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Disentangling a complex genus: systematics, biogeography and bioactivity of the genus *Phyllanthus* L. and related genera of tribe Phyllanthae (Phyllanthaceae)

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CHAPTER 1

General Introduction

Chapter 1

Background

Phyllanthaceae is a remarkable but understudied plant family that is classified in the Malpighiales, a difficult order often with families characterized by very small flowers, which has been the subject of several phylogenetic studies (Wurdack et al. 2004; Wurdack & Davis 2009; Xi et al. 2012). The order comprises roughly 42 families, with almost no clear apomorphies per clade and the distinctions and relations between families are still under discussion (Xi et al. 2012). One of the more difficult member families is the Euphorbiaceae, which in the past was classified in five major subfamilies: Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae (Webster 1994, 2014; Radcliffe-Smith 2001).

Following the results of APG II (APG 2003), Euphorbiaceae was found to be non-monophyletic and it was subsequently divided into five families: Euphorbiaceae s.s., Phyllanthaceae and Picrodendraceae (formerly subfamily Oldfieldioideae) (APG 2003; Wurdack et al. 2009), next to the non-related Pandaceae and Putranjivaceae. The most recent molecular study by Xi et al. (2012), identified a clade as the euphorbioids, which consists of the families Euphorbiaceae, Rafflesiaceae, Peraceae, Picrodendraceae, Phyllanthaceae, Linaceae and Ixonanthaceae). While support between the major clades varies between studies (see Xi et al. 2012; Sun et al. 2016), the Picrodendraceae and Phyllanthaceae are consistently retrieved as sister groups. Picrodendraceae and Phyllanthaceae represent what is commonly known as the phyllanthoids or bi-ovulate Euphorbiaceae and these families are still sometimes treated together with Euphorbiaceae (Webster 2014). Nevertheless, the phyllanthoids are an interesting clade and particularly the family Phyllanthaceae, which contains more than 2000 species (Hoffmann et al. 2006), characterized by unisexual flowers (with a few bisexual exceptions in *Aporosa* Blume), ecarunculate seeds (except in *Glochidion* J.R.Forst. & G.Forst. and *Margaritaria* L.f.) and generally capsular fruits (Fig. 1-1).

The latest classification of Phyllanthaceae divides the family into two subfamilies and ten tribes (Fig. 1-2). A further division of Phyllanthaceae into several families seems unwarranted (but see Chakrabarty & Balakrishnan 2018). Currently 58 genera are recognised and the majority of species are found in tribe Phyllanthae. The diversity of morphological characters within this tribe has caused several issues in its taxonomy, most notably in the species rich genus *Phyllanthus* L., which was found to be paraphyletic (Wurdack et al. 2004; Samuel et al. 2005; Kathriarachchi et al. 2006). Tribe Phyllanthae was originally defined by Dumort (1829) within the Euphorbiaceae (as Phyllanthae) containing the genera *Cluytia* Steud. (now Euphorbiaceae), *Xylophylla* L., *Phyllanthus*, *Kirganelia* A.Juss., *Cicca* L., *Andrachne* L. and *Bridelia* Willd. Several genera in this classification were merged with *Phyllanthus* in the second half of the 19th century (Müller 1863, 1865, 1866) and the classification

