylogeny, Co-speciation and Coevolution. pp. 310–341. University of Chicago Press, Chicago.

Conow C., Fielder D., Ovadia Y. and Libeskind-Hadas R. 2010. Jane: a new tool for the cophylogeny reconstruction problem. Algorithms Mol. Biol. **5**: 16. Available from https://doi.org/10.1186/1748-7188-5-16.

de Vienne D.M., Refrégier G., López-Villavicencio M., Tellier A., Hood M.E. and Giraud T. 2013. Co-speciation vs. host-shift speciation: methods for testing, evidence from natural associations and relation to coevolution. New Phytol. **198**: 347–385.

Doyle J.J. and Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11–15.

Edgar R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32** (5): 1792–1797.

Farris J.S., Källersjö M., Kluge A.G. and Bult C. 1995. Testing significance of incongruence. Cladistics **10**: 315– 319.

Hughes J., Kennedy M., Johnson K.P., Palma R.L. and Page R.D.M. 2007. Multiple Cophylogenetic Analyses Reveal Frequent Co-speciation between *Pelecaniform* Birds and *Pectinopygus* Lice. Syst. Biol. **56** (2): 232–251.

Ku C. and Hu J.M. 2014. Phylogenetic and cophylogenetic analyses of the leaf-nodule symbiosis in *Ardisia* subgenus *Crispardisia* (Myrsinaceae): evidence from nuclear and chloroplast markers and bacterial *rrn* operons. Int. J. Plant Sci. **17** (1): 92–109.

Kumar S., Stecher G. and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molec. Biol. Evol. **33** (7): 1870–1874.

Legendre P., Desdevises Y. and Bazin E. 2002. A statistical test for host-parasite coevolution. Syst Biol. **51**: 217–234.

Lemaire B., Smets E. and Dessein S. 2011a. Bacterial leaf symbiosis in *Ardisia* (Myrsinoideae, Primulaceae): molecular evidence for host specificity. Res. Microbiol. **162**: 528–534.

Lemaire B., Vandamme P., Merckx V., Smets E. and Dessein S. 2011b. Bacterial Leaf Symbiosis in Angiosperms: Host Specificity without Co-Speciation. PLoS ONE **6** (9): e24430. Available from https://doi.org/10.1371/journal.pone.0024430

Lemaire B., Janssens S., Smets E., Dessein S. 2012. Endosymbiont Transmission Mode in Bacterial Leaf Nodulation as Revealed by a Population Genetic Study of Psychotria leptophylla. Appl. Environ. Microbiol. **78** (1): 284–287. Available from htpps://doi:10.1128/AEM.06130-11.

Light J.E. and Hafner M.S. 2008. Codivergence in Heteromyid Rodents (*Rodentia*: Heteromyidae) and Their Sucking Lice of the Genus *Fahrenholzia* (Phthiraptera: Anoplura). Syst. Biol. **57** (3): 449–465.
Available from https://doi.org/10.1080/10635150802169610.

Martinez-Aquino A. 2016. Phylogenetic framework for coevolutionary studies: a compass for exploring jungles of tangled trees. Current Zoology. **62** (4): 393–403.
Available from https://doi.org/10.1093/cz/zow018.

Mendlová M., Desdevises Y., Civáňová K., Pariselle A. and Šimková A. 2012. Monogeneans of West African Cichlid Fish: Evolution and Cophylogenetic Interactions. PLoS ONE **7** (5): e37268.

Available from https://doi.org/10.1371/journal.pone.0037268.

Miller I.M. 1990. Bacterial leaf nodule symbiosis. Adv. Bot. Res. **17**: 163–234.

Miller I.M., Gardner I.C. and Scott A. 1983. The development of marginal leaf nodules in *Ardisia crispa* (Thunb.) A. DC. (Myrsinaceae). Bot. J. Linn. Soc. **86**: 237–252.

Page R.D.M. 2003. Introduction. *In*: Page R.D.M. (ed.) Tangled trees: Phylogeny, Cospeciation and Coevolution. pp. 1–21. University of Chicago Press, Chicago.

Peterson K.R., Donald H.P. and Charles D.B. 2010. Cophylogeny and biogeography of the fungal parasite *Cyttaria* and its host *Nothofagus*, Southern Beech. Mycologia **102** (6): 1417–1425.

Ronquist F., Teslenko M., Van Der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M.A. and Huelsenbeck J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**: 539–542.

Shaw J., Lickey E.B., Schilling E.E. and Small R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am. J. Bot. **94** (3): 275–288.

