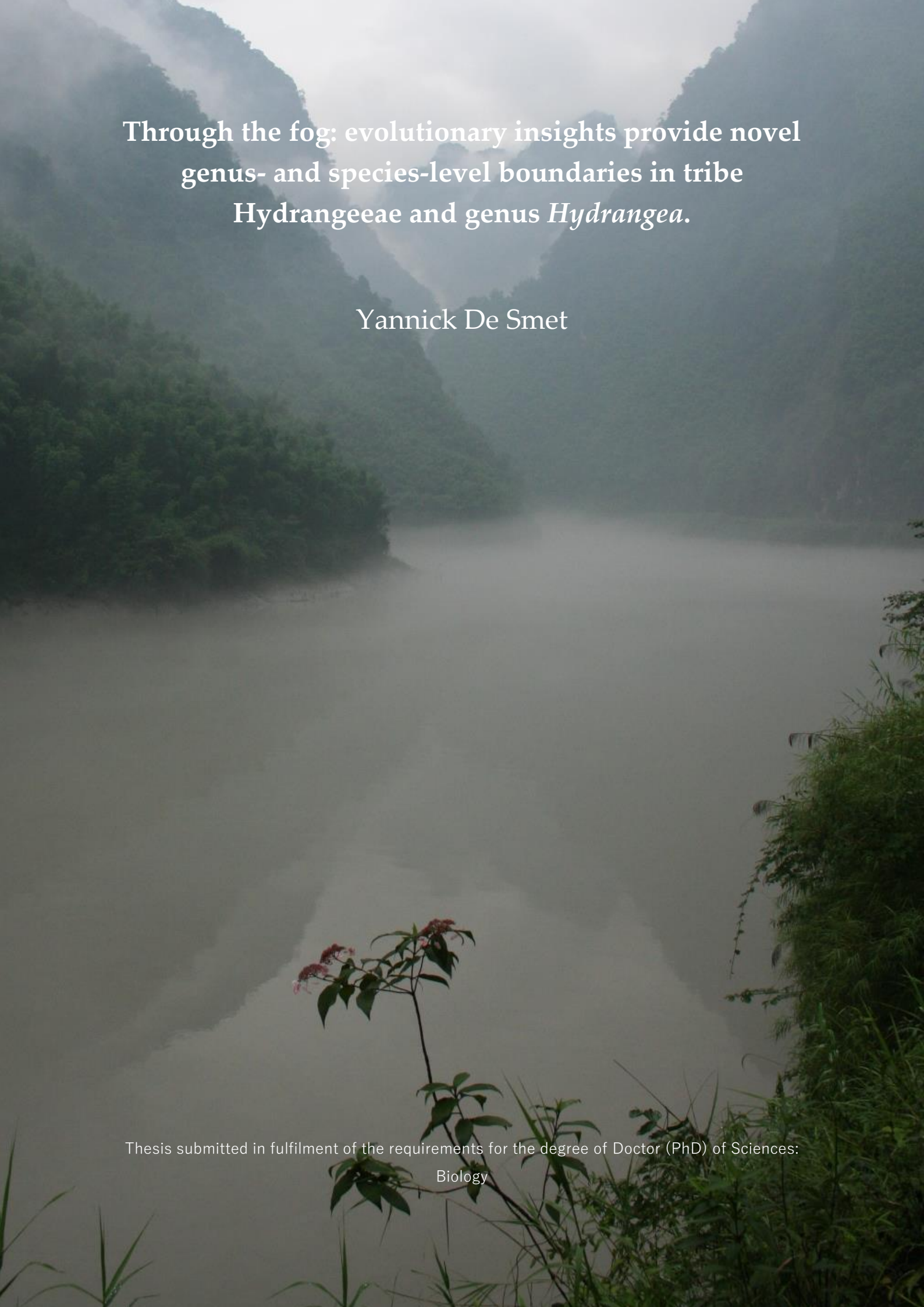

# Through the fog: evolutionary insights provide novel genus- and species-level boundaries in tribe Hydrangeeae and genus *Hydrangea*.

Yannick De Smet

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) of Sciences: Biology

Ghent University
Faculty of Sciences
Biology Department
Systematic and Evolutionary Botany Lab
K.L. Ledeganckstraat 35
B-9000 Gent

**Author:**
Yannick De Smet

**Supervisors**:
Prof. Dr. Marie-Stéphanie Samain (60%)
Prof. Dr. em. Paul Goetghebeur (20%)
Prof. Dr. Stefan Wanke, Dresden University of Technology (15%)
Prof. Dr. Lars Chatrou (5%)

**Members of the examination committee:**
Chair: Prof. Dr. Mieke Verbeken (Ghent University)
Secretary: (Ghent University)
Dr. Koen Camelbeke (Arboretum Wespelaar)
Prof. Dr. Eduardo Cires (University of Oviedo, Spain)
Dr. Carolina Granados Mendoza (Universidad Nacional Autónoma de México, Mexico)

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Both taken by Eduardo Cires Rodríguez, in Sichuan, China, during one of the field collection trips performed within the framework of this research.
Front: *Hydrangea aspera* in its natural habitat, a slope along the banks of a stream.
Back: The author on a collecting trip in Sichuan, China.

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Academic year 2020-2021

GHENT UNIVERSITY

FACULTY OF SCIENCES

SYSTEMATIC & EVOLUTIONARY BOTANY

# Dankwoord

# Contents

*"When the views entertained in this volume on the origin of species, or when analogous views are generally admitted, we can dimly foresee that there will be a considerable revolution in natural history. Systematists will be able to pursue their labours as at present; but they will not be incessantly haunted by the shadowy doubt whether this or that form be in essence a species. This I feel sure, and I speak after experience, will be no slight relief. The endless disputes whether or not some fifty species of British brambles are true species will cease. Systematists will have only to decide (not that this will be easy) whether any form be sufficiently constant and distinct from other forms, to be capable of definition; and if definable, whether the differences be sufficiently important to deserve a specific name. This latter point will become a far more essential consideration than it is at present; for differences, however slight, between any two forms, if not blended by intermediate gradations, are looked at by most naturalists as sufficient to raise both forms to the rank of species. Hereafter we shall be compelled to acknowledge that the only distinction between species and well-marked varieties is, that the latter are known, or believed, to be connected at the present day by intermediate gradations, whereas species were formerly thus connected. Hence, without quite rejecting the consideration of the present existence of intermediate gradations between any two forms, we shall be led to weigh more carefully and to value higher the actual amount of difference between them. It is quite possible that forms now generally acknowledged to be merely varieties may hereafter be thought worthy of specific names, as with the primrose and cowslip; and in this case scientific and common language will come into accordance. In short, we shall have to treat species in the same manner as those naturalists treat genera, who admit that genera are merely artificial combinations made for convenience. This may not be a cheering prospect; but we shall at least be freed from the vain search for the undiscovered and undiscoverable essence of the term species."*

On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life (1859). Charles Darwin (1809-1882)

**Chapter I**

# General introduction

## General background

Taxonomy and systematics are among the oldest fields of biology. They aim to satisfy one of mankind's most basic intellectual desires: to order the immense diversity of biological life into comprehensible, named units or "taxa". With the advent of evolutionary thinking, calls for reconciliation between these new insights and the traditional fields of taxonomy and systematics arose. Moving towards this reconciliation, however, the taxonomic world is faced with several challenges and ongoing debates. First, there is no unanimous agreement concerning the most desirable scheme of classification for all biological life. Taxonomists can range from total adherence to traditional, strictly morphology-based classifications, to purely genomic sequence-based organizations. Secondly, continuing development of new tools for inferring evolutionary relationships can overturn previously widely held believes concerning relationships between taxa. Finally, biologists tend to disagree on the definition of several key entities in biology. The most striking example of this can be found in the plethora of species concepts published in the 20$^{th}$ century. Several of these discussion points are highly relevant for the study presented in this thesis. Therefore, a general introduction is given into each of these, followed by an evolutionary and systematic background on the plant group under study.

## Importance of species and species concepts

### Species concepts

The taxonomic category of species represents one of the most fundamental and practically usable units in biology. Species names are used to identify plants in botanical gardens, or to communicate both within and outside of the confines of the scientific world. More importantly, they are used to predict the behavior and properties of organisms in medical, ecological, physiological, developmental, conservational and evolutionary contexts (Nelson, 1989). Laws on biodiversity conservation in the United States of America, for example, explicitly define a species concept in their legislation (Linder, 1995; Cracraft, 1997; Crandall et al., 2000; Allendorf et al., 2001). Therefore, the species category cannot be considered a mere abstraction only of interest to taxonomists. Despite this widespread acceptance of the importance of the species category, opinions abound concerning the nature of these entities. According to Mayr (1957), the origin of species concepts in biology lies with Linnaeus; since before his "Species plantarum" and 'Systema naturae", species were generally not believed to be stable entities (Wilkins, 2003). Older views on species (not only biological entities) are believed by Mayr to be heavily influenced by Plato's essentialism and are termed "typological species concepts" (Zachos, 2016). In this view, species are absolute and constant, but an abstract, artificial entity. In developing his theory of evolution, Darwin proposed a species concept that was significantly different from that of his predecessors. Two components are recognized in Darwin's species concept by de Queiroz (2011): an older taxonomic component, equating species to groups of organisms assigned to a particular rank in a taxonomic hierarchy, and a newer evolutionary component conceptualizing species as segments of population lineages. This latter component became increasingly accepted by post-Darwinian biologists, as adherence to an evolutionary worldview increased (de Queiroz, 2011). This becomes evident from the explicit equation of species to lineages in a number of middle and late 20$^{th}$ century species definitions (Simpson, 1961; Van Valen, 1976; Wiley, 1978). Apart from this evolutionary component, most of these species concepts also contained Darwin's other component, the idea of species as a rank in the hierarchy of taxonomic categories. This means that diverging lineages have to cross a threshold in a certain character or property (here called species criteria) in order to merit its recognition as a distinct species. As species are used in

various subdisciplines of Biology, these species criteria varied widely, ranging from sufficient morphological differences (Phenetic species concept: Michener, 1970; Sokal & Crovello, 1970; Sneath & Sokal, 1973) over monophyly in gene trees (monophyly version of the phylogenetic species concept: Rosen, 1979; Donoghue, 1985) and exclusive coalescence of alleles (genealogical species concept: Baum & Shaw, 1995) to strict reproductive isolation (biological species concept: Mayr, 1942; Dobzhansky, 1970). Since each of these criteria was deemed a necessary property of the taxonomic rank of species, they were believed to represent separate species concepts. In line with this idea, the last part of the 20th century saw an expansion of alternative "species concepts", mainly differing in the species criterion used to distinguish lineages as species (e.g. Michener, 1970; Sokal & Crovello, 1970; Sneath & Sokal, 1973; Donoghue, 1985; Baum & Shaw, 1995; de Queiroz, 2005a). Importantly, some of these concepts are incompatible, i.e. inferring different numbers of species given the same set of individuals.

In an attempt to reconcile these different concepts, de Queiroz (1998, 1999, 2005a,b,c, 2007, 2011) focused on the common idea shared by these species concepts: species represent (segments of) separately evolving metapopulation lineages (similar to Mayden, 1997). All species criteria which lead to conflicts between competing species concepts are deemed to be possible, not necessary, characters developed by diverging lineages during their differentiation (Figure 1.1). Seen in this light, these criteria remain important, since they still confer information regarding the diverging lineages under study. They no longer constitute, however, necessary properties for species recognition. Under this general lineage concept of species, or unified concept of species, delimiting species becomes the acquisition of different lines of evidence (species criteria from previous concepts), in order to strengthen the hypothesis that the lineages under study have diverged sufficiently to merit recognition as separately evolving metapopulation lineages (species). Since a significant portion of this work deals with the identification and delimitation of species within the genus *Hydrangea*, it would benefit from an explicitly defined species concept. Therefore, the general lineage concept of species is utilized, and all lines of evidence gathered towards species delimitation are compared and taken into account when proposing hypotheses concerning species boundaries. Alternative lines of evidence acquired in future studies can consequently corroborate or falsify the hypotheses developed here, resulting in a more objective discussion on species boundaries.

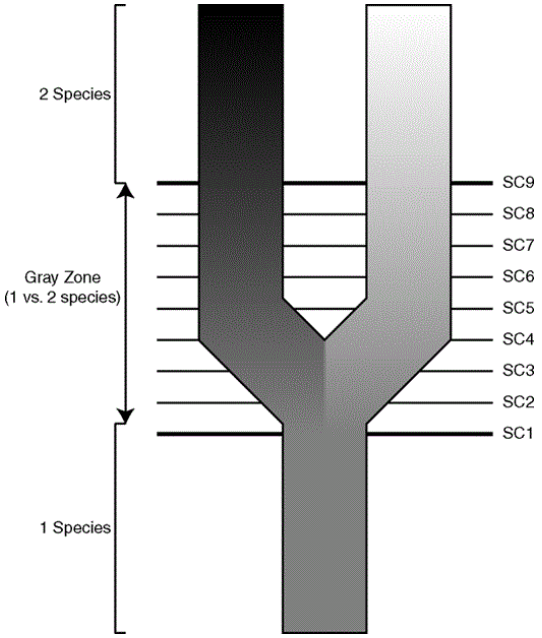


**Figure 1.1: The general lineage concept of species**. (after de Queiroz, 1998, 1999, 2005a). This highly simplified diagram represents a single lineage (species) splitting to form two lineages (species). The gradations in shades of gray represent the daughter lineages diverging through time, and the horizontal lines labeled SC (species criterion) 1 to 9 represent the times at which they acquire different properties (i.e., when they become phenetically distinguishable, diagnosable, reciprocally monophyletic, reproductively incompatible, ecologically distinct, etc.). The entire set of properties forms a gray zone within which alternative species concepts come into conflict. On either side of the gray zone, there will be unanimous agreement about the number of species. Before the acquisition of the first property, everyone will agree that there is a single species, and after the acquisition of the last property, everyone will agree that there are two. In between, however, there will be disagreement. The reason is that different contemporary species concepts adopt different properties (represented by the horizontal lines) as their species criteria—that is, as their cutoffs for considering a separately evolving lineage to have become a species (Figure and caption adapted from de Queiroz, 2007).

Species delimitation

Morphological data and approaches have necessarily dominated the field of species delimitation in its early days (Wiens & Servedio, 2000). Within an evolutionary framework, these characters present a number of difficulties for not accurately representing evolutionary relationships between taxa (Wiens & Penkrot, 2002). Indeed, similar morphologies can represent homoplasies, independent evolution of similar morphologies by, for example, adaptation to a similar environment or non-heritable variation (Mueller et al., 2004). Additionally, the importance attached to a certain morphological characteristic by a group of taxonomists might not coincide with its evolutionary significance, or rivaling interpretations (Sibley & Ahlquist, 1990). Part of these difficulties were alleviated with the introduction of genomic and molecular datasets in speciation studies (Wiens & Penkrot, 2002). Unlike phenotypic characters, genetic variation is heritable, which is a necessity in addressing relationships between and within lineages (Wiens & Servedio, 2000). Nevertheless, genetic data also have the potential to create misleading signals concerning divergence history. Incipient or recent divergence (De Smet et al., 2012), enduring gene flow (Petit & Excoffier, 2009) or low mutational rates can produce very low genetic variability (Hoelzer & Meinick,

1994), insufficient to identify separate evolutionary lines. Moreover, retention of ancient polymorphisms and incomplete lineage sorting can account for gene tree discordance, confusing evolutionary hypotheses derived from different molecular markers (Avise et al., 1983; Pamilo & Nei, 1988; Takahata, 1989; Doyle, 1992; Maddison, 1997; Rosenberg, 2002, 2003; Maddison & Knowles, 2006). The latter has been addressed by moving phylogenetic reconstruction and statistical species delimitation away from methods based on analyses of single genes (e.g. Pons et al., 2006), and towards the generation of "species trees", based on coalescent theory. This new approach merges properties of population genetics with phylogenetic tree reconstruction, in order to glean information on speciation events, processes of divergence and probability of evolutionary independence (Rannala & Yang, 2003; Edwards, 2009; Liu et al., 2009; Knowles & Kubatko, 2010). Application of these coalescent-based methods for species delimitation and phylogenetic reconstruction requires the acquisition of multiple independent molecular markers. Phylogenetic trees inferred from each of these markers (gene trees) will increase the knowledge on the evolutionary relationships of their containing taxa (species tree) (Figure 1.2). In the following two subchapters, the broad lines of coalescent theory and the acquisition of molecular markers used in this PhD are discussed, respectively.

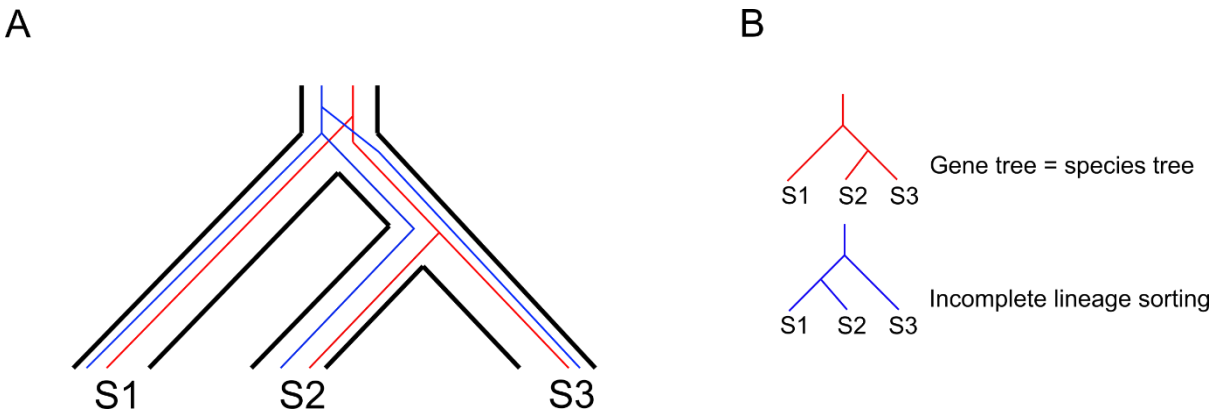


**Figure 1.2: Gene tree versus species tree.** The black outline in A represents the true species phylogeny, with S2 and S3 as sister taxa. The red and blue lines inside the outline represent gene trees underlying this species topology. The gene represented in red is congruent with the species, tree (B), while the blue gene experienced incomplete lineage sorting, resulting in a species tree gene tree conflict. Indeed, a phylogenetic tree based only on the blue gene will erroneously recover S1 and S2 as sister taxa. Figure created by the author.