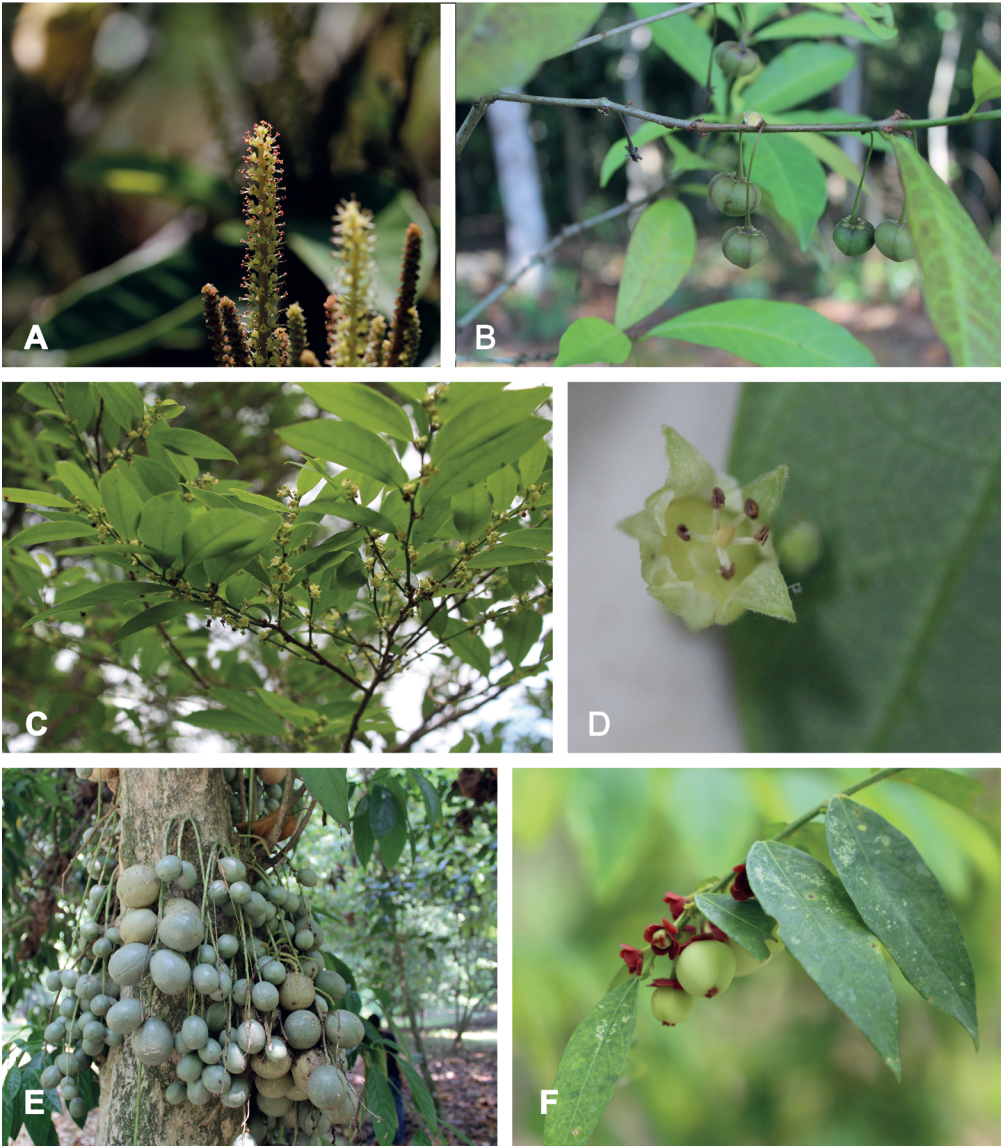


Figure 1-1. Examples of species of Phyllanthaceae: A. Flowers of *Antidesma bunioides* (L.) Spreng., B. Fruits and flower of *Actephila excelsa* (Dalzell) Müll.Arg.; C. flowering branch of *Bridelia* sp.; D. close-up of a staminate flower of a *Bridelia* sp.; E. fruits of *Baccaurea macrocarpa* (Miq.) Müll.Arg.; F. pistillate flowers and fruits of *Breynia androgyna* (L.) Chakrab. & N.P.Balakr. Photos: A, B, C, D, E by R.W.Bouman; F by R.-Y. Yu.

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Table 1-1. Genera included in tribe Phyllanthaceae by different authors.

Dumort (1829)	Hoffmann et al. (2006)	Van Welzen et al. (2014a)
<i>Cluytia</i>	<i>Phyllanthus</i>	<i>Breynia</i>
<i>Andrachne</i>	<i>Flueggea</i>	<i>Flueggea</i>
<i>Bridelia</i>	<i>Phyllanthus</i>	<i>Glochidion</i>
<i>Cicca</i>	<i>Heterosavia</i>	<i>Heterosavia</i>
	<i>Lingelsheimia</i>	<i>Lingelsheimia</i>
<i>Kirganelia</i>	<i>Margaritaria</i>	<i>Margaritaria</i>
<i>Xylophylla</i>	<i>Plagiocladus</i>	<i>Plagiocladus</i>
<i>Phyllanthus</i>	<i>Phyllanthus</i>	<i>Phyllanthus</i>
	<i>Phyllanthus</i>	<i>Synostemon</i>