Shaw J., Lickey E.B., Beck J.T. Farmer S.B., Liu W., Miller J., Siripun K.C., Winder C.T., et al. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot. **92** (1): 142–166.

Silvestro D. and Michalak I. 2012. raxmlGUI: a graphical front-end for RAxML. Org. Divers Evol. **12**: 335.

Spilker T., Baldwin A., Bumford A., Dowson C.G., Mahenthiralingam E., LiPuma J.J. 2009. Expanded multilocus sequence typing for *Burkholderia* species. J. Clin. Microbiol. **47**: 2607–2610.

Stamatakis A. 2008. RAxML-VI-HPC: a tool for phylogenetic analysis and post- analysis of large phylogenies. Bioinformatics **30**: 1312–1313.

Swofford D.L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods) version 4. Sinauer Associates, Sunderland, Massachusetts.

Szöllősi G.J., Tannier E., Daubin V. and Boussau B. 2014. The inference of gene trees with species trees. Syst Biol. **64**: 42–62.

White T.J., Bruns T.D., Lee S.H. and Taylor J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis M.A., Gefland D.H., Sninsky J.J., White T.J. (eds.) PCR protocols: A guide to methods and application. pp. 315–322. Academic Press, San Diego.

Table 3.1a. List of samples used in the host phylogenetic analysis. ID is the given sample's tube number.

No.	Genus/subgenus	Samples	Vouchers (coll. no., origin[a], herbarium[b])	ID
1	*Amblyanthopsis*	*Amblyanthopsis bhotanica*	KEA883, MYN, NYBG	517
2	*Amblyanthus*	*Amblyanthus* sp. vel aff. *multiflorus*	MY881, MYN, TNS	654
3	*Ardisia* subgen. *Crispardisia*	*Ar*. *corymbifera*	T907, THA, FU	250
4		*Ar. crenata* subsp. *crenata*	SNP37015, SBH, SNP	455a
5		*Ar*. *crenata* subsp. *obtusifolia*	T4630, THA, FU	271
6		*Ar*. *maculosa*	T3059, THA, FU	263
7		*Ar*. *maculosa*	T4760, THA, FU	273
8		*Ar*. *polysticta*	V1937, VNM, FU	303
9		*Ar*. *polysticta*	V3054, VNM, FU	361
10		*Ar*. *virens*	T3060, THA, FU	264
11		*Ar*. *virens*	IS274, SUM, FU	399
12		*Ar*. cf. *cadieri*	V1905, VNM, FU	302
13		*Ar.* cf. *tsangii*	V2415, VNM, FU	353
14		*Ar.* sp. vel aff. *clemensii*	V2741, VNM, FU	356
15		*Ar.* sp. vel aff. *vidalii*	V1955, VNM, FU	304
16		*Ar*. sp. vel aff. *villosa*	T2177, THA, FU	256

[a]Origin: MYN=Myanmar, SBH=Sabah, SUM=Sumatra, THA=Thailand, VNM=Vietnam

[b]Herbarium: KYU= Kyushu University, NYBG=New York Botanical Garden, SNP= Sabah National Park Herbarium, TNS= National Science Museum, Tsukuba.

Table 3.1b. List of samples used in the endosymbiont phylogenetic analysis. ID is the given sample's tube number, BS = bacterial symbiont.

Taxa	Voucher	Origin	ID
Endo. Amblyanthus sp. vel aff. *multiflorus*	MY881	Myanmar	BS16Aa
Endo. Amblyanthopsis bhotanica	KEA883	Myanmar	BS27
Endo. Ar. corymbifera	T907	Thailand	BS4
Endo. Ar. crenata subsp. *crenata*	SNP37015	Sabah	B22
Endo. Ar. crenata subsp. *obtusifolia*	T4630	Thailand	B2
Endo. Ar. maculosa	T4760	Thailand	B10
Endo. Ar. maculosa	T3059	Thailand	B7
Endo. Ar. polysticta	V1937	Vietnam	BS30
Endo. Ar. polysticta	V3054	Vietnam	BS15
Endo. Ar. virens	T3060	Thailand	BS6
Endo. Ar. virens	IS274	Sumatera	BS31
Endo. Ar. cf. *cadieri*	V1905	Vietnam	B32
Endo. Ar. cf. *tsangii*	V2415	Vietnam	B28
Endo. Ar. sp. vel aff. *clemensii*	V2741	Vietnam	B12
Endo. Ar. sp. vel aff. *vidalii*	V1955	Vietnam	B11
Endo. Ar. sp. vel aff. *villosa*	T2177	Thailand	BS8

Table 3.1c. Oligonucleotide primers for amplification and sequencing.