### Coalescent theory in species delimitation

The introduction of genetic data into species delimitation saw the rise of several sequence-based methods to identify species. Initially, these methods were mostly based on single genes, using reciprocal monophyly, diagnostic states (e.g. fixed differences) or differences in branching patterns (Brower, 1999; Davis & Nixon, 1999; Pons et al., 2006) as criteria to distinguish between lineages. Recently, it was postulated that many of these methods are flawed, in that single genes often do not represent the true evolutionary history of organisms. Not all alleles will reach reciprocal monophyly between related lineages, especially when their divergence is rather recent (Hudson & Coyne, 2002; Knowles & Carstens, 2007). Coalescent-based species delimitation circumvents these limitations by attempting to infer the true species tree, by extracting information from multiple independent gene trees, and then tests several hypotheses on lineage divergence on this inferred phylogenetic hypothesis (Figure 1.3). At the heart of this approach lies the coalescent, or the coalescent process; a mathematical model which randomly joins sampled gene lineages as they are followed back through time.

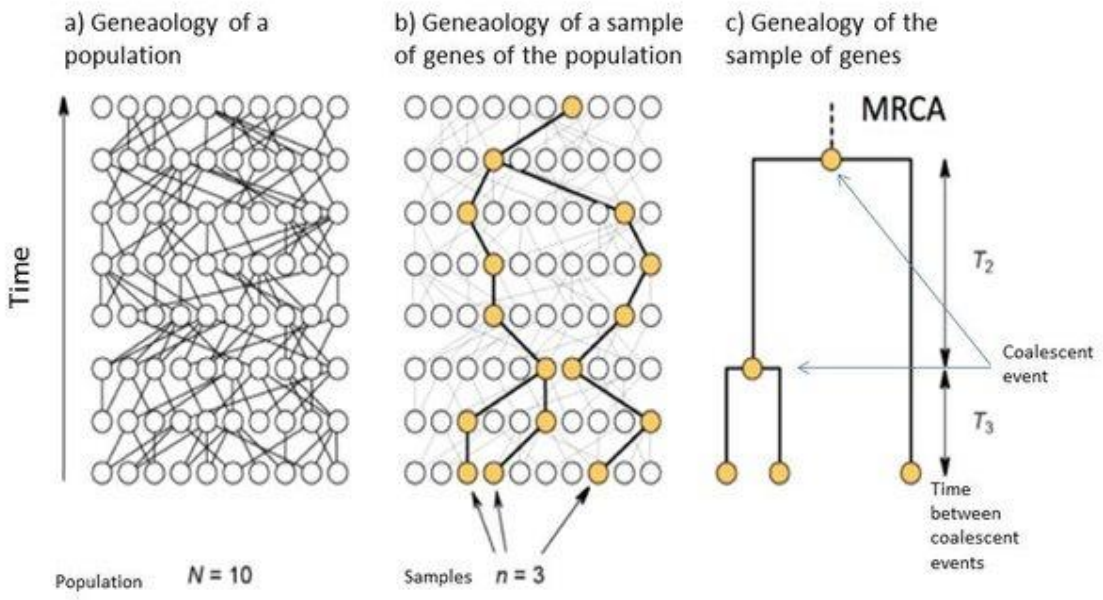


**Figure 1.3: The coalescent.** Neutral coalescence process running within a species tree, based on Klein (1998), Degnan & Rosenberg (2009) and Mailund (2009). Each dot represents an individual gene copy, each color a different allele, and each line connects a gene copy to its ancestor in the previous generation. Within a population, selection and/or drift will result in changing allele frequencies over time. In the initial stages of lineage splitting, sister species will largely share identical alleles, which has important consequences for species delimitation. In this example, constructing a gene tree at an early stage of speciation would result in none of the three species being monophyletic. Only after sufficient time has gone by, alleles will be completely sorted in each lineage, resulting in reciprocal monophyly for the each of the three species (Figure and caption adapted from Leliaert et al., 2014).

A full explanation of this model is beyond the scope of this introduction, but several comprehensive sources are available (Rosenberg, 2002; Knowles & Kubatko, 2010). In broad lines, the coalescent theory models the probability that two lineages find their most recent common ancestor (a coalescence event) within a certain time t. This can be expanded to include multiple lineages (Rosenberg, 2002), and from there, probabilities can be calculated associated with coalescent events within the branches of a species tree. When linking several of these branches together, this allows calculation of probability distributions associated with the gene tree topology (Pamilo & Nei, 1998; Rosenberg, 2002; Degnan & Salter, 2005). These gene tree topology distributions allow the calculation of a likelihood function, which can be used to estimate the species tree (e.g. Knowles & Carstens, 2007). Furthermore, this distribution of gene tree topologies for a given species tree has been used to evaluate the performance of previous methods of phylogenetic inference given multilocus data; concatenating independent loci (Kolaczkowksi & Thornton, 2004; Carstens & Knowles, 2007; Kubatko & Degnan, 2007). This evaluation aided in the realization that each independently evolving gene has its own branching history, contained within the one true species tree (Figure 1.3; Knowles & Kubatko, 2010). From these developments in coalescent theory, Rannala & Yang (2003) derived a framework to calculate the likelihood of multilocus data given a species tree: $f(D \mid S)$, by integrating over gene trees. Most recently used species tree reconstruction methods utilize this framework for calculating $f(S)$, the probability of a species tree, which includes the parameters $\mu$ (mutation rate) and $\tau$ (lineage divergence time). Extending this framework with a factor $\Lambda$ for species delimitation models allows for the estimation of the probability of a particular species delimitation given multilocus data (Yang & Rannala, 2010):

$$f(S, \Lambda \mid D) = 1 / f(D)\, f(D \mid S)\ f(S \mid \Lambda)\ f(\Lambda)$$

Where $f(S \mid \Lambda)$ is the prior distribution of species phylogenies and $f(\Lambda)$ denotes the prior distributions of delimitation models (Fujita et al., 2012). This represents the basis for Bayesian species delimitation as implemented in the program Bayesian Phylogenetics and Phylogeography (BP&P, Rannala & Yang, 2003). In this algorithm, a Reversible-jump Markov Chain Monte Carlo (rjMCMC) is used to move between different species delimitation models (obtained by collapsing nodes on a starting guide species tree, and thus differing in the number of species), calculating their posterior distribution. This distribution can then be used

to evaluate the different alternative species delimitation models, and thus identify the number of supported evolutionary lines in the data.

**Marker selection**

There remains an important contrast in the molecular markers useful in higher level phylogenies (genus and above) and phylogenies aiming to resolve relationships at lower levels (species and below). Traditionally, Angiosperm phylogenetic reconstruction has been dominated by the use of plastid markers, and a very limited set of nuclear markers such as the ribosomal ITS (Baldwin et al., 1995; Small et al., 1998; Shaw et al., 2005, 2007; Hughes et al., 2006). However, both can harbor undesirable characteristics for their application at or below the species level. Chloroplast markers are known to exhibit only limited amounts of variability, due to a low evolutionary rate in the plastid genome (Clegg et al., 1994). Furthermore, uniparental inheritance of chloroplasts in Angiosperms makes them inadequate for reconstructing patterns of hybridization, polyploidization and introgression (Naumann et al., 2011; Zimmer & Wen, 2012). The nuclear ribosomal marker ITS, which is popular in speciation studies (e.g. Zhao et al., 2018), can suffer from concerted evolution (Álvarez & Wendel, 2003), presence of pseudogenes (Mayol & Rosselló, 2001), and evolutionary constraints in ITS sequences related to the maintenance of secondary structures (Feliner & Rosselló, 2007). Since this realization, voices have gone up in the plant systematic community for the inclusion of low copy nuclear markers (LCNM) in low-level phylogenetic studies and species delimitation (Sang, 2002; Álvarez & Wendel, 2003; Small et al., 2004; Granados et al., in prep). However, great care has to be employed in their routine application, since LCNM can also be plagued by the presence of multiple copies, pseudogenes, and evolutionary constraint regions. Nevertheless, LCNM and single copy nuclear markers (SCNM) can represent an important source for orthologous sequence data for low level phylogenetic reconstruction. Ease of identification of these genomic regions has greatly increased with the advent of high-throughput sequencing, providing whole genomes or transcriptomes for non-model organisms. For Fungi, an algorithm is available to screen fungal genomic databases for SCNM, which could prove useful for studies at low taxonomic levels (PHYLORPH; Feau et al., 2011). Similar attempts at gleaning candidate SCNM from genomic data of *Arabidopsis thaliana*, *Populus trichocarpa*, *Vitis vinifera* and *Oryza sativa* by Duarte et al. (2010) have resulted

in 959 candidate regions. A subset of these regions has already been employed to generate serviceable primers for phylogenetic reconstruction in Hydrangeaceae by Granados et al. (2015).

The aforementioned shift towards multilocus analyses has instigated a search for independent molecular markers appropriate for low level phylogenetic studies. Even with the availability of databases listing potential LCNM and SCNM, primer design and the screening of loci for variability can be a time-consuming endeavor using traditional Sanger sequencing (McCormack et al., 2013). Partly for this reason, studies have turned towards high throughput sequencing to streamline multilocus data generation for non-model organisms (Lerner & Fleischer, 2010; Ekblom & Galindo, 2011; Cruaud et al., 2014). Where the application of these methods in other fields is mostly targeted at deep sequencing of a limited set of individuals, sequencing entire eukaryotic genomes is currently inefficient for phylogeographic, phylogenetic and ecological studies. These fields therefore focus their application of high throughput sequencing on acquiring sequence data for a (comparatively) small subset of the genome for a large set of individuals. These contrasts have led to the development of several library preparation methods focusing on a reduced representation of the genome, while allowing the pooling of many individuals in one sequencing effort. This genomic reduction can be achieved by digesting genomes with restriction enzymes (Baird et al., 2008; Davey et al., 2011) and sequencing a stretch of nucleotides adjacent to the cut-site or amplifying a subset of the genome by PCR (Binladen et al., 2007; Meyer et al., 2009). The resulting genomic fragments are labeled with barcodes (or "tags"), by ligation or as part of a PCR, allowing post-sequencing sample identification. One of the most popular library preparation techniques for studies at and below the species level is Restriction-site Associated DNA (RAD) sequencing (Baird et al., 2008). This method utilizes a restriction enzyme (or two different enzymes in the double digest version) to generate genomic fragments with known ends. These fragments are then sheared and size selected, after which a platform specific adapter with identifier barcode is added through ligation. Fragments generated from different individuals can then be pooled, and ran on a NGS-platform (Illumina in most published cases). RAD-seq has been successfully used in marker development (Miller et al., 2007), genome scans (Hohenlohe et al., 2010), population genetic studies (Hohenlohe et al., 2011; Massatti et al., 2016; Nazareno et al., 2018; Warschefsky & von Wettberg, 2019), phylogenetic reconstruction (Emerson et al., 2010;

Ahrens et al., 2017; Clugston et al., 2019) and its utility has been tested in species delimitation (e.g. Craud et al., 2014; Pante et al., 2015; Wagner et al., 2018; Dincă et al., 2019; Quattrini et al., 2019).

**Monophyly vs. paraphyly**

As discussed in the previous subchapter, the taxonomic category of species is generally accepted to represent a real, evolutionary relevant entity. A much stronger debate seems to exist regarding the nature and characteristics of higher taxa. In the past, some higher levels of taxonomic organization were ascribed the same level of reality and importance as species. Linnaeus, for example, in his establishment of binomial nomenclature, regarded genera and species alike as natural entities. All higher levels of organization he instead described as "art", being artificial constructs (Linnaeus, 1751). In the same line, Simpson (1953) developed theories recognizing higher taxa as discrete units, and studied the processes behind their formation (i.e. adaptive radiation into new adaptive zones). Following the introduction of cladistics, the general opinion on the nature of taxa changed considerably (Anderson, 1940; Barraclough & Humphreys, 2015), in favor of viewing only species as natural entities (Figure 1.4). Although recent efforts have been made to provide a theoretical background for the discrete nature of higher taxa, termed independently evolving higher evolutionary significant units (hESUs) (Barraclough, 2010; Humphreys & Barraclough, 2014; Barraclough & Humphreys, 2015), these views have not been widely adopted.

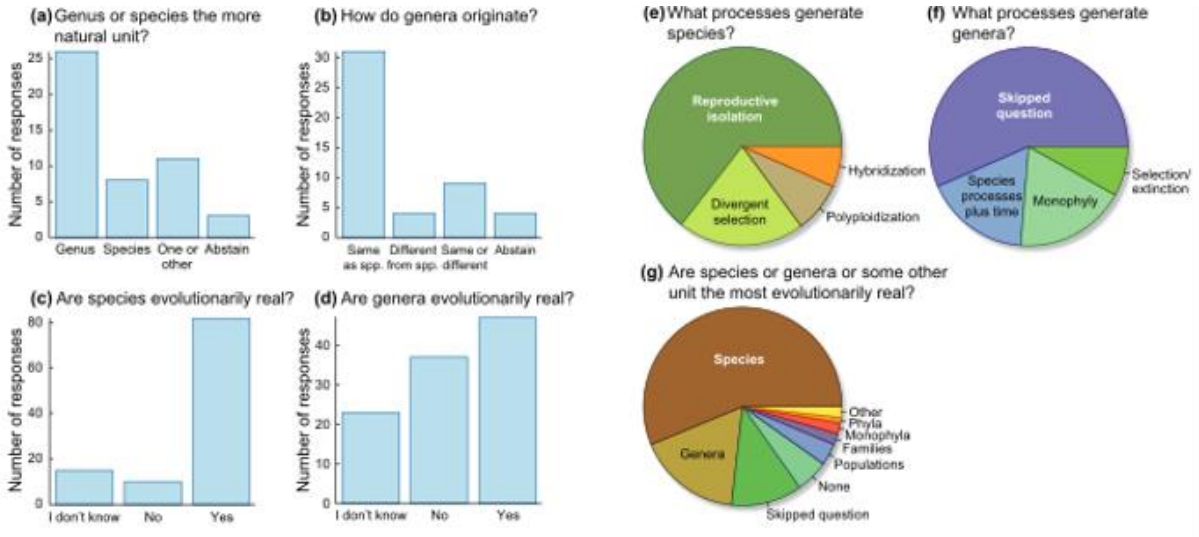


**Figure 1.4: Results of surveys on views regarding taxa and their evolutionary relevance.** Summary of responses (a, b) to the original Edgar Anderson (1940) survey and (c–g) the survey made by Barraclough

& Humphreys (2015). In (a) 'one or other' refers to respondents who felt that sometimes genera and sometimes species were the more natural unit. In (g) 'monophyla' includes the view that monophyletic groups are real – rank is irrelevant – and the view that 'only lineages' are real. 'Other' includes 'all taxa are equally real' and 'I don't know'. A few respondents said more than one rank, for example 'species and genera' or 'species and populations'. These have been scored in both mentioned categories (Figure and caption adapted from Baraclough & Humphreys, 2015).

Related to this, the introduction of molecular phylogenies led to another paradigm shift. Growing availability of sequence data across all levels of biological classifications produced a plethora of new hypotheses of evolutionary relationships, patterns and processes. This in turn led to the emergence of controversy regarding how taxonomists should incorporate the diversity of evolutionary patterns and processes into biological classification. Although most taxonomists seem to have embraced the importance of classifications reflecting common descent, strong debate still exists concerning the recognition of paraphyletic groups (Figure 1.5) as taxa in biological classifications (Stuessy, 1998; Brummit, 2002, 2006; Grant, 2003; Ebach & Williams, 2004; Nordal & Stedje, 2005; Hörandl, 2006, 2007, 2010, 2014; Hörandl & Steussy, 2010; Podani, 2010; Schmidt-Lebuhn, 2012, 2014; Stuessy & Hörandl, 2014). Accordingly, classifications (and systematists) have been labeled evolutionary or cladistic (phylogenetic), based on whether paraphyletic taxa are condoned or not, respectively. Integral to this discussion are the terms monophyletic and paraphyletic, and both schools of thought have at some point accused the other of misinterpreting these terms (e.g. Ebach &Williams, 2004; Hörandl, 2007). It can be argued that since Hennig's (1966) proposal of a cladistic classification, a monophyletic group has been identified as an assemblage of an ancestor and all of its descendants. Some proponents of evolutionary classifications argue against the use of this "inclusiveness" criterion (e.g. Ashlock, 1971; Hörandl, 2007), instead recognizing monophyletic groups in a broader sense, as any assemblage of taxa of common descent, regardless of the inclusion of all descendants of their latest common ancestor. Within this category, two other terms are proposed: holophyly, to represent Hennig's monophyletic groups, and paraphyly, to define non-inclusive groups; assemblages not containing all of the descendants of the most recent common ancestor of the group. Although this semantic discussion is part of the general disagreement between the cladistics and evolutionary schools for classifications, the real disagreement resides in which type of evolutionary entities

(monophyletic clades, polyphyletic clades, paraphyletic clades,…) are recognized in classifications. The following paragraphs are meant as an objective summary of the key characteristics of each school, juxtaposed with the contra-arguments by the opposing school.