has changed considerably (Table 1-1). *Phyllanthus* was defined as a broad genus with more than 40 sections (Müller 1863, 1865, 1866). Subsequent changes reinstated *Glochidion* (Kurz 1873) and *Margaritaria* (Webster 1957, 1979) as distinct genera. In the phylogenetic classification by Hoffmann et al. (2006), tribe Phyllanthaceae was divided into a limited number of genera and the authors recommended to combine a paraphyletic *Phyllanthus* with the genera *Breynia* J.R.Forst. & G.Forst., *Sauropus* Blume and *Glochidion*. However, some feel that this would push taxonomic problems with *Phyllanthus* to the subgeneric level while still not resolving them (Pruesapan et al. 2012; van Welzen et al. 2014a).

Phyllanthus represents an interesting paraphyletic taxon with a rich taxonomical history, divergent morphology and practically unknown ecology. In this thesis I hope to address several interesting questions in *Phyllanthus* while exploring the evolution of this diverse genus. Coincidentally, problems in the classification of *Phyllanthus* is not limited to only plants as it was also the name of a genus of birds (Cibois et al. 2018)). This monospecific genus has been recently subsumed (Cibois et al. 2018) in the larger (formerly paraphyletic) genus *Turdoides* Cretzschmar 1826 (Leiothrichidae). While problems are probably not attached to the name itself, it remains a peculiar coincidence.

Figure 1-2. Molecular phylogeny of Phyllanthaceae resulting from Bayesian and Maximum likelihood analysis on the dataset of Kathriarachchi et al. (2005). Subtribes, tribes and subfamilies follow Hoffmann et al. (2006) but genera of tribe Poranthereae incorporate changes from Vorontsova & Hoffmann (2008). The paraphyly of *Phyllanthus* is highlighted in red and polyphyly of *Cleistanthus* is highlighted in green. Figure shown on adjacent page.

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Chapter 1

Morphological diversity

With over 800 species (Chapter 3) originating from several evolutionary lineages, the genus *Phyllanthus* displays an enormous diversity of morphological characters (Fig. 1-3). Important characters within the genus and the rest of the tribe are the habit, branching system, flowers, fruits, seeds and pollen morphology.

The majority of species of *Phyllanthus* are woody plants varying from trees to shrubs, however herbaceousness has evolved several times independently (Chapter 7). Some well-known herbaceous species of *Phyllanthus* like *P. amarus* Schumacher & Thonn. and *P. urinaria* L. are often difficult to identify, which is complicated even more as a number of species have wide distributions and are invasive in many countries. By comparison, species of *Breynia* and *Glochidion* are usually shrubs to trees, while species of *Synostemon* F.Muell. are usually lower sprawling shrubs.

About 80% of the species of *Phyllanthus* exhibit a specialized branching system, which was coined by Webster (1956) as phyllanthoid branching. In this system, plagiotropic ultimate axes (side branches) resemble pinnate leaves, because they are deciduous and have a limited growth. These lateral branchlets are floriferous and bear laminate leaves while they are subtended by a scale-like leaf (cataphyll) and two cataphyllary stipules. Orthotropic axes typically only display laminate leaves in the first few nodes and switch to reduced leaves subtending the lateral axes in the upper nodes. Phyllanthoid branching is not present in *Phyllanthus* subgenera *Isocladus* G.L.Webster, *Macraea* (Wight) Jean F.Brunel and *Ceramanthus* (Hassk.) Jean F.Brunel, which are sister to the other clades in the genus (Kathriarachchi et al. 2006; Falcón Hidalgo et al. 2020) and it has been independently lost in several taxa (Chapter 6/7).

Inflorescences in *Phyllanthus* are generally axillary fascicles, unisexual or bisexual, with a varying number of flowers. Some taxa have more elaborate inflorescence structures like racemes (e.g. *Phyllanthus* subgenus *Gomphidium* (Baill.) G.L.Webster section *Nymanina* (K.Schum.) J.J.Sm.) or thyrses (e.g. *Phyllanthus* subgenus *Xylophylla* section *Epistylium* (Sw.) Griseb.). The flowers show a relatively remarkable variation in form even though they consist of only a few elements. Flowers of *Phyllanthus* are characterized by two whorls of tepals (with some discussion on the differentiation of sepals and petals, see Gama et al. 2016), a (nectar) disc and either the andro- or gynoecium. The sepal number often differs between staminate and pistillate flowers and varies per subgenus. Pistillate discs are usually entire (with exceptions) and variable in size, while staminate discs are more often segmented with varying shapes. No studies have focused on the nectar production of these flowers and how the sugar composition looks like.