Region	Primer	Primer sequence (5'-3')	Reference
ITS	ITS4-F	TCCTCCGCTTATTGATATGC	White et al. (1990)
	ITS5-R	GAAGTAAAAGTCGTAACAAGG	
*rpL*32-*trn*L$^{(UAG)}$	*rpL*32	CAGTTCCAAAAAAACGTACTTC	Shaw et al. (2005)
	*trn*L	CTGCTTCCTAAGAGCAGCGT	
trnS$^{(GCU)}$-*trn*G$^{(UUC)}$	*trn*S	AGATAGGGATTCGAACCCTCGGT	Shaw et al. (2007)
	*trn*G	GTAGCGGGAATCGAACCCGCATC	
*rec*A	*recA*-F (forward)	AGGACGATTCATGGAAGAWAGC	Spilker et al. (2009)
	recA-R (reverse)	GACGCACYGAYGMRTAGAACTT	
*gyr*B	*gyrB*-F (forward)	ACCGGTCTGCAYCACCTCGT	Spilker et al. (2009)
	gyrB-R (reverse)	YTCGTTGWARCTGTCGTTCCACTGC	
*lep*A	*lepA*-F (forward)	CTSATCATCGAYTCSTGGTTCG	Spilker et al. (2009)
	lepA-R (reverse)	CGRTATTCCTTGAACTCGTARTCC	

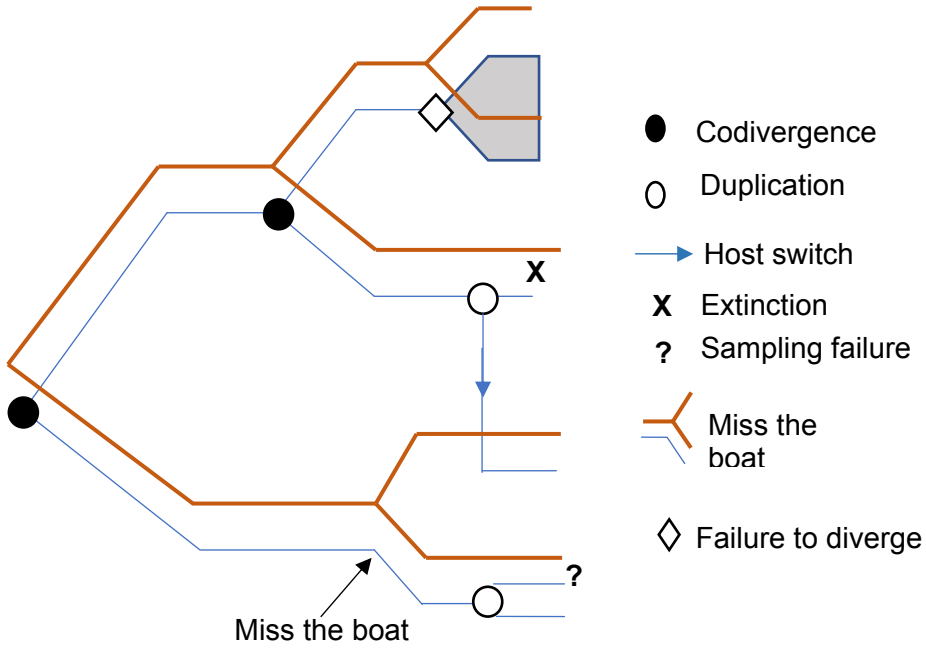

Figure 3.1 The coevolutionary events that explain relationships between the host and their parasites. Codivergence and duplication are shown in solid and open circles respectively; a host switch is shown by an arrow from source to target, and loss is implied by the existence of just one child lineage persisting on one of two recently diverged hosts. The three processes that lead to loss are missing the boat, sampling failure ("?") and extinction ("X"). Failure to diverge is shown in diamond shape with a grey area covering both host lineages that the parasite continues to infect.