The evolutionary school of classification adheres to the idea that evolutionary information must be the basis for natural classifications (Stuessy & Hörandl, 2014). In this view, only taxa maximizing the information content of the classification are desirable. Phylogenies are accepted as the basis for recognizing taxa, but more aspects than the branching pattern are considered as important. This translates to a recognition of monophyletic (s.l., so including both holophyletic and paraphyletic groups) taxa, which can be defined by a sufficient amount of divergence in any other character than the branching pattern in phylogenetic trees. Importantly, this includes paraphyletic taxa, but only if they are deemed to maximize information content of the classification. Distinctness from parental taxa and the recognition of the evolutionary processes leading to this distinctness are generally quoted as the most important criteria for this information content. Degree of distinctness or divergence can be measured from phylogenetic analyses (e.g. branch length), or non-sequence-based analyses (Stuessy & Hörandl, 2014). Evolutionary processes quoted to cause these divergences are cladogenesis, anagenesis and reticulate evolution (Hörandl, 2006). A common critique from the evolutionary school of classification against cladistic systematics is that the latter system does not account for the latter two processes of evolution (especially see Hörandl's 2007 paper "Neglecting evolution is bad taxonomy"). A rebuttal to the central argument in evolutionary classifications (higher information content) can be found in the work of Schmidt-Lebuhn (2012, 2014). This author argues that a classification trying to combine different sources of information (e.g. phylogenetic relationships and phenetic similarity) to delimit taxa, will not contain reliable information of either source. Indeed, some taxa will be evolutionary nested within each other, making it impossible to infer evolutionary relationships directly from the classification. The same difficulties arise if one aims to extract information on phenetic patterns (evolutionary divergence) from the classification.

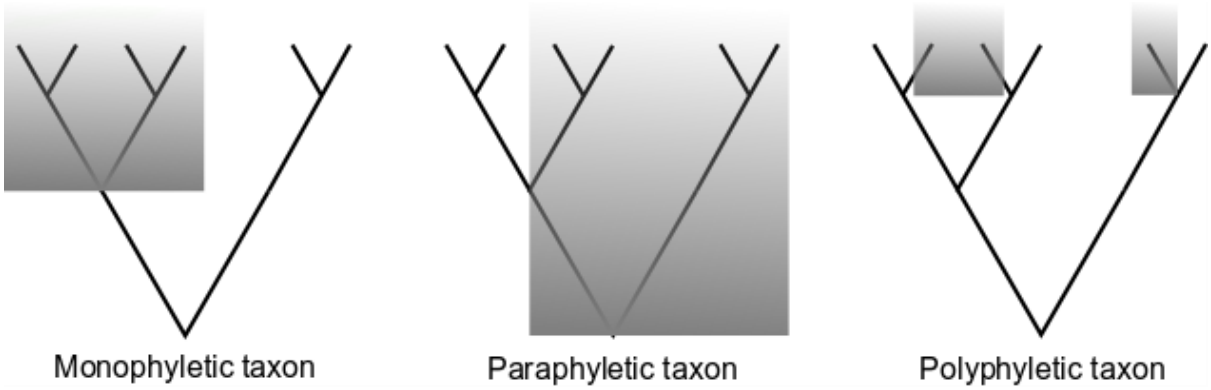


**Figure 1.5: Monophyly versus paraphyly and polyphyly.** A monophyletic taxon contains the most recent common ancestor of a group of organisms and all of its descendants. A paraphyletic taxon includes the most recent common ancestor, but not all of its descendants. Finally, a polyphyletic taxon can be defined as a group not containing the common ancestor of all of its members. Figure created by the author.

In cladistic or phylogenetic classifications, the only necessary property to recognize a taxon is for it to include all descendants from a certain common ancestor. This idea was first put forward in the book *Phylogenetic Systematics* (Hennig, 1966), and has since won immense grounds in the systematic community. It is argued by proponents of this school that monophyly (s.s., holophyly sensu evolutionary systematists) is the only testable, reproductive, objective and universal criterion for the circumscription of supraspecific taxa (Schmidt-Lebuhn, 2012). In this view, the characters used to decide on sufficient divergence (also called distinctness, degree of distinctness) in an evolutionary classification are deemed subjective, and not always testable. Several critiques on the cladistics school of classification can be found in the literature (e.g. Hörandl & Stuessy, 2010; Timm, 2010; Stuessy & Hörandl, 2014), but the most common ones are based on the inclusion of all evolutionary processes in classification. This criticism is, for example, brought forward by Hörandl (2007), in saying that by using monophyly (s.s.) as the exclusive criterion for classification, certain evolutionary processes cannot be reflected in classifications.

An in-depth discussion of pro- and contra arguments for each school is beyond the scope of this introduction. The aim of the above is to illustrate the ongoing debate concerning the acceptance of paraphyletic taxa. Both sides, however, agree on two key points: 1) phylogenetic hypotheses can, and should, be used to create more natural classifications, 2) polyphyletic taxa cannot be accepted in a natural classification, since they do not signify common descent (Schmidt-Lebuhn, 2012; Hörandl, 2014). Furthermore, attempts have been made in reconciling

both sides of the argument, either by explicitly mentioning the genealogical nature of each unit in a classification (paraphyla and holophyla: Timm, 2012) or by creating an overarching "consensus" classification, which can be revised over a period of time (Catalogue of Life, CoL: Ruggiero et al., 2015).

**Evolutionary and systematic framework of the thesis**

Evolutionary history of the Hydrangeaceae

The Angiosperm family Hydrangeaceae has been plagued with systematic, taxonomic and evolutionary uncertainties at both high and low taxonomic levels. The name was first published by the Belgian botanist Barthélemy Charles Joseph Dumortier in 1829, to include the genera *Hydrangea* and *Deutzia*. This name, however, overlapped with the already published Hortensiaceae (Martinov, 1820), based on *Hortensia opuloides*, one of the many synonyms of the ornamentally important *Hydrangea macrophylla*, but has been conserved as the more well-known name (McNeill et al., 2006).

At the family level, the evolutionary affinities of Hydrangeaceae have been unclear, mainly until the advent of molecular tools. Traditionally, the family was associated with the herbaceous members of the Saxifragaceae (Engler, 1890; Schulze-Menz, 1964; Cronquist, 1981). Other authors, however, considered the group to be closely related to the Cornaceae, placing it in the order Cornales (e.g., Hutchinson, 1927; Huber, 1963; Hufford, 1992; Thorne, 1992; Takhtajan, 1997). Recent morphology and sequence based phylogenetic analyses have provided support for this second view, placing the Hydrangeaceae firmly within the order Cornales (Downie & Palmer, 1992; Hufford, 1992; Chase et al., 1993; Morgan & Soltis, 1993; Olmstead et al., 1993, 2000; Xiang et al., 1993; Savolainen et al., 2000) and in a well-supported sister relationship with the Loasaceae (Hempel et al., 1995; Soltis et al., 1995, 2000; Xiang et al., 1998, 2011; Samain et al., 2010; Stevens, 2012). Despite this high support for the evolutionary placement of Hydrangeaceae, there has been some uncertainty regarding the inclusion of the monogeneric family Hydrostachyaceae within the Hydrangeaceae. The genus *Hydrostachys*, containing ~23 aquatic species restricted to Madagascar, tropical and southern Africa, is notorious for its difficult evolutionary placement. The highly specialized morphological adaptations present in this group (e.g., a tuberous-thickened stem, a basal hold-fast, fibrous roots, a cluster of basal, often pinnitifid or pinnate leaves, inaperturate pollen tetrads, and the

lack of stomates, vessels, and many common secondary compounds (Cronquist, 1981; Scogin, 1992) render morphology-based assessment of its closest relatives challenging. Furthermore, these taxa exhibit elevated rates of nucleotide changes, possibly ascribable to the habitat shift into a novel (aquatic) environment, and accompanying factors as elevated mutation rates, selection and genetic drift (Xiang et al., 2011). These particular characteristics of nucleotide diversity within the Hydrostachyaceae result in different evolutionary placements of this family, both within and outside of the Cornales. In part, these differences in resolution of evolutionary relationship are caused by artefacts introduced by methods for phylogenetic reconstruction; most notably the sensitivity of Maximum Parsimony methods to long branch attraction (LBA). Methods less sensitive to LBA tend to place the Hydrostachyaceae inside the Cornales, albeit on long braches and at differing positions, mostly in or near Hydrangeaceae and Loasaceae, sometimes near the base of Cornales (Xiang, 1999; Albach et al., 2001; Xiang et al., 2002; Fan & Xiang, 2003; Schenk & Hufford, 2010). In the most elaborate study of Cornales evolutionary relations to date, Xiang et al. (2011) find strong support for the placement of Hydrostachyaceae as sister to a clade containing both Hydrangeaceae and Loasaceae. Yet the authors urge that the exact position of the Hydrostachyaceae within the Cornales remains uncertain, since the tree placing the family within the Hydrangeaceae was not significantly worse at explaining their sequence data than the tree placing the family sister to the Hydrangeaceae + Loasaceae clade according to Shimodaira-Hasegawa tests.

At a lower level of organization, several infrafamilial classifications have been proposed within Hydrangeaceae (Table 1.1). Several authors centered their classifications around two alliances: the *Philadelphus*-like genera on the one hand, and *Hydrangea*-like genera on the other hand, recognizing these groups as either tribes (Hydrangeeae and Philadelpheae) or subfamilies (Hydrangeoideae and Philadelphoideae). The genus *Kirengeshoma* is the only Hydrangeaceae genus morphologically anomalous enough to be recognized as a separate systematic grouping on par with the abovementioned *Philadelphus*-like or *Hydrangea*-like alliances. The most recent infrafamilial classification of the Hydrangeaceae was proposed by Hufford et al. (2001). This classification is based on monophyletic groups recovered in a combined analysis of *matK*, *rbcL* and morphological characters, accepting well-supported nodes as evidence for evolutionary relevant groupings of genera. This classification differs from previous attempts in the erection of the subfamily Jamesioideae, to reflect the consistent

placement of the genera *Jamesia* and *Fendlera* as sister to the rest of the Hydrangeaceae. Subfamily Hydrangeoideae (Table 1.1) is then subdivided into tribe Philadelpheae, and the focal group of this study: tribe Hydrangeeae, in line with the previously proposed dichotomy of *Hydrangea*-like and *Philadelphus*-like taxa. In this circumscription of Hydrangeaceae, the family contains 17 genera, distributed across warm-temperate and tropical regions of Europe, Asia, America and Oceania (Cronquist, 1981; Takhtajan, 1997; Hufford, 2004; Samain et al., 2010). The deciduous shrubby genera of the aforementioned subfamily Jamesioideae are restricted to North America, while subfamily Hydrangeoideae comprises a more geographically and morphologically diverse assemblage, containing deciduous and evergreen, shrubby, herbaceous and root-climbing growth forms, which are distributed across America, Asia and Europe.

**Table 1.1: Hydrangeaceae classifications.**

| Engler (1890) | Hutchinson (1927) | Takhtajan (1997) | Hufford et al. (2001), Hufford (2004) |
|---|---|---|---|
| SAXIFRAGACEAE | HYDRANGEACEAE | HYDRANGEACEAE | HYDRANGEACEAE |
| **Hydrangeoideae** | **Hydrangeoideae** | **Hydrangeoideae** | **Hydrangeoideae** |
| Hydrangeeae | Hydrangeeae | Hydrangeeae | Hydrangeeae |
| *Hydrangea* | *Hydrangea* | *Hydrangea* | *Hydrangea* |
| *Broussaisia* | *Decumaria* | *Decumaria* | *Broussaisia* |
| *Cardiandra* | *Pileostegia* | *Pileostegia* | *Cardiandra* |
| *Decumaria* | *Schizophragma* | *Platycrater* | *Decumaria* |
| *Deinanthe* | | *Schizophragma* | *Deinanthe* |
| *Dichroa* | | Cardiandreae | *Dichroa* |
| *Pileostegia* | | *Cardiandra* | *Pileostegia* |
| *Platycrater* | | *Deinanthe* | *Platycrater* |
| *Schizophragma* | | | |
| | Kirengeshomeae | **Kirengeshomoideae** | |
| | *Cardiandra* | *Kirengeshoma* | |
| | *Deinanthe* | | |
| | *Kirengeshoma* | | |
| | **Philadelphoideae** | **Philadelphoideae** | |
| Philadelpheae | Philadelpheae | Philadelpheae | Philadelpheae |
| *Philadelphus* | *Philadelphus* | *Philadelphus* | *Philadelphus* |
| *Carpenteria* | *Broussaisia* | *Carpenteria* | *Carpenteria* |
| *Deutzia* | *Deutzia* | *Fendlera* | *Deutzia* |
| *Fendlera* | *Dichroa* | *Fendlerella* | *Fendlerella* |
| *Jamesia* | *Neodeutzia* | *Jamesia* | *Kirengeshoma* |
| *Whipplea* | *Platycrater* | *Whipplea* | *Whipplea* |
| | Carpenterieae | Deutzieae | |
| | *Carpenteria* | *Broussaisia* | |
| | *Fendlera* | *Deutzia* | |
| | *Fendlerella* | *Dichroa* | |
| | *Jamesia* | | |
| | *Kania* | | |
| | *Whipplea* | | |
| | | | **Jamesioideae** |
| | | | *Jamesia* |
| | | | *Fendlera* |

Evolutionary relationships within tribe Hydrangeeae

The monophyletic Hydrangeeae (Soltis et al., 1995; Hufford et al., 2001) consists of a basal clade *Deinanthe* + *Cardiandra*, in a sister position with the "*Hydrangea* clade" (Hufford et al., 2001) which contains the ornamental genus *Hydrangea* and allied genera *Broussaisia*, *Decumaria*, *Dichroa*, *Pileostegia*, *Platycrater* and *Schizophragma*. The first studies providing insight into evolutionary relationships within the tribe were mainly focused at a different taxonomic level, being the elucidation of family relations within Cornales (Morgan & Soltis, 1993; Olmstead et al., 1993; Xiang et al., 1993; Xiang et al., 1998). Therefore, the earliest phylogenetic information for tribe Hydrangeeae is limited to a subset of the genera currently included. However, once sample size of the tribe in these studies increased, a consistent pattern of a para- or polyphyletic genus *Hydrangea* emerged. This was first established, albeit in a largely unsupported phylogenetic hypothesis, based on *rbcL* by Soltis et al. (1995). Subsequent studies elaborated on this analysis by sequencing different plastid markers (*rps16-trnK* and *trnK-psbA* spacers, *trnK* intron, *trnK* exon and *matK* gene: Samain et al., 2010; *matK* and *rbcL*: Hufford et al., 2001) or combining plastid markers with nuclear and anonymous sequences (*accD-psa1*, *matK*, *psbA-trnH*, ITS: Jacobs, 2010), amassing more evidence for the polyphyletic nature of *Hydrangea*. Furthermore, phylogenetic resolution within the tribe increased, allocating the nine constituting genera to two large clades (Samain et al., 2010); Hydrangea I and Hydrangea II (Figure 1.6). Despite strong molecular support for these clades in later studies (Granados et al., 2013), no morphological characters seem to reflect this split in tribe Hydrangeeae.

Hydrangea I as proposed by Samain et al. (2010) contains the genera *Cardiandra*, *Deinanthe*, *Pileostegia*, *Schizophragma*, *Decumaria*, *Platycrater* and *Deinanthe*. All studies including multiple specimens of these traditionally recognized genera recovered them as monophyletic, but nested within a polyphyletic *Hydrangea*. Intergeneric relationships in Hydrangea I were largely resolved by Granados et al. (2013), in a phylogenetic hypothesis based on a set of 13 plastid markers and a limited but representative sample of individuals. Noticeably, the position of *H. arborescens*, the type species of the genus, remains unresolved. This species was recovered in different positions in the two most recent phylogenetic hypotheses of Hydrangeeae, being either sister to *Cardiandra* + *Deinanthe* (Samain et al., 2010), or in a grade with *H. quercifolia* and sister to a clade consisting of *H*. subsect. *Calyptranthe*, *H*. sect. *Cornidia*,

the genus *Platycrater* and *H*. subsect. *Asperae* (Granados et al., 2013). The latter authors furthermore provided statistical support for the placement of the *Cardiandra* + *Deinanthe* clade inside of tribe Hydrangeeae, while previous studies were unable to fully support this hypothesis (Samain et al., 2010), or suggested a sister relationship between this clade and the rest of the Hydrangeeae (Hufford, 1997, 2001). Other intergeneric relationships supported in the analysis by Samain et al. (2010) are corroborated by Granados et al. (2013). Hydrangea II consists of the genera *Broussaissia* and *Dichroa*, nested within the remaining taxa of *Hydrangea* s.s.. Evolutionary relationships inferred for this clade were concordant between Samain et al. (2010) and Granados et al. (2013) and are fully resolved.

These phylogenetic hypotheses for tribe Hydrangeeae are, at least in part, incompatible with the current infrageneric classification of *Hydrangea* s.s., as devised by McClintock (1957). Apart from representing a polyphyletic assemblage, the genus *Hydrangea* was divided into a hierarchy of taxa of which some do not represent the evolutionary relationships within the genus. Most notably, McClintock divided the genus into two sections: *Hydrangea* section *Hydrangea*, and *Hydrangea* section *Cornidia*, while there seems to be no evolutionary justification for separating section *Cornidia* from the rest of *Hydrangea* at this level. Furthermore, monophyly for several subsections (subsect. *Asperae*, subsect. *Americanae* and subsect. *Macrophyllae*) could not be confirmed (Figure 1.6; Samain et al., 2010; Jacobs, 2010) or is rejected with high support (subsect. *Asperae* in Granados et al., 2013). Despite these strong indications against McClintock's classification, it remains the most influential system in contemporary herbaria, botanical gardens and scientific studies. This indicates the need for a new classification which brings these infrageneric taxa in line with the available evolutionary data.