Micromorphological characters have had a large influence on the classification of *Phyllanthus*. Webster's (1956, 1957, 1958) seminal work on the

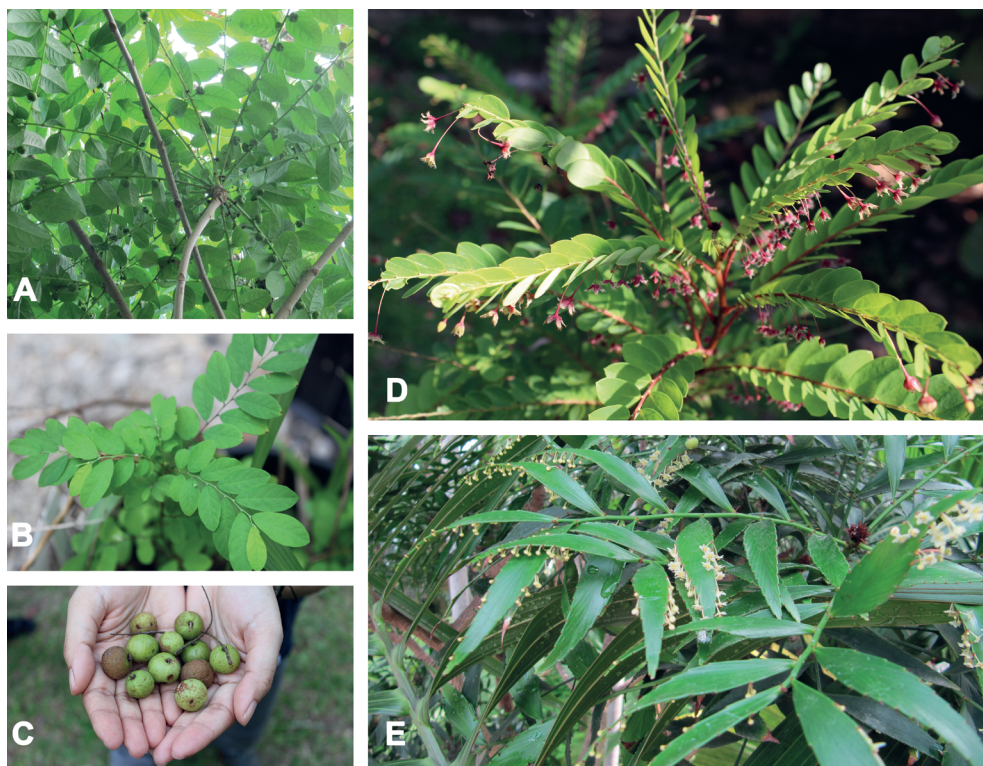


Figure 1-3. Examples of various species of *Phyllanthus*: A. *Phyllanthus juglandifolius* Willd. in fruit; B. *Phyllanthus Tenellus* Roxb.; C. fruits of *Phyllanthus emblica* L.; D. *Phyllanthus pulcher* Wall. ex Müll.Arg. in flower; E. *Phyllanthus arbuscula* (Sw.) J.F.Gmel. showing its characteristic phylloclades with flowers along the margins. Photos by R.W.Bouman.

infrageneric classification of *Phyllanthus* was expanded upon and complemented with a series of palynological studies from various authors (e.g. Punt 1967, 1972, 1975, 1980; 1987; Brunel 1987; Lobreau-Callen et al. 1988; Webster & Carpenter 2002, 2008; Sagun & van der Ham 2003; Santiago et al. 2004; Chen et al. 2009; Wu et al. 2016).