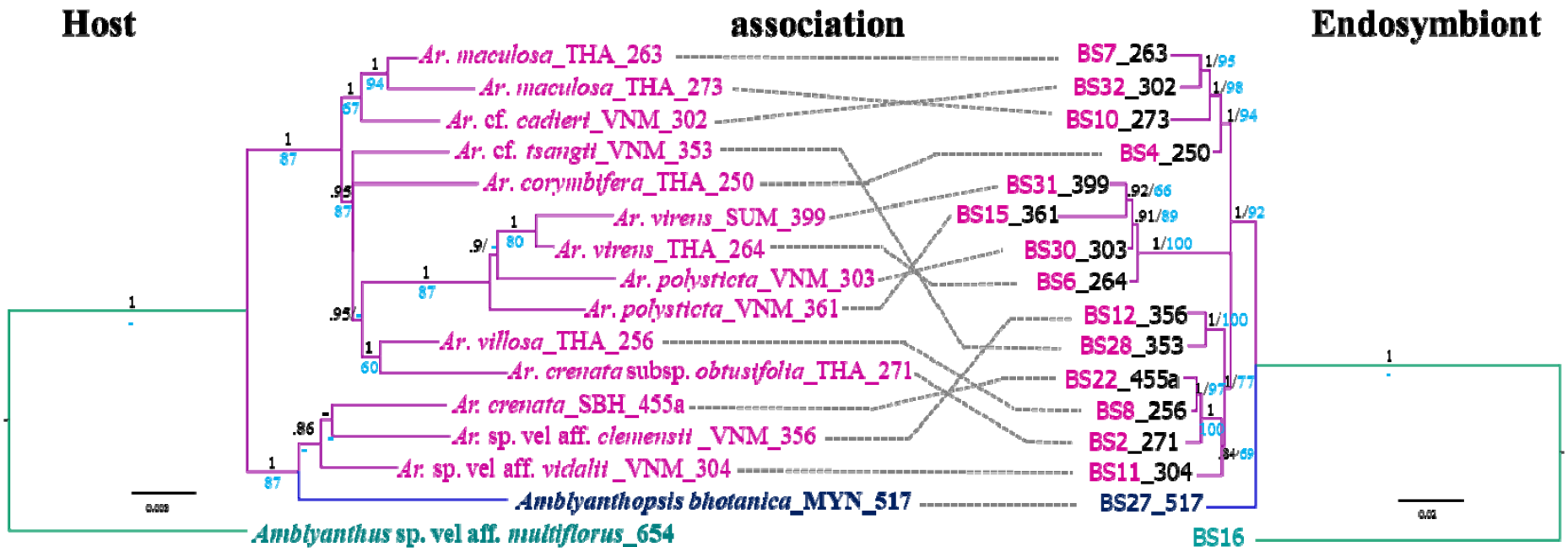

Figure 3.2a. Comparison of host and endosymbiont phylogeny in Myrsinoideae. The myrsinoid host (left) and endosymbiont (right) phylogenies were reconstructed from nrITS, plastid *rpL*32-*trn*L, *trn*S-*trn*G and bacterial *gyr*B and *lep*A, respectively. Posterior probabilities ≥ 5.0 are in black and bootstrap supports ≥ 50% (maximum likelihood) in blue. The '-' indicates bootstrap value <50%, BS = bacterial symbiont.