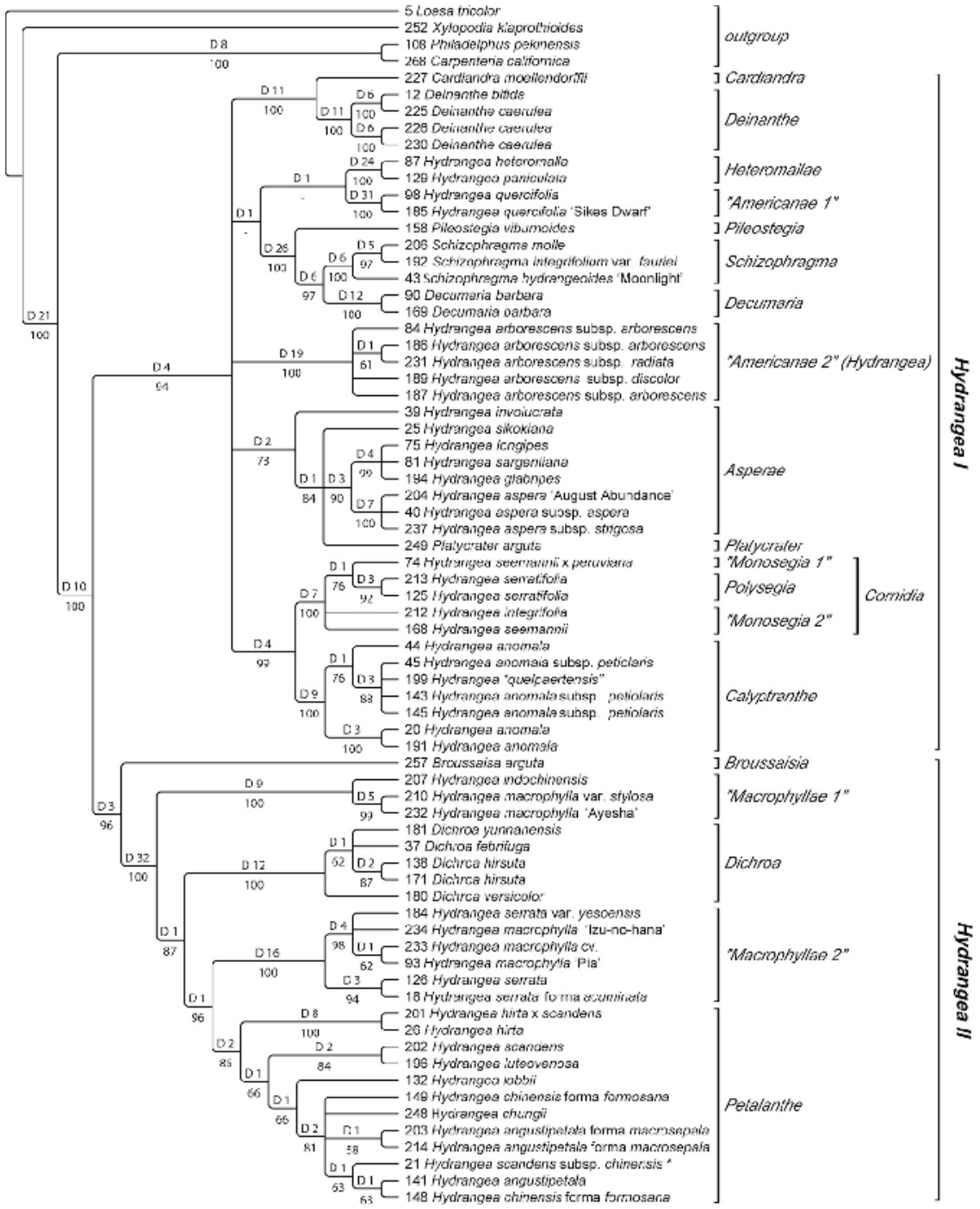


**Figure 1.6: Phylogenetic hypothesis for tribe Hydrangeeae.** Strict consensus tree inferred from the complete chloroplast dataset and coded length mutations (*trnK* intron, *matK* gene) by Samain et al. (2010). To evaluate nodes, the Bremer support (Decay value) has been labeled above the branch. In addition, a bootstrap analysis has been performed using 1000 replicates. These values are indicated below the respective branch. The infrageneric clade names of *Hydrangea* s. s. follow McClintock (1957). *Hydrangea scandens* subsp. *chinensis* * = *Hydrangea scandens* subsp. *chinensis* forma *angustipetala* (Figure and caption adapted from Samain et al., 2010).

**Tribe Hydrangeeae Morphology**

The genera attributed to tribe Hydrangeeae (sensu Hufford, 2001) form a morphologically diverse assemblage (Figure 1.7). Woody shrubs and small trees dominate the tribe, occurring in the genera *Platycrater*, *Broussaisia*, *Dichroa* and part of *Hydrangea* s.s. (Fosberg, 1939; Hufford et al., 2001, 2004; Samain et al., 2010). The other genera represent either herbaceous perennials (*Cardiandra* and *Deinanthe*), or root-climbing lianas (*Pileostegia*, *Schizophragma* and *Decumaria*, *H*. subsect. *Calyptranthe* and *H*. sect. *Cornidia*). This last category contains scandent to climbing shrubs using unbranched adventitious roots to cling to boulders or other plants. The anatomical and morphological peculiarities of this growth-form have been researched in detail for *H.* sect. *Cornidia* by Granados et al. (2014). Phyllotaxis for most genera in tribe Hydrangeeae is opposite (*Decumaria*, *Pileostegia*, most *Hydrangea* s.s. *Platycrater*, *Dichroa*), some members of *Hydrangea* s.s. have verticillate leaves, while in *Cardiandra* leaves are alternating. Genera can be both deciduous and evergreen, with leaves ranging from membranous to thickly coriaceous.

Inflorescences in tribe Hydrangeeae are predominantly terminal, with few exceptions (e.g. *H. luteovenosa*, most taxa in *H*. sect. *Cornidia*) and are composed of a corymbose cyme, corymbose panicle, umbellate cyme or thyrse. Most taxa of the focal tribe present a higher number of flowers per inflorescence compared to other members of the Hydrangeaceae, but individual flowers are notably smaller (Hufford, 2001). The genera *Deinanthe* and *Platycrater* present exceptions to this situation, in producing a limited number of larger flowers. Representatives of tribe Hydrangeeae are well-known for their production of showy marginal flowers (often incorrectly termed sterile flowers: e.g. Gurung et al., 2018) in conjunction with smaller, less conspicuous central flowers. These flowers have been suggested to contribute to attraction of pollinators (Sato & Kato, 2019). This floral dimorphism could possibly represent a synapomorphism for the tribe, being absent only in several *Hydrangea* s.s. taxa, *Dichroa*, *Decumaria* and *Broussaisia*. Cultivated plants of the Japanese *H. macrophylla*, often display inflorescences existing solely of these showy, colorful marginal flowers. The enormous horticultural success of these morphotypes is largely due to the presence of these large, showy inflorescences.

Dioecism is rare in tribe Hydrangeeae, only occurring in the monotypic *Broussaisia* (Klink, 1995; Hufford, 2001; Ronse de Craene, 2010) and the majority of the taxa of *Hydrangea* section *Cornidia* (Nevling & Gómez-Pompa, 1968; Samain et al., 2014, 2019). Other taxa of the tribe present monoecious individuals.

Hydrangeaceae are typical Cornales in showing a valvate calyx aestivation. The imbricate aestivation seen in *Deinanthe* represents an exception within the family, possibly linked to another situation in this genus unique for tribe Hydrangeeae; entirely free sepals. This contrasts with the other genera of the tribe, where the sepals are generally slightly joined along their base (Hufford, 2001). Corolla aestivation for the tribe is mostly valvate, with the exception of an imbricate aestivation in the genera *Deinanthe* and *Cardiandra*. Petals always develop as free in tribe Hydrangeeae, but fuse along their margins postgenitally in *Pileostegia* and *Hydrangea anomala*. Merosity of floral organs is highly variable in tribe Hydrangeeae. Tetramerous, pentamerous or hexamerous perianths are common, while the octamerous organization of *Cardiandra* and dodecamerous organization of e.g. *Decumaria* are rather unusual for the tribe. The androecium of all genera in tribe Hydrangeeae is diplostemonous and/or polystemonous, with the diplostemonous and haplostemonous species of *Dichroa* forming the exception. Carpels in the focal tribe generally number two to six, with *Decumaria* presenting an aberrant 12 carpels. Gynoecia among members of Hydrangeeae differ in style morphology. Most genera have simple styles, while others present multiple free, postgenitally connate (*Deinanthe*) or branched (*Dichroa*) styles. Position of the ovules can be horizontal, erect or pendant, while position of the ovary varies the complete scale between completely superior and fully inferior. The berries produced by *Broussaisia* and *Dichroa* present an aberrant fruit form within the tribe, where all other genera develop capsular fruits. The latter dehisce apically (*Deinanthe*, *Cardiandra*, *Hydrangea* s.s and *Platycrater*) or by fragmentation of the lateral walls (i.e. *Schizophragma*, *Pileostegia* and *Decumaria*) (Hufford, 2004; Hufford et al., 2001). Most genera in tribe Hydrangeeae produce numerous, winged seeds, with the exception of *Dichroa*, *Broussaisia* and several species of *Hydrangea* s.s. (Wei & Bartholomew, 2001).

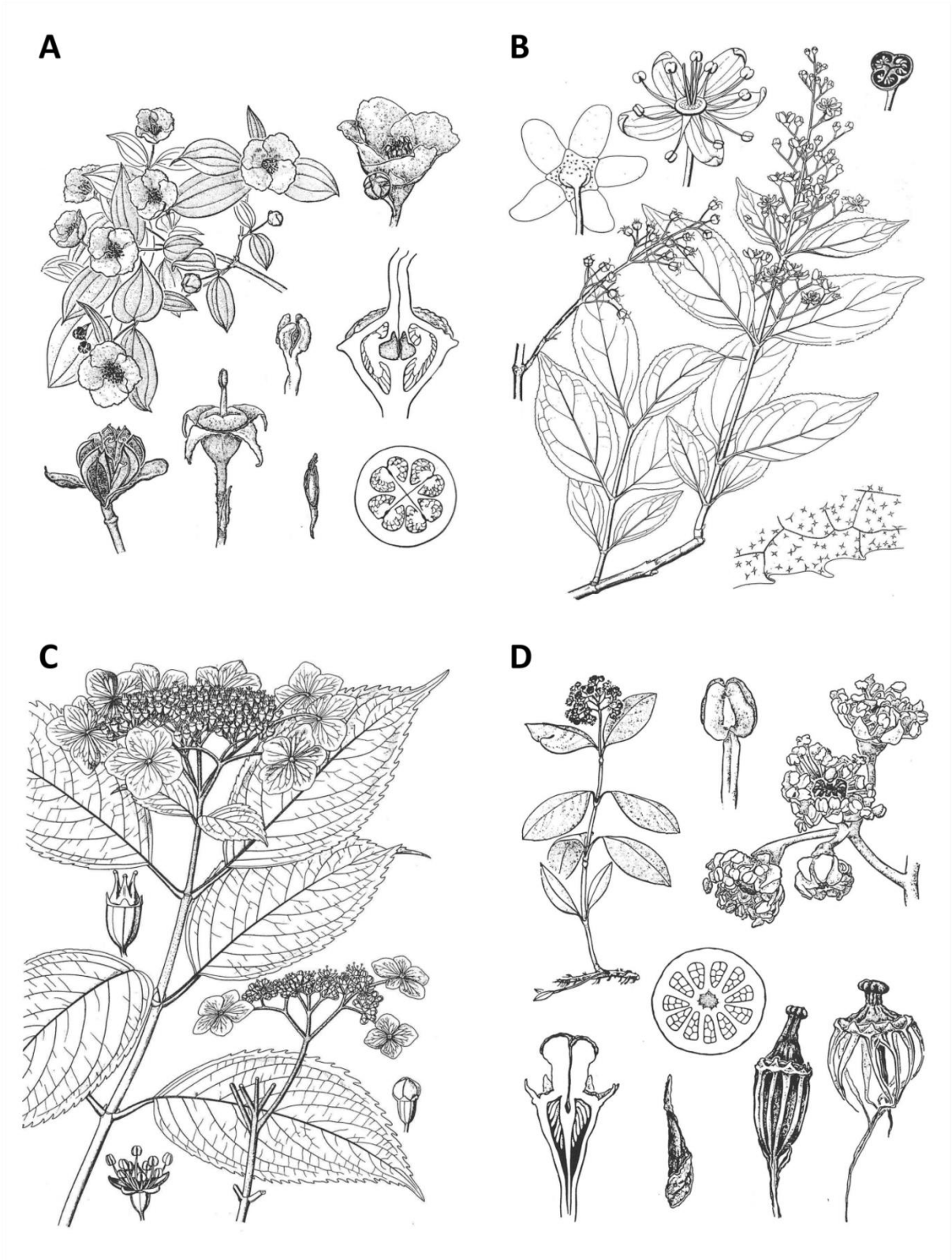


**Figure 1.7: Representatives of Hydrangeaceae subfamily Hydrangeoideae**. A. *Philadelphus inidorus* and B. *Deutzia paniculata* of tribe Philadelpheae. C. *Hydrangea serrata* and D. *Decumaria barbara* from the tribe Hydrangeeae (Drawings adapted from Hufford, 2004).

### *Hydrangea aspera* complex and allied taxa

*Hydrangea aspera* was first described in 1825 by David Don, from specimens collected in the Himalayas by Francis Buchanan-Hamilton and Nathaniel Wallich. The following years, several allied taxa were collected and described, ranging from the eastern Himalayas across western and southern central China as well as Taiwan, Sumatra, Java and Japan. These species were first placed within series *Piptopetalae*, along with several other eastern Asiatic species, based on their deciduous, separately falling petals, erect habitat and caudate seeds (Maximowicz, 1867). Later, as the number of described species allied to *H. aspera* grew, they were relegated to subsection *Asperae* by Rehder, in his 1911 revision of the specimens collected by E.H. Wilson in China. This subsection was chiefly characterized by "the inferior ovary developing into a hemispheric or turbinate capsule truncate at the apex" (Figure 1.9), and contained 12 putative species, of which five newly described. In this work, Rehder often compares the Chinese specimens under his scrutiny to the Nepalese or Indian species described by his predecessors, describing new species or varieties based on differences in leaf shape and pubescence. These differences in leaf morphology are subsequently considered intraspecific variation within a single widespread species, *H. aspera*, in the most recent worldwide revision of the genus (McClintock, 1957). As such, this monograph considers only three species (Figure 1.8) within subsection *Asperae*: the morphologically variable and geographically widespread *H. aspera*, and the two Japanese species *H. involucrata* and *H. sikokiana*. McClintock does, however, recognize the presence of four subspecies within *H. aspera*, which she distinguishes based on pubescence of the abaxial leaf surface, and shape of leaves and petioles: *H. aspera* subsp. a*spera*, subsp. s*trigosa*, subsp. *robusta* and subsp. s*argentiana*. Revising the Chinese representatives of *Hydrangea* for the latest edition of the Flora of China, authors Wei and Bartholomew (2001) disagree with McClintocks interpretation of species boundaries within this subsection (Figure 1.8). Remarkably, both authors disagree on the number of species to be recognized within this group, as is illustrated by several footnotes appended by one of the authors (Bartholomew), arguing for the lumping of several taxa recognized by his co-author. As is evident from this history, species boundaries within subsection *Asperae* are not well understood, and different interpretations seem to hinge mainly on the importance of certain morphological traits for species recognition. We therefore

use the term "*H. aspera* species complex" to include all eastern Asian representatives of subsect. *Asperae*, with the exclusion of the Japanese taxa and *H. longifolia*.

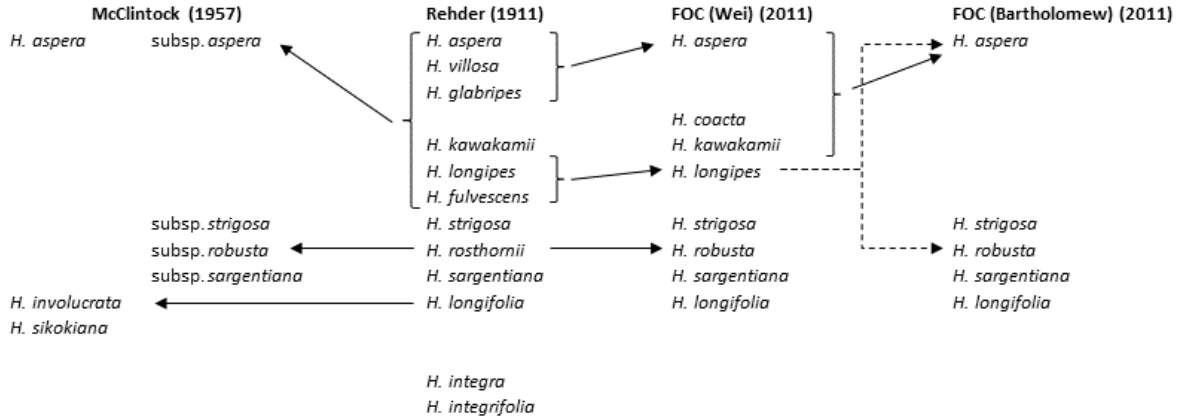


**Figure 1.8: *Hydrangea aspera* species complex and allied taxa throughout revisions.** The nominal taxa classified in *H.* sect. *Asperae* have been merged and split according to subsequent authors. Full arrows indicate a merger of different taxa into a recognized species, whole dotted lines represent the splitting of a previously recognized species. Rehder places both *H. integra* and *H. integrifolia* in this group, while subsequent authors relocated them to different sections or subsections. For the Flora of China (FOC) both authors where unable to agree upon the number of species in the section. Their interpretation of species boundaries is presented separately here.

Despite not being discussed in some of the abovementioned revisions (Rehder, 1911; Wei & Bartholomew, 2001), the Japanese species enjoy a much more taxonomically stable history, being recognized as separate species by most authors, probably owing to their distinct morphology and geographical occurrence. Indeed, *H. sikokiana* is the only member of the subsection showing lobed leaves, and is endemic to the Japanese island of Shikoku. The other Japanese species, *H. involucrata*, is remarkable within the subsection for the presence of involucral bracts covering the young inflorescence. This character, which is always present in McClintock's subsect. *Cornidia*, the sister clade of subsect. *Asperae* (Samain et al., 2010), is only described for one other putative species within this group, which is often synonymized with *H. involucrata* (McClintock, 1957); the Taiwanese taxon *H. longifolia*.

Apart from the abovementioned morphological variation, *H.* subsection *Asperae* is characterized by cytogenetic variation unique within the genus *Hydrangea*. While exploring genome sizes, base composition and chromosome numbers within the entire genus, Cerbah et al. (2001) found that most representatives show 2n = 2x = 36. However, the specimens identified as members of subsect. *Asperae* showed chromosome numbers of 2n = 30, 34 and 36.