Similar to other taxa in Phyllanthaceae and Euphorbiaceae, most species of *Phyllanthus* are characterized by small schizocarpic fruits. Ornamentation and number of locules may be differentiative characters between species, but often the fruit morphology is similar between species. Drupaceous fruits have evolved independently in a few taxa and are mainly found in *Phyllanthus* subgenus *Kirganelia*, while some species are also cultivated for their edible fruits (e.g. *P. acidus* (L.) Skeels and *P. emblica* L.). The seeds are usually trigonal, but show differences between subgenera in their ornamentation.

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Ecology and geography

Phyllanthus species can be found in a wide range of areas from a few desert species to the tropics. Only some invasive species are recorded in Mediterranean climate. The distribution of *Phyllanthus* and related genera has not been studied in detail. Early investigations based on the subgeneric classification from Webster (1956, 1957, 1958) indicated multiple dispersal events between continents (Holm-Nielsen 1979), but these have not been studied in a phylogenetic context. Similarly, while elevation or substrate is sometimes noted on collection labels, this has not been correlated between species or to specific distributions. Some well known calciferous species are known, but for example the amount of rheophytes is probably highly underestimated.

With the remarkable diversity in floral characters such as flower shape (open and disc-like to more closed and tube-shaped), disc shape and the variation in fusion and numbers of stamens and sepals, the cause could be inferred from pollination. Various systems are found and the ancestral system has been hypothesized based on observations in *Flueggea* (basal in the phylogeny) to be generalist bees or flies (Kawakita 2010). Kato et al. (2003) discovered a fascinating pollination system in *Glochidion*, which involves a mutualism with parasitic moths of the genus *Epicephala* Meyrick 1880. The female moths actively gather pollen from staminate flowers and visit pistillate flowers to pollinate them, afterwards they lay an egg in the flower and the developing larvae consume a portion of the seeds and receive protection from the plant. Host specificity and a close relation between plant and moth species are hypothesized to have caused a rapid co-evolution in *Glochidion* (see also Hembrey et al. 2013, 2018) and variations on this system are found in *Breynia* (Kawakita & Kato 2004b; Zhang et al. 2012) and various clades in *Phyllanthus* (Kawakita et al. 2009, 2019; Luo et al. 2011b; Kawakita & Kato 2017). This area of study is being further explored in terms of floral scent (Svensson et al. 2010), flower abortion (Goto et al. 2010), sharing and selection of hosts (Zhang et al. 2012) and seems to explain part of the rapid speciation of *Glochidion*. Similarly, in *Phyllanthus* this system has been related to the high species number found in subgenus *Gomphidium* (>100) which occurs mainly in New Caledonia, Australia and Papua New Guinea (Kawakita & Kato 2004a). However, comparable species numbers are not found in every clade associated with *Epicephala* moths and other factors should probably be included (Chapter 8).

As most species of *Phyllanthus* are characterized by schizocarpic fruits, the seeds are mostly dispersed autochorously. However, this mechanism and the distances traveled by seeds have not been studied for *Phyllanthus*. There are some indications that small fruits can disperse seeds about one to two meters (personal observation), but this has not been appropriately tested in the lab or the field. Similarly, dormancy of seeds has been a neglected study within the genus, but the presence of several

invasive species suggests they can establish readily with an appropriate travel vector. Fleshy fruits have evolved several times independently within tribe Phyllanthae (Chapter 7) and these are probably mostly dispersed by birds. Observations of bird dispersal have been sporadic in *Flueggea*, but has not been published for *Phyllanthus*. Similarly in *Glochidion*, the fruits dehisce to expose seeds with a brightly colored sarcotesta that is probably attractive to avian dispersers.

An interesting facet of a few species is their adaptation to areas with high metal concentrations in the ground. Recent studies in the biological accumulation of metals in plants have identified several species of *Phyllanthus* (Van der Ent et al. 2013; Nkrumah et al. 2016), which occur in ultramafic areas.