Legend

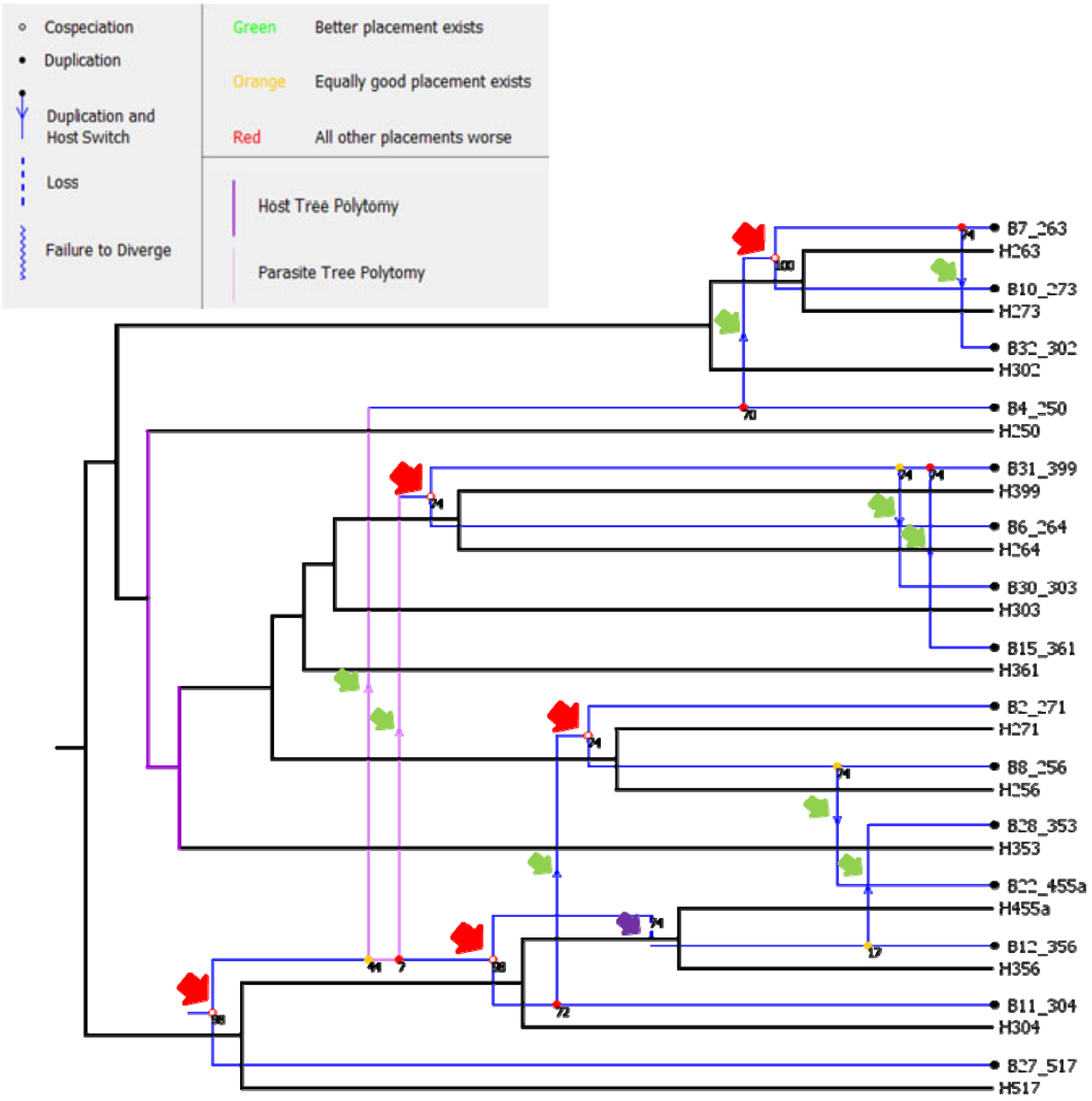

Figure 3.2b Reconciliation reconstructions of Myrsinoideae-nodulated host and their endosymbionts with JANE 4. Black branches represent the host phylogeny and blue branches represent the symbiotic bacteria phylogeny. The cost regime used for the reconstruction was by following default event costs: co-speciation=0, duplication=1, duplication and host switch=2, loss or lineage sorting=1 and failure to diverge=1. The best fit reconciliation of the Myrsinoideae-nodulated host and their endosymbionts trees included 5 co-speciation (red arrows), 9 duplications and host switches (green arrows), and 1 losses or lineage sorting (purple arrow).

GENERAL DISCUSSION

In general, the results of this study reveal evolution of leaf nodulation involved more complex interaction among different groups of plants and bacteria. The dynamic interaction involving vertical and horizontal transmission of bacterial symbionts in the symbiotic association of Myrsinoideae-host and their endosymbionts may suggest that co-speciation may not be kept always in an obligate symbiotic association.

GENERAL CONCLUSION

The current obtained results were not only improved our understanding on evolution of bacterial leaf nodule symbiosis in Myrsinoideae but also in angiosperms generally. With additional data sought from this study, the bacterial leaf nodule symbiosis is now known evolved at least five times independently in angiosperms: in the genus *Pavetta*, *Psychotria* and *Sericanthe* of Rubiaceae and in the *Amblyanthus* and *Amblyanthopsis* + *Ardisia* subgenus *Crispardisia* of Myrsinoideae (Primulaceae). Additionally, the closely related between leaf nodulating endosymbiont of *Amblyanthus* to leaf nodulating endosymbiont of *Psychotria* provide further evidence of genetically closely related *Burkholderia* endosymbiont between nodulated genera of Myrsinoideae and Rubiaceae. Moreover, my research provides certainly information to answers some basic questions need to be address for example the endosymbionts identity and confirmation of the presence of symbiotic bacteria associated with nodulated taxa within Myrsinoideae. The documentation of this information can be used later in other studies that might interest other researchers such as investigating the function of endosymbionts to the host or to clarify further transmission mechanism of endosymbionts to the host.

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