These results were corroborated and expanded on by Mortreau et al. (2010), by measuring DNA content and chromosome mapping of 5S and 18S-5.8S-26S rDNA by fluorescent *in situ* hybridisation (FISH). This showed that there is variation in genome size and FISH banding within the *H. aspera* complex, exceeding that which is to be expected within a species. With this the authors suggest that the single species model proposed by McClintock (1957) for the *H. aspera* complex does not match the cytogenetic and genomic properties of the taxa in their study. They therefore prefer the splitting of the *H. aspera* complex into multiple species, but do not offer a full examination of all species described within this complex.

Finally, variation in geographical distribution seems to be prevalent within *Hydrangea* subsection *Asperae*, containing both widespread and narrowly endemic taxa. Certain taxa are described from Northern India to Eastern China, like *H. aspera*, covering a vast geographical range. Other taxa, however, have only been collected from a single population, such as *H. sargentiana*, an interesting situation from a conservationist standpoint, on which is divulged in chapter 5 (Box 5.1). Furthermore, the subsection under study here presents putative species in varying degrees of isolation from one another; the Japanese taxa *H. involucrata* and *H. sikokiana* are clearly isolated from the taxa described from the mainland, while *H. kawakamii* and *H. longifolia* are isolated to some degree on the island of Taiwan. Other putative species - *H. aspera* (in the sense of Wei & Bartholomew, 2001), *H. robusta* and *H. strigosa* for example- are described to occur in varying degrees of sympatry, often making contact along altitudinal clines (McClintock, 1957).

The presence of these different types of variation within *Hydrangea* subsection *Asperae*, and especially the *H. aspera* species complex, makes for an interesting case-study regarding species boundaries. As of now, it is unclear how much of this variation falls within intraspecific variation, and which part of this can be attributed to differences between species or indeed, evolutionary lineages. These questions are addressed in chapters 3, 4 and 5 of this thesis.


**Figure 1.9: *Hydrangea longipes* and *H. aspera*.** 1-5: *H. longipes*. 1: fruiting branch. 2: leaf blade portion adaxial view, showing hairs. 3: leaf blade portion abaxial view showing hairs. 4: fruit, capsule with truncate apex and two styles. 5: seed. 6-8 *H. aspera*. 6: fruiting branch. 7: fruit, capsule with truncate apex and two styles. 8: seed (Figure adapted from Wei & Bartholomew, 2001).

**Conservation and taxonomy**

The region where *Hydrangea* sect. *Asperae* shows its highest species diversity, central China, is known for heavy anthropogenic pressures on species diversity through ecosystem degradation (Li, 2004). Many of these threats are caused by China's large population increase and associated rise in agricultural and infrastructural demands on the environment. Mitigating these detrimental effects on biodiversity requires strong conservation and restoration efforts (Isbell et al., 2017; Li, 2004), some of which have already been implemented (e.g. The Natural Forest Protection Program and the Returning Farmland to Forest Program; Robbins & Harrell, 2014; Wang et al., 2007), albeit under certain levels of criticism (Hua et al., 2016). These threats to biodiversity are exacerbated by the fact that China is one of the most species-rich countries in the world. Indeed, the country is home to over 33.000 species of vascular plants, among which almost half have been designated as endemic (Huang et al., 2011). This high amount of endemics is linked to the presence of Quaternary glacial period refugia situated in Chinese mountain ranges (López-Pujol et al., 2006). These refugia, along with secondary contact zones and recolonization, might have contributed to the high biodiversity in the country. Nevertheless, patterns of post-glacial hybridization could contribute to reticulate evolution, forming of species complexes, and thus difficulties in formulating stable species boundaries (e.g. De Smet et al., 2012).

Taxonomy and systematics, being the sciences involved in identifying, naming and classifying the world's biodiversity are inextricably linked to conservation efforts. Indeed, high level policy in the field of biodiversity conservation is informed by estimates of species richness, biodiversity and vulnerability of taxa. Since species are the units of conservation in most legislations (e.g. CITES, EUTR, Lacey-act), their correct identification is pivotal to correct implementation and enforcement. As pointed out by several authors (Mace, 2004; Garnett & Christidis, 2019), taxonomic changes have the potential to negatively impact conservation efforts by obscuring the correct natural entity to place under governance or protection. Applied to *Hydrangea*, the lack of stable species boundaries renders identifying possibly vulnerable taxa nearly impossible. Generating a stable classification, with clearly delineated species based on multiple explicitly documented operational criteria constitutes a first step towards conservation of *Hydrangea* taxa.

**Framework of this PhD**

Hydrangeaceae represent one of the taxonomic groups for which the Research Group Spermatophytes directed by Prof. Dr. Paul Goetghebeur at Ghent University obtained international renown. The base for this research line was laid at the international *Hydrangea* conference in 2007, which received a wide variety of breeders and academics from across the world. Once the challenges faced by this taxonomic group were clearly identified, they were initially outlined in the 2010 paper by Samain et al., entitled "Unraveling Extensive Paraphyly in the Genus *Hydrangea* s. l. with Implications for the Systematics of Tribe Hydrangeeae". From this paper, several research lines emerged, resulting in two PhD studies. The first, undertaken by Dr. Carolina Granados Mendoza, encapsulated two levels of research. At the level of the tribe Hydrangeeae breeding potential and molecular marker development was targeted. At a lower taxonomic level, systematics and biomechanics of the New World *Hydrangea* section *Cornidia* was tackled. Taxonomy and systematics of this section continue to be one of the main research lines of the research group of Dr. Marie-Stéphanie Samain at the Instituto de Ecología, A.C. in Mexico.

The current manuscript is the result of the second PhD study into tribe Hydrangeeae phylogenetics and systematics. In this work, the focus is placed on the resolution of two main issues: the paraphyletic nature of the genus *Hydrangea* (s.s.) and the unclear species boundaries in *Hydrangea* subsect. *Asperae*. Pursuing these aims was made possible through the presence of a large body of acquired experience and knowledge at the abovementioned Research Group, providing the necessary background to taxonomic and systematic research, collections in the field and lab work. Due to the nature of taxonomic and systematic studies, and the close link to conservation science, gaining insight into the natural state of the studied taxa, as well as collecting wild samples was pivotal to the thesis presented here. Two main field trips were planned for this work (Figure 1.10), one to the Chinese province of Sichuan and to Taiwan, and a second to Hubei province and Japan. In addition to hands-on experience with collecting and assessing natural populations, these fieldtrips allowed setting up new research contacts. One of these contacts, Tatsuya Uemachi, invaluable for collecting wild populations in Japan, would also contribute to the publication of one of the chapters part of this thesis. Apart from the value of *in situ* collection of plants, the study of herbarium material

and collections amassed by breeders or plant enthusiasts greatly contributed to the present work. It is only through the meticulous notes of plant collectors and breeders that some rare morphotypes of *Hydrangea* can be traced back to their original type locations (e.g. *H. sargentiana*, De Smet et al., 2015b).

Processing the collected specimens and acquiring the genetic data necessary for phylogenetic study was possible through the presence of a molecular lab in which the Research Group participated, the Center for Molecular Phylogeny and Evolution (CeMoFe). The expertise available here, as well as in related Research Groups, supplemented with high quality workshops, conferences and personal study, made the molecular portion of this study possible.


**Figure 1.10: Field collections of *Hydrangea*.** A: different specimens ready for pressing and drying. B: freshly collected specimen. C: leaf sample of *H. sargentiana* to be dried on silica-gel for DNA-extraction.

**Objectives and outline**

In this thesis an attempt is made to improve the understanding of evolutionary relationships within tribe Hydrangeeae, and to use this information to create a stable classification and taxonomy for the group. Tackling these issues will inevitably touch upon several ongoing discussions concerning the reconciliation of new and traditional views in taxonomy. This study therefore aims to provide examples of how these ongoing discussions translate to the empirical field. Tribe Hydrangeeae presents an ideal case study for: 1) resolution of paraphyletic or polyphyletic genera, 2) rate of acceptance for taxonomic changes in a well-known ornamental plant group. At a lower taxonomic level, *Hydrangea* subsection *Asperae* presents an interesting case to evaluate the utility of recent species delimitation algorithms to stabilize shifting species demarcations. Absence of model organisms in the group furthermore provide the opportunity to explore different methods for obtaining sufficiently variable sequence data. Consequently, the main research lines of this thesis are:

- Inferring a robust phylogenetic hypothesis for tribe Hydrangeeae, using a representative sampling of described taxa, in order to evaluate the previously suggested paraphyletic or polyphyletic nature of genus *Hydrangea* (addressed in chapter 2).

- Proposing a new classification scheme for tribe Hydrangeeae, addressing the controversy surrounding the recognition of paraphyletic or polyphyletic taxa (addressed in chapter 2).

- Providing molecular markers containing sufficient variability for species level studies within the genus *Hydrangea*, using both traditional Sanger sequencing (chapter 3), and High-throughput sequencing (chapter 4).

- Amassing several independent lines of evidence to generate stable species boundaries for *Hydrangea* subsection *Asperae* within the framework of the general lineage concept of species (chapters 3, 4 and 5)

Chapter 1

This chapter provides the philosophical, taxonomical and phylogenetic background for the rest of the thesis. Morphological features specific to the studied groups are discussed, as is the systematic and taxonomic history. As several novel molecular methods are utilized in the next chapters, the theoretic background of these algorithms, and the justification for using them is briefly touched upon. Finally, several ongoing debates in evolutionary biology, classification and species delimitations are presented, as they bear relevance on presenting a novel classification for tribe Hydrangeeae and species delimitation in *Hydrangea* sect. *Asperae*. As a full discussion of these concepts (e.g. species concepts, phylogenetic classifications/taxonomy) is beyond the scope of this work, indeed, would justify a thesis in itself, only aspects relevant for the other chapters are summarized here. *This chapter was written by YDS.*

Chapter 2

This chapter builds on previous studies identifying the genus *Hydrangea* as polyphyletic (Samain et al., 2010) and providing new plastid markers for phylogenetic reconstruction in tribe Hydrangeeae (Granados Mendoza et al., 2013). Assembling a representative sample of tribe Hydrangeeae containing individuals for all satellite genera and sections, this chapter presents the most comprehensive phylogenetic hypothesis for the tribe to date. Using the evolutionary relationships defined by this hypothesis, a new classification is proposed. In order to generate a classification concordant with evolutionary history, the eight satellite genera (*Broussaisia*, *Cardiandra*, *Decumaria*, *Deinanthe*, *Dichroa*, *Pileostegia*, *Platycrater* and *Schizophragma*) were merged into *Hydrangea*, alleviating the undesirable polyphyletic nature of the latter. In order to promote acceptance of this new classification by the broader public, the recognizable names of the previously recognized genera are conserved at the section level where possible. *Lab work was performed by YDS, PA and CGM, analyses and manuscript drafting by YDS, formal taxonomic changes by MS, PG and YDS, field collections by YDS, KB, CGM, MS*.

Chapter 3

Species boundaries in the genus *Hydrangea* were previously based exclusively on morphological variation. Consequently, subsequent revisions fluctuated widely the number of recognized species, based on their interpretation of diagnostic features. In order to create stable species hypothesis in *Hydrangea* section. *Asperae*, a range of molecular species delimitation methods was applied, utilizing several specifically developed low copy nuclear

markers. These results were integrated with morphological features such as abaxial leaf pubescence to create species delimitations based on multiple lines of evidence. Following this approach, several well-supported evolutionary lineages were identified within the notoriously difficult *H. aspera* species complex. *Lab work, analyses and manuscript drafting by YDS, field collections by YDS, KB and ER.*

Chapter 4

Developing low copy nuclear markers specific to a group under study can be a cost and time intensive endeavor. With the availably of high-throughput sequencing methods, other approaches for generating polymorphic markers used in phylogenetic and species delimitation studies became available. In this chapter the utility of RADseq for phylogenetic reconstruction and species delimitation is compared to that of low copy nuclear markers. Utilizing the same data set as chapter 3, an easy comparison between these two methods is possible. In addition to this comparison, the acquired data provide additional lines of evidence to be used in construction well-supported species hypotheses in *H*. sect. *Asperae*. *Lab work by PA and YDS, analyses and manuscript drafting by YDS, field collections by YDS, KB and ER.*

Chapter 5

In order to formalize the species hypothesis proposed in the two previous chapters, chapter 5 provides formal descriptions for the recognized species in *H*. sect. *Asperae*. In order to link these taxa to the molecular research, each taxon is accompanied by both a morphological description and summarized references to their evolutionary history. *This chapter was written by YDS.*

Chapter 6

The advances made in Hydrangeeae classification, taxonomy and evolutionary knowledge are outlined in this chapter. Since several research lines inevitably encountered conflicts arising from reconciliating traditional taxonomy with molecular based taxonomy, challenges associated with these conflicts are also presented. In addition, future perspectives and research lines are outlined. *This chapter was written by YDS.*

CGM: Carolina Granados Mendoza, ER: Eduardo Cires Rodríguez, KB: Kenneth Bauters, MS: Marie-Stéphanie Samain, PA: Pieter Asselman, PG: Paul Goetghebeur, YDS: Yannick De Smet.

**Chapter II**

# Molecular phylogenetics and new (infra)generic classification to alleviate polyphyly in tribe Hydrangeeae (Hydrangeaceae, Cornales)

"Nothing in biology makes sense except in the light of evolution"

Theodosius Dobzhansky (1900-1975)

**Abstract**

Tribe Hydrangeeae of Hydrangeaceae currently contains nine morphologically diverse genera, many of which are well-known garden ornamentals. Previous studies have shown eight of these genera to be phylogenetically nested within *Hydrangea*, rendering the latter polyphyletic. To clarify the phylogeny of tribe Hydrangeeae, the present study sequenced four chloroplast regions and ITS for an extensive set of taxa, including the type species for all nine genera involved. The resulting phylogenetic hypotheses corroborate the polyphyly of *Hydrangea*. Since polyphyletic taxa are deemed unacceptable by both sides in the ongoing debate concerning the adherence to strict monophyly in biological classifications, a new (infra)generic classification for tribe Hydrangeeae is proposed. In order to create a stable, evolutionary informative classification a broader circumscription of the genus *Hydrangea* is proposed, to include all eight satellite genera of the tribe. Such treatment is considered highly preferable to an alternative where *Hydrangea* is to be split into several morphologically potentially unidentifiable genera. To facilitate the acceptance of the new classification proposed here, and in order to create a classification with high information content, the familiar generic names were maintained as section names where possible.

Adapted from: De Smet, Y., Granados Mendoza, C., Wanke, S., Goetghebeur, P., Samain, M.-S. 2015. *Molecular phylogenetics and new (infra)generic classification to alleviate polyphyly in tribe Hydrangeeae (Hydrangeaceae, Cornales).* Taxon 64 (4): 741-753.

## Introduction

Over the past few decades, rapid advances in DNA technologies have brought about an increase in the use of phylogenetic hypotheses in taxonomy (e.g. phylogenetic systematics, Hennig, 1966). Indeed, the majority of contemporary taxonomic studies attempt to establish natural, genealogy-based classifications, guided by phylogenetic hypotheses. Therefore, a consensus seems to have arisen that common descent should play a major role in biological classification (Xiang et al., 2012). Disagreements, however, still exist with respect to the treatment of paraphyletic taxa, with two sides locked in ongoing debate (reviewed in: Hörandl & Stuessy, 2010; Schmidt-Lebuhn, 2012). On the one hand, the school of evolutionary systematics advocates a classification system with a high information content (Stuessy, 1987; Van Wyk, 2007; Hörandl, 2010; Mayr & Bock, 2002) and practicability (Brickel et al., 2008; Brummit, 2002), reflecting natural processes. In this philosophy, shared descent is viewed as an important character for grouping taxa, but an emphasis is placed on degrees of divergence and similarity between elements of a certain taxon (Hörandl & Stuessy, 2010). As a consequence, evolutionary systematists advocate the recognition of paraphyletic taxa, as these are argued to reflect similarity, high information content and practicability. The school of phylogenetic (or cladistic) systematics, on the other hand, proposes strict adherence to monophyletic (holophyletic) taxa, recognized by the presence of synapomorphic characters. This school argues that monophyletic groups are objective entities, considering all taxa above species level as human-devised, artificial constructs. Therefore, since paraphyletic taxa are based on a subjective idea of what is "divergent enough" (Schmidt-Lebuhn, 2012), these entities are rejected as artificial classes created to emphasize particular characters or divergence (Ebach et al., 2006; Donoghue & Cantino, 1988). Here, some of the prominent discussion points between both schools are illustrated with the taxonomy of Hydrangeaceae tribe Hydrangeeae. This group provides an interesting case study for solving complex classification problems due to the presence of 1) paraphyletic groups both at genus level and below, 2) a large polyphyletic assemblage, and 3) important horticultural representatives with very distinct morphology.


**Figure 2.1: Genera of tribe Hydrangeeae.** A. *Broussaisia arguta* Gaudich. B. *Hydrangea aspera* Buch.-Ham. ex D. Don. C. *Decumaria barbara* L. D. *Deinanthe bifida* Maxim. E.*Cardiandra alternifolia* (Siebold) Siebold & Zucc. F.*Dichroa febrifuga* Lour. G. *Pileostegia viburoides* Hook. F. & Thomson H. *Platycrater arguta* Siebold & Zucc. I. *Schizophragma hydrangeoides* Siebold & Zucc.

The asterid family Hydrangeaceae (Cornales) is an assemblage of 17 currently recognized genera, containing ca 270 accepted species. In the most recent revision of the classification of Hydrangeaceae, Hufford et al. (2001) combined results from previous morphological (Hufford, 1997) and molecular (Soltis et al., 1995) studies to support the split of Hydrangeaceae into subfamilies Jamesioideae and Hydrangeoideae. The 15 genera contained in subfamily Hydrangeoideae were classified in tribes Philadelpheae and Hydrangeeae. The focal group of the present study, tribe Hydrangeeae, represents a heterogeneous assembly of nine genera (*Broussaisia* Gaudich., *Cardiandra* Siebold & Zucc., *Decumaria* L., *Deinanthe* Maxim., *Dichroa* Lour., *Hydrangea* L., *Pileostegia* Hook. f. & Thomson, *Platycrater* Siebold & Zucc. and *Schizophragma* Siebold & Zucc.), encompassing warm temperate to tropical species (Table 2.1) with shrubby, herbaceous or root-climbing growth forms (Figure 2.1). Many representatives of this tribe have inflorescences with large, showy marginal flowers, to which these plants owe their popularity as garden ornamentals.