Genetics

Molecular systematics of *Phyllanthus* have mostly focused on resolving the relation between various taxa both at or above species level. First indications of paraphyly were found by Wurdack et al. (2004) and were subsequently confirmed by Samuel et al. (2005) and Kathriarachchi et al. (2006). Hoffmann et al. (2006) based an updated classification of Phyllanthaceae on the phylogeny from these previous studies. A molecular study of tribe Poranthereae and subsequent changes in classification on the groupings resulted in the recognition of eight genera (Vorontsova et al. 2007; Vorontsova & Hoffmann 2009). Pruesapan et al. (2008, 2012) argued that the classification of Hoffmann et al. (2006) would push taxonomic problems only to subgeneric levels while in turn creating a giant heterogeneous *Phyllanthus*. Increased sampling efforts of tribe Phyllanthae with a focus on *Breynia* and *Sauropus*, suggested that they should be combined, but could be kept separate from *Phyllanthus* while also reinstating the genus *Synostemon*. Full plastome sequences are only available for four taxa of tribe Phyllanthae: *Glochidion chodoense* C.S. Lee & Im (Cheon et al. 2019), *Flueggea virosa* (Roxb. ex Willd.) Baill. (Wang et al. 2020), *Breynia fruticosa* (L.) Müll. Arg. (Zhou et al. 2020) and *P. emblica* (record NC_047477.1 on Genbank). There are some indications of a possible genome duplication occurring in tribe Phyllanthae, but data on chromosomal numbers or genome sizes are severely lacking for many taxa (see Webster & Ellis 1962). Sampling of *Phyllanthus* has previously covered about 10% of the genus, but some subgenera and sections have not yet been included. A more thorough sampling could investigate the relationship between major lineages of *Phyllanthus* and test the monophyly of the current classification of subgenera and (sub) sections within the genus.

Medicinal effects and metabolites

Two species are common in cultivation because they are renowned for their edible fruits (*P. acidus* and *P. emblica*), which are high in vitamin C (Liu et al. 2008). Aside

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from their nutritional value, a long list of species are also used in traditional medicine or hunting practices (Unander et al. 1990, 1991, 1992, 1995), however only a small number of species have been included in studies towards bioactive compounds. This is where botanical gardens, which harbour an enormous diversity of plant species, provide an opportunity to study more species.

Outline and aims of this thesis

Several gaps in knowledge have been highlighted in the introduction above, the largest of which is the current paraphyly of the genus *Phyllanthus*. With this thesis, I aim to expand our knowledge of *Phyllanthus* and discuss broader evolutionary patterns in the context of their morphology and phylogenetics. To begin studying the diversity within *Phyllanthus*, which has been described across hundreds of papers, this information needed to be summarized and the system of subgenera and sections was an ideal candidate that needed to be applied to the rest of *Phyllanthus* (Chapter 2 & 3). From this review, several taxonomic problems were identified and some subsequently treated (Chapter 4 & 5). With the rising use of *Phyllanthus* in traditional medicine, we wished to test several lineages for their bioactive compounds, to identify possible further interesting groups (Chapter 6). As the classification was based on morphology, we wanted to expand the current sampling for phylogenetic studies of *Phyllanthus* to see whether all groups were monophyletic (Chapter 7). The phylogeny allowed us to map broad distribution patterns of various clades and study where *Phyllanthus* originated and how it dispersed to the rest of the world (Chapter 8). Finally, a decision needed to be made on the paraphyly of *Phyllanthus*, which will have taxonomic consequences for an enormous amount of species (Chapters 9).

Chapter 2

In the preparation of this thesis, several new species of *Phyllanthus* were discovered through herbarium or molecular work. *Phyllanthus rufuschaneyi* Welzen, R.W.Bouman & Ent is the first to be described (Bouman et al. 2018a) and its general affinities are discussed. What makes this species all the more interesting, is the fact that it was grown at Kinabalu parks (Sabah, N. Borneo) for several years and studied for its extraordinary accumulation of metals. Therefore, this species is a strong candidate for its use in the upcoming field of agromining, which aims to use plants for the collection of metals from contaminated soils or those naturally rich in heavy metals.

Chapter 3

Breynia, *Synostemon* and *Glochidion* were found to be nested within *Phyllanthus*, but the genera were upheld by van Welzen et al. (2014a). *Phyllanthus* is therefore a

paraphyletic genus and to explore whether it is possible to split the genus, each species needs to be placed in a morphological and phylogenetic framework. In chapter 3, a table is presented that summarizes the taxonomic history of *Phyllanthus* and updates the classification system to cover all accepted species (Bouman et al. 2018b). Several taxonomic problems are highlighted for future study and a provisional key to the subgeneric classification of *Phyllanthus* is provided.

Chapter 4

One of the remaining taxonomical anomalies presented in chapter 3 concerns *Phyllanthus* subgenus *Isocladius* and its originally broad treatment by Webster (1956). Following his treatment would result in a polyphyletic subgroup, but the alternative, a classification by Brunel (1987) has often been ignored. Subgenus *Macraea* was originally placed in subgenus *Isocladius*, but was shown to be phylogenetically distinct (Kathriarachchi et al. 2006). This group of plants is here revised over its entire distribution and its taxonomic rank and affinity are discussed extensively to highlight morphological (dis)similarities with other plants of the genus *Phyllanthus*.

Chapter 5

Due to a lack of material and incomplete descriptions, not all species could be confidently assigned in chapter 3 to a specific subgeneric group. Two species were described by Koorders (1904) for the island of Sulawesi (Indonesia), but the initial description only mentioned the habit and which species it resembled. With no mention of the morphology of the flowers, the affinity of these species was initially unknown. During a recent trip during a Flora Malesiana Symposium in the region, I had the chance to see type material collected by Koorders himself that is currently stored at the herbarium of Bogor (BO) in Cibinong (Java, Indonesia). In a short revision, which includes flower descriptions, these species are assigned to *Phyllanthus* subgenus *Eriococcus* and their closest affinity seems to be to species that occur in the Philippines. The biogeographical implications are discussed and a key to the species of *Phyllanthus* on Sulawesi is presented. As Sulawesi remains drastically understudied, this small contribution opens the path to finding new species.

Chapter 6

To further explore the medicinal effects of various species of *Phyllanthus*, I sampled material from the living collections of the Hortus botanicus Leiden and studied their antimicrobial and antifungal effects in correlation with their metabolite content. Some species were found to have antimicrobial effects, but we could not determine which compounds were responsible for this.

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Chapter 7

As past morphological work resulted in classifications that were not strictly monophyletic, the framework presented in chapter 3 needs to be complemented with a phylogeny with adequate sampling. The previous phylogenetic study that focused on *Phyllanthus* (Kathriarachchi et al. 2006) included about 10 % of the whole genus. Increased sampling efforts by Pruesapan et al. (2008, 2012) already elucidated the structure of the genus *Breynia* (with *Sauropus* found to be nested within). Here, I increased the sampling efforts for the genus *Phyllanthus* itself in an attempt to include the majority of morphological variation and to add some understudied areas such as Australia. With increased sampling, I hope to confirm how *Phyllanthus* can be split in monophyletic genera and how they can be morphologically recognized.

Chapter 8

Most of the work on this thesis has been geared towards the goal of creating a new classification for the genus *Phyllanthus*. Here together with experts of different floras, we incorporate the results from all previous chapters into a new classification of tribe Phyllanthae. In this chapter I reinstate several genera to create a new classification of monophyletic taxa, some with a more restricted distribution. This new classification shows that *Phyllanthus* is restricted to the neotropics with only some cultivated species more widely distributed. With this, we propose a possible solution to the problem of paraphyly within the tribe.

Chapter 9

Now turning from taxonomy to its distribution, another aspect of why *Phyllanthus* is so remarkable is studied in this chapter. *Phyllanthus* occurs in all tropics and subtropics with a few species reaching temperate areas. However, how this distribution came to be has never been explored extensively. Initial surveys that incorporated older subgeneric classifications were minimal and only an extensive discussion existed for Africa, which did not incorporate any phylogenetics. By using the phylogeny of chapter 7 in conjunction with an expanded dataset of the distribution of included species and a dataset of fossil findings, we calibrate the phylogeny to determine how old the nodes on the phylogenetic tree are and reconstruct how the group might have reached its current distribution. By analysing this, I hope to correlate this with known events of plate tectonics and environmental conditions that could have allowed for the dispersal and colonization of new areas by the genus through time.

Chapter 10 General conclusions

In the last chapter of this book, I discuss the knowledge gained on tribe Phyllanthae

and its evolution and diversification. New problems are still created and several areas remain poorly studied for this interesting group of species. With a new appreciation of each individual clade and its new constituent genera, new problems are highlighted that offer interesting case studies for several evolutionary subjects. Some considerations are presented that would serve as a continuation of this work.