**Table 2.1: Genera of tribe Hydrangeeae**

| Genus | Author | # of species | Distribution |
|---|---|---|---|
| *Broussaisia* | Gaudich. | 2 | Hawaii |
| *Cardiandra* | Siebold & Zucc. | 9 | East Asia |
| *Decumaria* | L. | 7 | China, North America |
| *Deinanthe* | Maxim. | 2 | East Asia |
| *Dichroa* | Lour. | 23 | East Asia |
| *Hydrangea s.s.* | L. | 140 | East and Southeast Asia, New World |
| *Pileostegia* | Hook. f. & Thomson | 6 | China, East India, Japan |
| *Platycrater* | Siebold & Zucc. | 1 | East Asia |
| *Schizophragma* | Siebold & Zucc. | 17 | East Asia |
| *Hydrangea s.l.* | L. | 208 | East and Southeast Asia, New World |

A small but representative sampling of Hydrangeeae was included in studies addressing the evolutionary relationships within the Hydrangeaceae using both morphological (Hufford et al., 1997) and molecular (Soltis et al., 1995; Hufford et al., 2001) data. In addition to suffering from low statistical support, these studies resulted in different phylogenetic hypotheses. Sequencing a series of chloroplast regions for an extensive sampling of specimens, Samain et al. (2010) were able to identify two well-supported clades in tribe Hydrangeeae. A first clade, termed Hydrangea I, contained *Cardiandra*, *Deinanthe*, *Pileostegia*, *Schizophragma*, *Decumaria*

and several representatives of *Hydrangea*. Relationships among these genera remained mainly unresolved. In the second major clade, termed Hydrangea II, *Dichroa* and *Broussaisia* were in a grade with two separate clades of *Hydrangea* representatives. Therefore, the results obtained by Samain et al. (2010) suggest that *Hydrangea* is a polyphyletic assemblage, with the remaining eight genera of Hydrangeeae phylogenetically nested within *Hydrangea*. Moreover, this study suggested that the infrageneric classification of *Hydrangea* proposed by McClintock (1957) is in need of revision. In a more recent study, Granados Mendoza et al. (2013) tested the utility of 13 plastid markers using a reduced sampling for resolving backbone relationships within tribe Hydrangeeae (*Broussaisia* not included). A highly supported phylogenetic hypothesis was recovered for Hydrangea I and II, offering better resolution within the first clade, and only leaving the position of *H. arborescens* L. unsupported. Furthermore, *Hydrangea* was once more recovered as a polyphyletic assemblage, corroborating the findings by Samain et al. (2010).

In the present study, a comprehensive phylogeny of tribe Hydrangeeae is presented, sampling all major evolutionary clades retrieved in previous studies, using four plastid markers selected according to their phylogenetic informativeness (Granados Mendoza et al., 2013) and ITS. Using the resulting phylogenetic hypothesis, we address the polyphyletic nature of *Hydrangea* and evaluate the merits of creating a monophyletic *Hydrangea*. Finally, a new infrageneric classification is proposed, incorporating the inferred relationships among and within subclades Hydrangea I and II. Throughout the manuscript, all section names used are those of the here-proposed classification of *Hydrangea s.l.*, the broad circumscription of *Hydrangea*, including the other eight genera of tribe Hydrangeeae. In contrast, *Hydrangea s.s.* refers to the previously recognized, polyphyletic *Hydrangea*, not including the eight satellite genera.

**Material and methods**

Taxon sampling

Taxa pertaining to all major clades and subclades recovered in Samain et al. (2010), all sections and subsections proposed in McClintock's (1957) infrageneric classification, as well as the eight allied genera *Broussaisia*, *Cardiandra*, *Decumaria*, *Dichroa*, *Deinanthe*, *Schizophragma*, *Pileostegia*, *Platycrater* were sampled. For all genera under study, a specimen representing the

type species was included. Two species of Loasaceae (*Loasa tricolor* Ker Gawl. and *Xylopodia klaprothioides* Weigend) and two species of Hydrangeaceae tribe Philadelpheeae (*Philadelphus mexicanus* Schltdl. and *Philadelphus pekinensis* Rupr.) were used as outgroups. Material used for DNA extraction consisted of silica-gel dried leaf tissue of wild collected accessions, while fresh leaves were used for material originating from botanical gardens.

Molecular methods and alignments

Total genomic DNA was extracted from leaf tissue using a modified CTAB method (Doyle & Doyle, 1987). Four noncoding plastid regions, previously shown to be phylogenetically informative for tribe Hydrangeeae (Granados Mendozaet al., 2013), were utilized in this study. The *rpl32-ndhF* intergenic spacer (IGS), *trnV-ndhC* IGS, *trnL-rpl32* IGS and the *ndhA* intron were sequenced for all accessions. Primer sequences and protocols for PCR amplification were taken from Granados Mendoza et al. (2013), with the exception of the amplification of the *ndhA* intron for the *Asperae* clade, which required the design of the additional primers *ndhA*-asp-F (GATTCGTTGAGACATAAATT) and *ndhA*-asp-R (GTACATGAGATTTTCACCT). These plastid markers are non-overlapping and distributed across the large and short single copies of the chloroplast genome (Granados Mendoza et al., 2013). In order to rule out incorrect conclusions based on incongruence between plastid and nuclear phylogenies, ITS was sequenced for a subset of taxa, representing all major clades found in the plastid analyses. Sequencing of this region was performed using primers ITS1 and ITS4 with PCR conditions as described in White et al. (1990). Raw sequences were edited in Sequencher 5.0.1 (Gene Codes Corporation), and aligned with Muscle 3.8.1 (Edgar, 2004). The obtained alignments were subsequently evaluated manually, excluding regions of uncertain homology such as mononucleotide repeats (for a list of excluded regions, see Table S2.1 in Appendix 1). Insertions and deletions (indels) were coded following the simple indel coding scheme of Simmons & Ochoterena (2000) available in SeqState version 1.4.1 (Müller, 2005).

Phylogenetic analysis

The most appropriate model for nucleotide evolution was selected with the Akaike information criterion (AIC) in JModeltest 2.1.3 (Darriba et al., 2012). This procedure selected the TVM+G model for all regions except for the *trnL-rpl32* IGS, for which GTR+G was

preferred. Bayesian inference analysis was run in MrBayes 3.2.1 (Ronquist et al., 2012), for each of the four plastid regions and ITS separately, a concatenated matrix containing all four plastid regions, and a concatenated matrix combining the plastid regions with ITS. The concatenated dataset was generated to examine the impact of the information in the ITS dataset on the phylogenetic relationships recovered, and only attempted since there were no supported (PP > 0.95) incongruences. For each of the abovementioned alignments, two analyses were run; one with and one without indels coded. All analyses were run using the GTR+G model, since the TVM model is not implemented in MrBayes. The analyses of the concatenated matrices were run with partitions for each region, unlinking model parameters for each partition. The Markov Chain Monte Carlo (MCMC) was run using four simultaneous runs with four chains each, for a total of five million generations, sampling trees every 100 generations. Parameter sampling was checked in Tracer v1.6 (Rambaut & Drummond, 2014) to ensure stationarity for each run. Discarding the first 12500 trees as burn-in, the remaining trees were used to calculate the posterior probabilities (PP) of clades using the majority rule consensus. The Cyber infrastructure for Phylogenetic Research (Cipres Science gateway; www.phylo.org; Miller et al., 2010) was used to run all Bayesian analyses. A maximum likelihood analysis in RAxML7.2.8 (Stamatakis et al., 2005) was performed on both concatenated datasets (plastid and plastid + ITS) without indel coding, using the GTRGAMMA model for sequence evolution, with the dataset partitioned according to marker regions, and 1000 rapid bootstrap replicates (Stamatakis et al., 2008).

### Phylogenetic hypothesis testing

Bayesian phylogenetic inference did not resolve the evolutionary position of three taxa: *Broussaisia arguta* Gaudich., *Hydrangea arborescens* and *H. quercifolia* W. Bartram. Therefore, all possible resolutions of the unsupported branches in the phylogenetic hypothesis (M1-M9, Figure 2.2) were statistically compared using Bayesian inference and the combined plastid dataset with indels coded. The marginal likelihoods for each possible resolution were calculated using the stepping stone algorithm (Xie et al., 2011), as implemented in MrBayes 3.2.2 (Ronquist et al., 2012). For each hypothesis under study, a phylogenetic tree with all major clades constrained to match the phylogenetic hypothesis was used as a prior (Figure 2.2), in accordance with the preferred approach of Bergsten et al. (2013). The stepping stone algorithm was run for 10 million generations over 50 steps, with the first step as burn-in for

four independent runs. The marginal likelihoods for each hypothesis were then compared using Bayes Factors (Kass & Raftery, 1995).

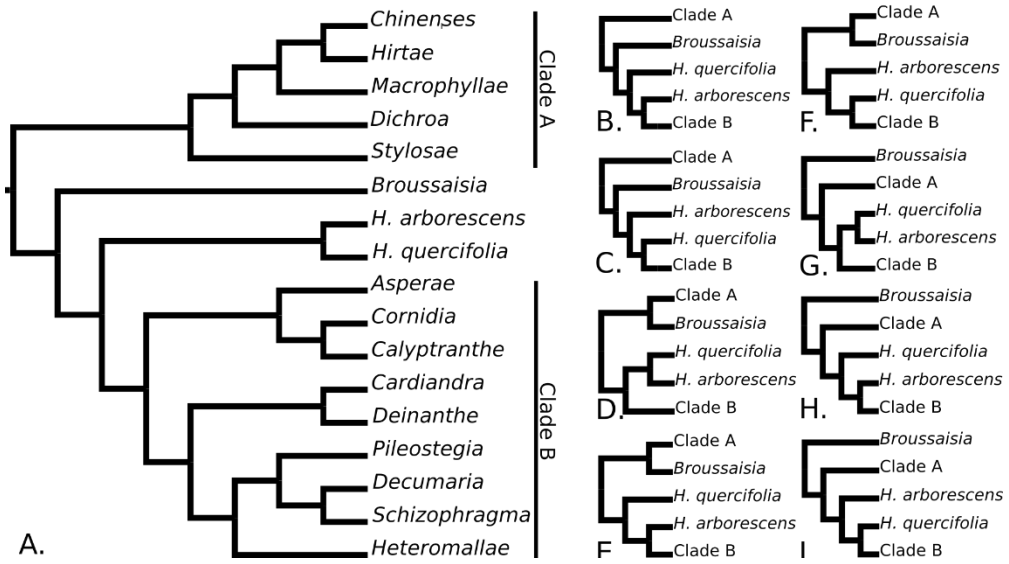


**Figure 2.2: Phylogenetic hypothesis used for Bayesian hypothesis testing.** A: the full tree corresponding to model M1, monophyly of all sections was constrained, as were all depicted nodes. B-I: alternative hypotheses, clade A and B are constrained as depicted in figure 2A, positions of *Broussaisia, H. quercifolia* and *H. arborescens* differ between models (B: model M2, C: model M3, D: model M4, E: model M5, F: model M6, G: model M7, H: model M8, I: Model M9).

### Estimating phylogenetic informativeness

The online application PhyDesign (López-Giráldez & Townsend, 2011) was used to calculate the net phylogenetic informativeness (Townsend, 2007) for each marker used in this study. This calculation used an ultrametric tree generated from the combined plastid and ITS dataset without indel coding. Substitution rates were estimated in HyPhy (Pond et al., 2005). Phylogenetic informativeness profiles for each individual region were compared to the reference ultrametric tree. Maximum net phylogenetic informativeness (PImax) was documented for each separate region, in order to determine the point in time at which each region is phylogenetically most informative.

## Results

### Data matrices

Final alignments for the plastid regions contained 1704, 1553, 1188, 1283 and 664 nucleotide characters for the *rpl32-ndhF* IGS, *trnV-ndhC* IGS, *trnL-rpl32*IGS, *ndhA* intron and ITS region, respectively. Simple indel coding (Simmons & Ochoterena, 2000) resulted in the addition of 112, 90, 76, 72 and 53 binary characters, respectively. The *trnV-ndhC* IGS for *Broussaisia arguta* contained two unique deletions of 169 and 1062 bp, respectively. These deletions were confirmed by resequencing both accessions twice.

Phylogenetic inference

In the plastid combined analysis (concatenated chloroplast nucleotide dataset, including indel data) (Figure 2.3), sect. *Dichroa* is sister to a grade of the monophyletic *Hydrangea* sects. *Macrophyllae*, *Hirtae* and *Chinenses*. Section *Stylosae* is recovered as sister to this entire assemblage, completing a clade congruent with Hydrangea II without *Broussaisia arguta*. This latter taxon is sister to a strongly supported clade (PP: 1) coinciding with Hydrangea I. This sister relationship, however, remains weakly supported (PP: 0.61). Within Hydrangea I, *H. arborescens* and *H. quercifolia* are grouped in a weakly supported clade (PP: 0.52), and are sister to the rest of Hydrangea I. In this major clade, sect. *Pileostegia* is sister to a clade containing the monophyletic sects. *Schizophragma* and *Decumaria*, while sect. *Heteromallae* is sister to this entire assemblage (PP: 0.7). Section *Cardiandra* is recovered as monophyletic and in a sister relationship with a monophyletic sect. *Deinanthe*, while this assemblage is sister to the clade comprising sects. *Heteromallae-Schizophragma-Decumaria-Pileostegia*. All these sections are in turn sister to a clade containing sects. *Asperae*, *Cornidia*, *Calyptranthe* and *Platycrater arguta*. The last is phylogenetically nested within sect. *Asperae*, which in turn is sister (PP: 1) to a clade (PP: 1) containing the two highly supported monophyletic sister sects. *Cornidia* and *Calyptranthe*. Analysis of the indel coded concatenated dataset including the ITS region recovered a similar phylogenetic hypothesis, the only topological difference being the position of *Broussaisia arguta*. This taxon is sister to a well-supported clade (PP: 1) consisting of sects. *Chinenses*, *Hirtae*, *Macrophyllae*, *Dichroa* and *Stylosae*. Furthermore, support for the deeper nodes is reduced by adding ITS to the analysis (Figure 2.4).

Including the data from the simple indel coding scheme generally improved clade support in the Bayesian analysis for the separate regions. Topology was not affected by inclusion of these characters, except for the position of *Broussaisia arguta* in the analysis of the *rpl32-ndhF* IGS

and the concatenated dataset (Figures S2 & S3A). For the *rpl32-ndhF* region, *B. arguta* was sister to the Hydrangea II clade with weak support (PP: 0.82) when only nucleotide data were analyzed (not shown), while this relationship was not recovered when indel data were added to the analysis (Figure S2.2 in Appendix 1). A parallel pattern for this taxon occurred in the combined plastid analysis, with *B. arguta* sister to Hydrangea II for the nucleotide data (PP: 0.80; Figure S2.1 in Appendix 1), and sister to Hydrangea I (PP: 0.62) when indel data were included in the analysis (Figure 2.1). Bayesian analysis of the datasets combining plastid and ITS data recovered *B. arguta* as sister to Hydrangea II (PP: 0.90, not shown) when indels were not coded, while this relationship was not supported when indels were coded (PP: 0.67, Figure 2.4).

Analyses of separate regions did not yield well-supported conflicts. The position of *H. arborescens* and *H. quercifolia* remains unresolved in all single gene trees and the combined analyses. However, these taxa are recovered as part of a well-supported clade with the representatives of Hydrangea I in the combined analyses (with and without indel data, Figures 2.3, 2.4 and S2) and the single gene trees for *rpl32-ndhF* IGS and *trnV-ndhC* (Figure S2.2 in Appendix 1). Phylogenetic hypotheses resulting from the ML analyses did not show any supported topological differences with those generated with Bayesian inference (Figure S2.3 in Appendix 1).

### Hypothesis testing

Comparing the marginal likelihoods obtained from the stepping stone algorithm for each of the nine hypotheses (Figure 2.2) showed four hypotheses (M3-6) to be strongly preferred over the alternatives (Table 2.2). Models placing *Broussaisia arguta* sister to the rest of Hydrangea II are preferred over alternative models with the same configuration for *H. arborescens* and *H. quercifolia*. Between models sharing the same placement of *B. arguta* (Figure 2.2: A, B, C; D, E, F and G, H, I), the model placing *H. quercifolia* sister to the rest of Hydrangea I shows the highest marginal likelihood. Bayes Factor analysis only shows this difference to be strongly supported for model M3 over M2 and M1, and for M9 over M8 and M7.

### Phylogenetic informativeness

The phylogenetic informativeness profiles of all sequenced regions are plotted below the ultrametric tree based on the concatenated dataset with ITS and plastid regions, without indel coding in Figure 2.4. The profile for the ITS region reaches a clear maximum at time 0.35, which is prior to the divergence of tribe Hydrangeeae at time 0.43, and sharply declines towards more ancient times. The plastid regions show lower, flatter profiles, steadily increasing in informativeness towards deeper nodes. Of the plastid regions, the *ndhF-rpl32* IGS reaches the highest informativeness, followed by *trnV-ndhC*, *trnL-rpl32* and finally the *ndhA* intron, respectively.

**Table 2.2: Comparison of the nine different hypotheses presented in Figure 2.2 using Bayes factors.** Bayes factors calculated with the stepping stone algorithm for comparison of the nine alternative phylogenetic hypotheses presented in Fig. 2, with H1 in the first column, and H2 in the top row. Values > 3 but < 10 signify a significant support for H1 over H2, values > 10 signify strong support for H1 over H2 (Jeffreys, 1961).

| | **M1** | **M2** | **M3** | **M4** | **M5** | **M6** | **M7** | **M8** | **M9** |
|---|---|---|---|---|---|---|---|---|---|
| **M1** | 1.00 | 12.68 | 0.01 | 0.02 | 0.02 | 0.01 | 34.47 | 32.79 | 1.27 |
| **M2** | 0.08 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 2.72 | 2.59 | 0.10 |
| **M3** | 79.84 | 1012.32 | 1.00 | 1.23 | 1.62 | 0.51 | 2751.77 | 2617.57 | 101.49 |
| **M4** | 64.72 | 820.57 | 0.81 | 1.00 | 1.31 | 0.41 | 2230.54 | 2121.76 | 82.27 |
| **M5** | 49.40 | 626.41 | 0.62 | 0.76 | 1.00 | 0.32 | 1702.75 | 1619.71 | 62.80 |
| **M6** | 156.02 | 1978.31 | 1.95 | 2.41 | 3.16 | 1.00 | 5377.61 | 5115.34 | 198.34 |
| **M7** | 0.03 | 0.37 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 0.95 | 0.04 |
| **M8** | 0.03 | 0.39 | 0.00 | 0.00 | 0.00 | 0.00 | 1.05 | 1.00 | 0.04 |
| **M9** | 0.79 | 9.97 | 0.01 | 0.01 | 0.02 | 0.01 | 27.11 | 25.79 | 1.00 |

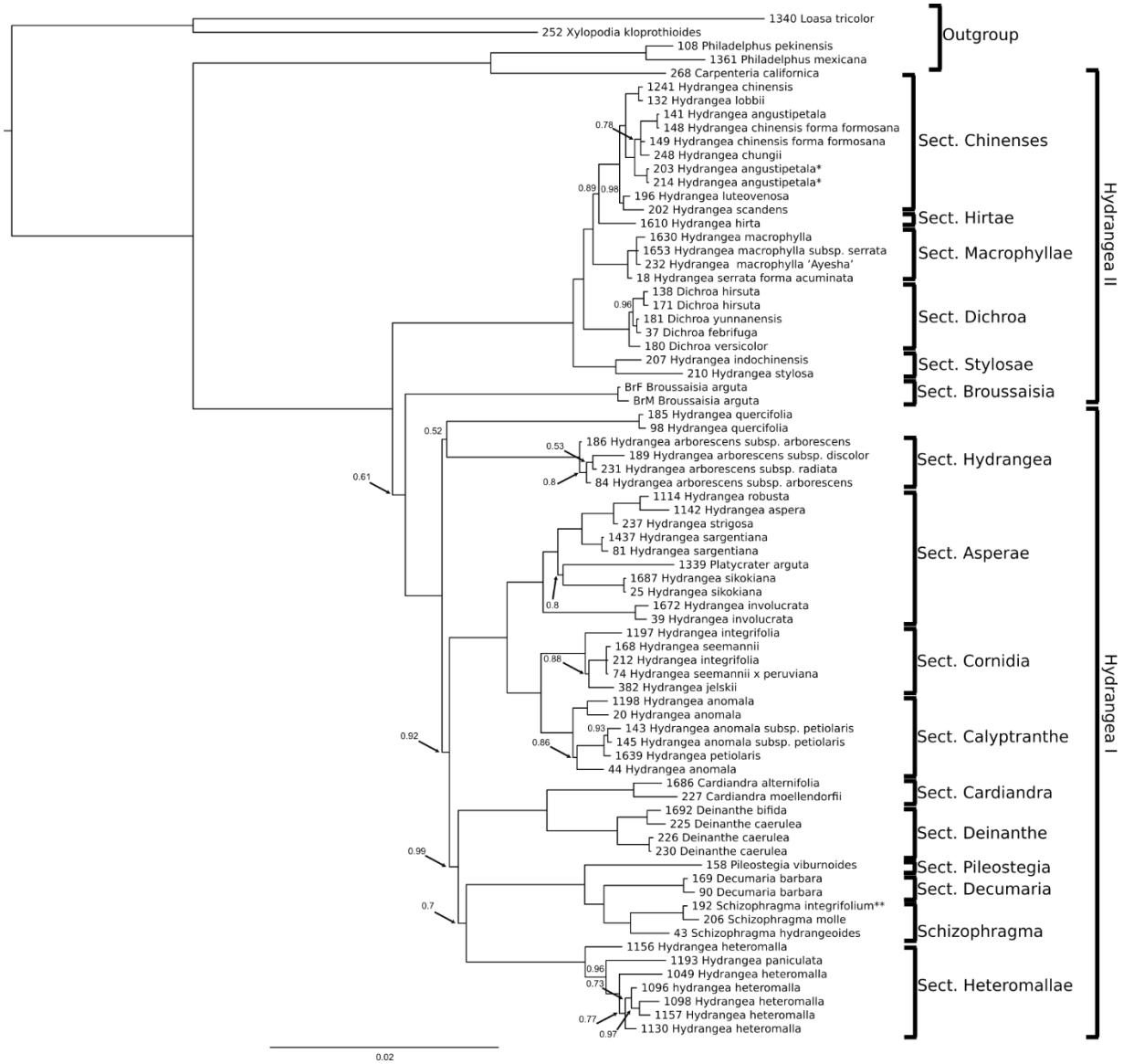


**Figure 2.3: Phylogenetic hypothesis based on plastid dataset.** The 50% majority rule consensus tree based on the combined plastid dataset with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. Section names according to the new infrageneric classification presented here. *Hydrangea angustipetala** = *Hydrangea angustipetala* forma *macrosepala*. *Schizophragma integrifolium*** = *Schizophragmaintegrifolium* var. *fauriei*.

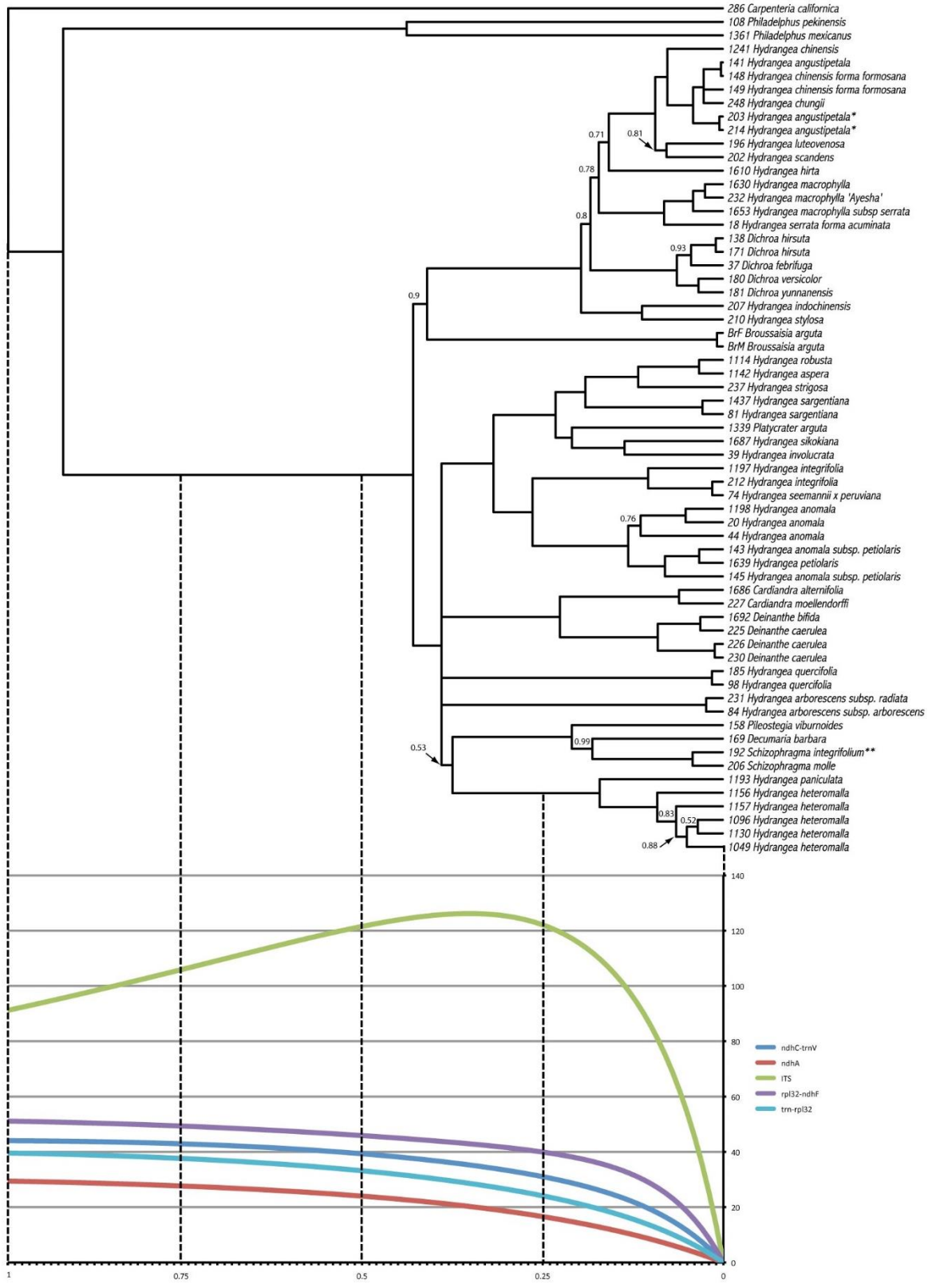


**Figure 2.4: Phylogenetic informativeness plot.** Net phylogenetic informativeness across time for all four sequenced regions, plotted against the ultrametric phylogenetic tree based on ITS and plastid sequences, excluding indel data. Posterior probabilities for drawn branches only displayed if below 1.

## Discussion

### Generic relationships, congruences and conflicts in tribe Hydrangeeae.

This study presents the most comprehensive phylogenetic hypothesis for tribe Hydrangeeae to date. Single gene trees for the ITS region (Figure S2.2E in Appendix 1) showed the same major clades as the chloroplast markers. Resolution for the deeper nodes remained much lower than in the combined plastid analysis. Furthermore, inclusion of ITS into the concatenated analysis drastically reduced support for evolutionary relationships among large clades (sections) within Hydrangea I (Figure 2.4). The inclusion of the ITS data therefore introduced noise into the dataset, as can be deduced from the phylogenetic informativeness profile in Figure 2.4. The maximum phylogenetic informativeness of ITS is reached more recently (t=0.34) than the divergence of the major clades in Hydrangea I. This region was therefore fairly uninformative for resolving evolutionary relationships prior to this time, as more recent changes in its sequence might obscure signals that have arisen within the time interval of the divergence of these major Hydrangea I lineages (Townsend, 2007). The more uniform informativeness profiles of the plastid markers, the better suited they are for resolving deeper nodes in tribe Hydrangeeae. Consequently, the new classification presented here is discussed using the phylogenetic tree based on the concatenated chloroplast regions (Figure 2.3), as this is the most complete dataset, with best support for relationships among sections. In this phylogenetic hypothesis, the morphologically diverse genera *Broussaisia*, *Cardiandra, Decumaria*, *Deinanthe, Dichroa, Pileostegia, Platycrater* and *Schizophragma* were recovered as monophyletic, but nested within the larger polyphyletic *Hydrangea* (Figure 2.1). These findings were in general agreement with earlier studies (Samain et al., 2010, Granados Mendoza et al., 2013). A combined analysis of 13 chloroplast regions by Granados Mendoza et al. (2013) recovered *H. quercifolia* in a grade with *H. arborescens* and a clade containing sect. *Asperae* (plus *Platycrater*) as sister to the sister sects. *Calyptranthe* and *Cornidia*. The short branch subtending *H. arborescens*, however, remained unsupported in Granados Mendoza et al. (2013). In the present study, phylogenetic placement of *H. arborescens* and *H. quercifolia* was only partly resolved (with low support) for the combined plastid dataset with indels coded and both analyses of the *rpl32-ndhF* IGS (Figure S2.2A in Appendix 1). Furthermore, the Bayesian test of phylogenetic hypotheses did not prefer one configuration of these taxa over

alternative configurations. The reason for this absence of resolution is the presence of deep, short branches connecting the two North American taxa to the rest of the tribe, combined with long branches subtending these monophyletic species. Resolving such short branches positioned deep in a phylogeny is considered a difficult endeavour (Townsend & Leuenberger, 2011), and requires multiple genes of high phylogenetic signal and demonstrated absence of incongruence (Salichos & Rokas, 2013), or loci highly informative on that specific time scale (Townsend, 2007). Moreover, resolving the position of *H. arborescens* is of pivotal importance as this taxon is the type species of *Hydrangea*.

A second conflict between the present and previous studies was the position of the Hawaiian endemic *Broussaisia arguta*. The phylogenetic hypothesis generated by Samain et al. (2010) placed this taxon sister to Hydrangea II with high support (bootstrap 96, PP: 0.98). The current study, however, recovered a weakly supported sister relationship (PP: 0.62) between *B. arguta* and Hydrangea I in the plastid concatenated analysis incorporating indel data, while *B. arguta* was sister to Hydrangea II (PP: 0.80) when indels were not coded. When ITS was added to the concatenated dataset, *B. arguta* was recovered as sister to Hydrangea II whether or not indel data were included, although higher support was achieved with the inclusion of indel data (PP: 0.90 compared to 0.67; Figure 2.4). Comparison of marginal likelihoods for the different positions of *B. arguta* (Figure 2.2, Table 2.2) preferred the sister relationship with Hydrangea II over the alternative positions, which is congruent with the results shown in Samain et al. (2010). The contrasting position of *B. arguta* in the phylogenetic analysis of the concatenated data with indels coded might therefore be heavily influenced by the presence of large indels within the *trnV-ndhC* IGS. The long branches subtending this species might indicate an accelerated rate of molecular change, obscuring the evolutionary relationships of *Broussaisia*. A similar pattern was recovered in the Cornales family Hydrostachyaceae (Xiang et al., 1998; Xiang, 1999; Fan & Xiang, 2003; Xiang et al., 2011), where the difficulties of reconstructing relationships in this group were suggested to be caused by an acceleration of evolution in molecular and morphological characters. Shifts into novel environments, followed by selection, increased mutation rates and genetic drift were suggested as likely to have caused this accelerated accumulation of variation. Similarly, the long branches subtending *B. arguta*, as well as its deviating molecular sequences might be caused by its isolated geographic location, as the only member of tribe Hydrangeeae endemic to the Hawaiian Islands.

From a polyphyletic *Hydrangea* s.s. to a monophyletic *Hydrangea* s.l.

Unraveling the polyphyletic nature of *Hydrangea* is a necessity, as neither of the large schools of systematics accepts polyphyletic taxa (Hörandl & Stuessy, 2010; Schmidt-Lebuhn, 2012). Phylogenetic hypotheses resulting from the present study suggest two possible resolutions: 1) creating new genera to accommodate monophyletic groups of *Hydrangea* not directly related to the type *H. arborescens*, retaining the eight satellite genera as separate entities, or, 2) including the eight satellite genera into *Hydrangea*, creating a broadly described, monophyletic *Hydrangea* s.l. The first approach would entail splitting *Hydrangea*, with the description of minimally seven new genera, of which two would be monotypic. Furthermore, splitting *Hydrangea s.s.* would result in morphologically very similar taxa which would be very difficult to distinguish. Several degrees of splitting can be proposed, depending on the acceptance of monotypic and paraphyletic genera. For example, in order to retain the genus *Platycrater*, McClintock's subsect. *Asperae* would have to be split into three genera, two of them monotypic. The second approach entails the creation of a large genus *Hydrangea*, containing all species of the eight satellite genera, among which several taxa would require new specific epithets. Furthermore, the newly created *Hydrangea* s.l. would display wide variation in morphology, losing the practicability of classifying morphologically aberrant taxa as separate (satellite) genera.

It is argued here that a splitting approach, creating several new genera, would complicate Hydrangeeae taxonomy, resulting either in a large amount of monotypic genera or multiple morphologically very variable, and hence potentially unrecognizable, taxa. Furthermore, small changes in relationships between clades potentially recovered in future studies may possibly require new changes in number and configuration of genera. Therefore, a broad circumscription of *Hydrangea* to include *Broussaisia*, *Cardiandra, Decumaria*, *Deinanthe, Dichroa, Pileostegia, Platycrater* and *Schizophragma* would best serve the science of taxonomy, in creating a stable classification.

We do recognize the point made by evolutionary systematists that a classification should carry information about similarities between its constituents. Therefore, a new infrageneric classification is proposed, which is expected to facilitate the acceptance of the taxonomical changes in horticulture. By circumscribing the previous satellite genera as distinct sections,

these entities remain recognizable for the broader public, with already well-known names, albeit at a different taxonomic level.

**Taxonomic treatment**

***Hydrangea*** L., Sp. Pl. 1: 397. 1753 – Type: *Hydrangea arborescens* L.

= *Decumaria* L., Sp. Pl. (ed. 2) 2: 1663. 1763 – Type: *Decumaria barbara* L.

= *Dichroa* Lour., Fl. Cochinch. 1: 301. 1790 – Type: *Dichroa febrifuga* Lour.

= *Broussaisia* Gaudich., Voy. Uranie: 479. 1830 – Type: *Broussaisia arguta* Gaudich.

= *Schizophragma* Siebold & Zucc., Fl. Jap. 1: 58. 1838 – Type: *Schizophragma hydrangeoides* Siebold & Zucc.

= *Platycrater* Siebold & Zucc., Fl. Jap. 1: 62. 1838 – Type: *Platycrater arguta* Siebold & Zucc.

= *Cardiandra* Siebold & Zucc., Fl. Jap. 1: 119. 1839 – Type: *Cardiandra alternifolia* (Siebold) Siebold & Zucc.

= *Pileostegia* Hook. f. & Thomson, J. Proc. Linn. Soc., Bot. 2: 57. 1858 – Type: *Pileostegia viburnoides* Hook. f. & Thomson.

= *Deinanthe* Maxim., Mém. Acad. Imp. Sci. Saint Pétersbourg, Sér. 7 ser. 7. 10(16): 2. 1867– Type: *Deinanthe bifida* Maxim.

A new infrageneric classification of *Hydrangea*, including new sections and combinations

The eight satellite genera of *Hydrangea* are recognized as distinct sections, with the exception of *Platycrater*, which is placed in sect. *Asperae* in order to avoid the creation of a polyphyletic *Asperae*. The subsections in the classification of McClintock (1957) are raised to section level. Assignment of all currently recognized Hydrangeeae species names to their respective section is provided in Appendix 1, Table S2.2.

**1. *Hydrangea* sect. *Asperae*** (Rehder) Y. De Smet & Samain, **stat. nov. ≡** *Hydrangea* subsect. *Asperae* Rehder, Plantae Wilsonianae. 1: 39. 1911 – Type: *Hydrangea aspera* D. Don.

***Hydrangea platyarguta*** Y. De Smet & C. Granados, **nom. nov**. for *Platycrater arguta* Siebold & Zucc., Fl. Jap. 1: 64. 1835, non *Hydrangea arguta* (Gaudich.) Y. De Smet & C. Granados (this paper).

**2. *Hydrangea* sect. *Broussaisia*** (Gaudich.) Y. De Smet & Samain, **comb. et stat. nov.**

**≡** *Broussaisia* Gaudich., Voy. Uranie 479. 1830 – Type: *Hydrangea arguta* (Gaudich.) Y. De Smet & C. Granados, **comb**. **nov**. ≡ *Broussaisia arguta* Gaudich., Voy. Uranie 479, t. 69. 1830.

**3. *Hydrangea* sect. *Calyptranthe*** Maxim., Mém. Acad. Imp. Sci. Saint Pétersbourg, Sér. 7, 10 (16): 6. 1867 – Lectotype (designated here): *Hydrangea scandens* Maxim., Mém. Acad. Imp. Sci. Saint Pétersbourg, Sér. 7, 10(16): 16. 1867.

Maximowicz assigned two species to this section; *Hydrangea scandens* Maxim. (newly described) and *Hydrangea altissima* Wallich (with a short note). Both names are now considered synonyms of *Hydrangea anomala* D. Don (1825) sensu lato.

**4. *Hydrangea* sect. *Cardiandra*** (Siebold & Zucc.) Y. De Smet & Samain, **comb. et stat. nov. ≡** *Cardiandra* Siebold & Zucc., Fl. Jap. 1: 119. 1839 – Type: *Hydrangea alternifolia* Siebold, Nov. Act. Nat. Cur. 14(2): 692. 1829.

***Hydrangea amamiohsimensis*** (Koidz.) Y. De Smet & C. Granados, **comb. nov. ≡** *Cardiandra amamiohsimensis* Koidz., Pl. Nov. Amami-Ohsim. 10. 1928.

***Hydrangea densifolia*** (C.F. Wei) Y. De Smet & C. Granados, **comb. nov. ≡** *Cardiandra densifolia* C.F. Wei., Acta Bot. Austro Sin., 10: 9, f. 1. 1995.

= *Cardiandra formosana* Hayata, Bot. Mag. (Tokyo) 20(231): 54–55. 1906, *non Hydrangea formosana* Koidz., Bot. Mag. Tokyo. 43: 394. 1929.

**5. *Hydrangea* sect. *Chinenses*** Y. De Smet & Samain, **sect. nov. –** Type: *Hydrangea chinensis* Maxim., Mém. Acad. Imp. Sci. Saint Pétersbourg, Sér. 7, 10(16): 16. 1867 = *Hydrangea* sect. *Petalanthae* Maximowicz (1867: 6), *nom. illeg*., including the type species of the genus.

Small shrubs with rather small and narrow leaves, inflorescences rather numerous, scattered over many branchlets, with enlarged marginal flowers

Section *Petalanthae* as proposed by Maximowicz (1867) is illegitimate, as it contains the type species of *Hydrangea*. Here this section is renamed as sect. *Chinenses*.

**6. *Hydrangea* sect. *Cornidia*** (Ruiz & Pav.) Engl., Nat. Pflanzenfam. 3(2a): 76. 1891 ≡ *Cornidia* Ruiz & Pav., Fl. Peruv. Prodr. 53, pl. 35. 1794 – Type: *Hydrangea preslii* Briq. Annuaire Conserv. Jard. Bot. Genève 20: 40–410. 1919.

**7. *Hydrangea* sect. *Decumaria*** (L.) Y. De Smet & Samain**, comb. et stat. nov.** ≡*Decumaria* L., Sp. Pl. (ed. 2) 2: 1663. 1763 – Type: *Hydrangea barbara* (L.) B. Schulz, Gehölzbestimmung im Winter: 285. 2013.

***Hydrangea obtusifolia*** (Hu) Y. De Smet & C. Granados, **comb. nov.** ≡ *Schizophragma obtusifolium* Hu., Bull. Fan Mem. Inst. Biol. 5: 309. 1934 = *Decumaria sinensis* Oliv., Hooker's Icon. Pl. 18(2): pl. 1741. 1888, *non Hydrangea chinensis* Maxim., Mém. Acad. Imp. Sci. Saint Pétersbourg, Sér. 7, 10(16): 7. 1867.

**8. *Hydrangea* sect. *Deinanthe* (Maxim.)** Y. De Smet & Samain, **comb. et stat. nov.**

≡ *Deinanthe* Maxim., Mém. Acad. Imp. Sci. Saint Pétersbourg, Sér. 7, 10(16): 2. 1867

– Type: *Hydrangea bifida* (Maxim.) Y. De Smet & C. Granados, **comb. nov.** ≡ *Deinanthe bifida* Maxim., Mém. Acad. Imp. Sci. Saint Pétersbourg, Sér. 7, 10(16): 3. 1867.

***Hydrangea caerulea*** (Stapf) Y. De Smet & C. Granados, **comb. nov.** ≡ *Deinanthe caerulea* Stapf, Bot. Mag. 137, t. 8373. 1911.

**9. *Hydrangea* sect. *Dichroa*** (Lour.) Y. De Smet & Samain, **comb. et stat. nov.** ≡

*Dichroa* Lour., Fl. Cochinch. 1: 301. 1790 – Type: *Hydrangea febrifuga* (Lour.) Y. De Smet & C. Granados, **comb**. **nov**. ≡ *Dichroa febrifuga* Lour., Fl. Cochinch. 1: 301. 1790.

***Hydrangea hirsuta*** (Gagnep.) Y. De Smet & C. Granados, **comb. nov.** ≡ *Dichroa hirsuta* Gagnep. in Lecomte, Fl. Indo-Chine 2: 690. 1920.

***Hydrangea mollissima*** (Merr.) Y. De Smet & C. Granados, **comb. nov.** ≡ *Dichroa mollissima* Merr., Philipp. J. Sci. 23(3): 245. 1923.

***Hydrangea yaoshanensis*** (Y.C. Wu) Y. De Smet & C. Granados, **comb. nov.** ≡ *Dichroa yaoshanensis* Y.C. Wu., Bot. Jahrb. Syst. 71(2): 180. 1940.

***Hydrangea daimingshanensis*** (Y.C. Wu) Y. De Smet & C. Granados, **comb. nov.** ≡ *Dichroa daimingshanensis* Y.C. Wu., Bot. Jahrb. Syst. 71(2): 179. 1940.

**10. *Hydrangea* sect. *Heteromallae*** (Rehder) C.F.Wei, Guihaia 14(2): 111. 1994 ≡ *Hydrangea* subsect. *Heteromallae* Rehder, Plantae Wilsonianae 1: 37. 1911 – Type: *Hydrangea heteromalla* D. Don, Prodr. Fl. Nepal.: 211. 1825.

**11. *Hydrangea* sect. *Hirtae*** Y. De Smet & Samain, **sect. nov. –** Type: *Hydrangea hirta* (Thunb.) Siebold, Flora 11: 757. 1828 ≡ *Viburnum hirtum* Thunb., Fl. Jap.: 124. 1784.

Small shrubs with conspicuously dentate leaves, inflorescence a compact corymb, on a short peduncle, and enlarged marginal flowers absent.

**12. *Hydrangea* sect. *Hydrangea*** – Type: *H. arborescens* L., Sp. Pl. 1: 397. 1753.

The type species of *Hydrangea*, *H. arborescens*, was classified in subsect. *Americanae* (McClintock, 1957), together with another North American species, *H. quercifolia*. In this classification, sect. *Hydrangea* only consists of the morphologically very variable *H. arborescens*, while *H. quercifolia* remains unclassified. The latter is due to the unresolved relationships of this taxon in all phylogenetic hypotheses published to date.

**13. *Hydrangea* sect. *Macrophyllae*** (E.M. McClint.) Y. De Smet & Samain, **stat. nov.** ≡ *Hydrangea* subsect. *Macrophyllae* E. M. McClint., J. Arnold Arbor. 37: 374. 1956 – Type: *Hydrangea macrophylla* (Thunb.) Ser., Prodr. 4: 15. 1830 ≡ *Viburnum macrophyllum* Thunb., Fl. Jap.: 125. 1784.

In accordance with previous studies (Samain et al., 2010), subsect. *Macrophyllae* as recognized by McClintock (1957) was recovered here as polyphyletic, forming two well-supported clades. The clade containing *Hydrangea macrophylla* will remain as *Macrophyllae*, raised from

subsection to section level. For the other clade, containing *H. indochinensis* and *H. stylosa*, a new name is provided (see below).

**14. *Hydrangea* sect. *Pileostegia*** (Hook. f. & Thomson) Y. De Smet & Samain, **comb. et stat. nov.** ≡ *Pileostegia* Hook. f. & Thomson, J. Proc. Linn. Soc., Bot. 2: 57. 1858 – Type: *Hydrangea viburnoides* (Hook. f. & Thomson) Y. De Smet & C. Granados, **comb.nov**. ≡ *Pileostegia viburnoides* Hook. f. & Thomson, J. Proc. Linn. Soc. 2: 76, pl. 2. 1858.

***Hydrangea tomentella*** (Hand.-Mazz.) Y. De Smet & C. Granados, **comb. nov.** ≡ *Pileostegia tomentella* Hand.-Mazz., Akad. Wiss. Wien, Math.-Naturwiss. Kl. Anz. 59: 55. 1922.

**15. *Hydrangea* sect. *Schizophragma*** (Siebold & Zucc.) De Smet & Samain, **comb. et stat. nov.** ≡ *Schizophragma* Siebold & Zucc., Fl. Jap. 1: 58. 1838 – Type: *Hydrangea hydrangeoides* (Siebold & Zucc.) B. Schulz, Gehölzbestimmung im Winter: 285. 2013 ≡ *Schizophragma hydrangeoides* Siebold & Zucc., Fl. Jap.1: 59, pl. 26. 1835.

***Hydrangea ampla*** (Chun) Y. De Smet & C. Granados, **comb. nov.** ≡ *Schizophragma amplum* Chun, Acta Phytotax. Sin. 3(2): 165–166. 1954.
= *Schizophragma integrifolium* Oliv., Hook. Icon. Pl. 20(2): pl. 1934. 1890, *non Hydrangea integrifolia* Hayata, J. Coll. Sci. Imp. Univ. Tokyo 22: 131. 1906.

***Hydrangea corylifolia*** (Chun) Y. De Smet & C. Granados, **comb. nov.** ≡ *Schizophragma corylifolium* Chun, Acta Phytotax. Sin. 3(2): 170–172, pl. 21. 1954.

***Hydrangea crassa*** (Hand.-Mazz.) Y. De Smet & C. Granados, **comb. nov.** ≡ *Schizophragma crassum* Hand.-Mazz., Akad. Wiss. Wien, Math.-Naturwiss. Kl., Anz. 59: 247. 1922.

*Hydrangea fauriei* (Hayata) Y. De Smet & C. Granados **comb. nov.** ≡ *Schizophragma fauriei* Hayata, J. Coll. Sci. Imp. Univ. Tokyo 22: 131. 1906.

***Hydrangea glaucescens*** (Rehder) Y. De Smet & C. Granados, **comb. nov.** ≡ *Schizophragma glaucescens* (Rehder) Chun, Acta Phytotax. Sin. 3: 166. 1954

= *Schizophragma hypoglaucum* Rehder, Sargent, Plantae Wilsonianae 1: 43. 1911, *non Hydrangea hypoglauca* Rehder, Plantae Wilsonianae 1(1): 26. 1911.

***Hydrangea schizomollis*** Y. De Smet & C. Granados, **nom. nov.** for *Schizophragma integrifolia* var. *molle* Rehder, Plantae Wilsonianae 1: 42. 1911, *non Hydrangea mollis* (Rehder) W.T. Wang, Bull. Bot. Res., Harbin 1(1–2): 54. 1981. ≡ *Hydrangea heteromalla* var. *mollis* Rehder Plantae Wilsonianae. 1: 151. 1912.

**16. *Hydrangea* sect. *Stylosae*** Y. De Smet & Samain, **sect. nov.** – Type: *Hydrangea stylosa* Hook. F. & Thomson, J. Proc. Linn. Soc., Bot. 2: 75. 1857.

Small shrubs with rather small and narrow leaves, inflorescences with enlarged marginal flowers, their sepals conspicuously dentate, capsules globose, with usually 4 prominent styles.

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**Chapter III**

# Multilocus coalescent species delimitation to evaluate traditionally defined morphotypes in *Hydrangea* sect. *Asperae* (Hydrangeaceae)

*"Systematists will have only to decide (not that this will be easy) whether any form be sufficiently constant and distinct from other forms, to be capable of definition; and if definable, whether the differences be sufficiently important to deserve a specific name."*

Charles Darwin (1809-1882)

**Abstract**

The number of species recognized in section *Asperae* of the flowering plant genus *Hydrangea* differs widely between subsequent revisions. This variation is largely centered around the *H. aspera* species complex, with numbers of recognized species varying from one to nearly a dozen. Despite indications of molecular variation in this complex, no sequence-based species delimitation methods have been employed to evaluate the primarily morphology-based species boundaries. In the present study, a multi-locus coalescent based approach to species delimitation is employed in order to identify separate evolutionary lines within *H.* sect. *Asperae*, using four chloroplast and four nuclear molecular markers. This algorithm supports eight lineages within the focal group, of which five correspond with named morphotypes. The other three lineages illustrate different types of conflict between molecular species delimitation and traditional morphology-based taxonomy. One molecular lineage represents two named morphotypes, which possibly diverged recently enough to not have developed sufficient molecular divergence. A second conflict is found in *H. strigosa*. This morphotype is recovered as a separate lineage when occurring in geographic isolation, but when occurring in sympatry with two other morphotypes (*H. aspera* and *H. robusta*), the coalescent species delimitation lumps these taxa into a single putative species.

Adapted from: De Smet, Y., De Clerck, O., Uemachi, T., Granados Mendoza, C., Wanke, S., Goetghebeur, P., Samain, M.-S. 2017. Multilocus coalescent species delimitation to evaluate traditionally defined morphotypes in *Hydrangea* sect. *Asperae* (Hydrangeaceae). Molecular Phylogenetics and Evolution 114: 415-425.

**Introduction**

Species are held to be fundamental biological units, on par in importance with fundamental units at lower levels of organization such as cells and organisms (Mayr, 1982). Despite the importance of the species category, the second half of the 20th century has seen widespread controversy concerning its definition. However, since the publication of Darwin's "On the Origin of Species", all species concepts formulated within an evolutionary worldview have shared a common central idea. This core idea can be traced back with a few minor modifications to Darwin's own vision of species as branches in the lines of descent (de Queiroz, 2011). The proliferation of species concepts, however, originated from the idea that these lines of descent need to develop a specific property in order to be recognized as species ("species criterion", e.g. reproductive isolation, reciprocal monophyly, etc.). In an attempt to create a unified species concept, de Queiroz (1998, 1999, 2007) proposed to eliminate these species criteria, effectively reducing the alternative species concept to their common denominator: the evolutionary component first proposed by Darwin. Under this unified species concept, species are independently evolving metapopulation lineages. These lineages may or may not develop the properties used to delimit species in previous species concepts (e.g. reproductive isolation, distinct ecological niche, etc.) in the early stages of divergence. Moreover, these properties underlying the differences between alternative species concepts remain important in this unified species concept in at least three ways (de Queiroz, 2011). First, all of these properties represent different lines of evidence to recognize certain entities as separately evolving lineages. Secondly, explicitly mentioning the properties that differ between a set of recognized species can offer insights into the processes that cause or maintain lineages separation. Finally, these secondary properties can be used to distinguish subcategories of the species category based on the species criteria they satisfy, resulting in more objective and informative subcategories.

Despite the conceptual elegance of this unified species concept, contrasting different types of data can be challenging. Most, if not all, operational criteria for species delimitation are prone to misinterpret species diversity in certain circumstances. The often-used operational criterion of reciprocal monophyly, for example, is prone to misinterpretation of evolutionary lines due to incomplete lineage sorting (Maddison, 1997) or introgressive hybridization (Nosil et al.,

2009). Because of these difficulties associated with molecular data, many species-delimitation studies have turned to methods for analyzing DNA sequence data in a coalescent-based framework, capable of accounting for confounding processes such as incomplete lineage sorting (ILS; Bagley et al., 2015). The algorithm for species validation implemented in the Bayesian Phylogenetics and Phylogeography program (BP&P; Yang & Rannala, 2010), for example, tests different species hypotheses based on a species tree. The latter is generated from a sample of multiple, unlinked molecular markers, allowing for gene tree incongruence caused by ILS. Generation of gene trees or guide trees, however, generally requires an a priori assignment of individuals to species (but see: Bryant et al., 2012). The majority of studies employing Bayesian algorithms for species delimitation seem to focus on morphologically cryptic radiations, validating molecularly divergent, but morphologically similar lineages as separate species. In this study, however, we aim to utilize a coalescent approach to species delimitation in a species complex consisting of several morphotypes of uncertain species status. This approach, i.e. comparing traditional morphological species delimitations with a molecular-based species hypothesis has the advantage of potentially validating morphological characters useful for identifying molecularly diverged lineages. Such diagnostic characters are highly valuable, for instance, in the identification of threatened or commercially valuable independent lineages.

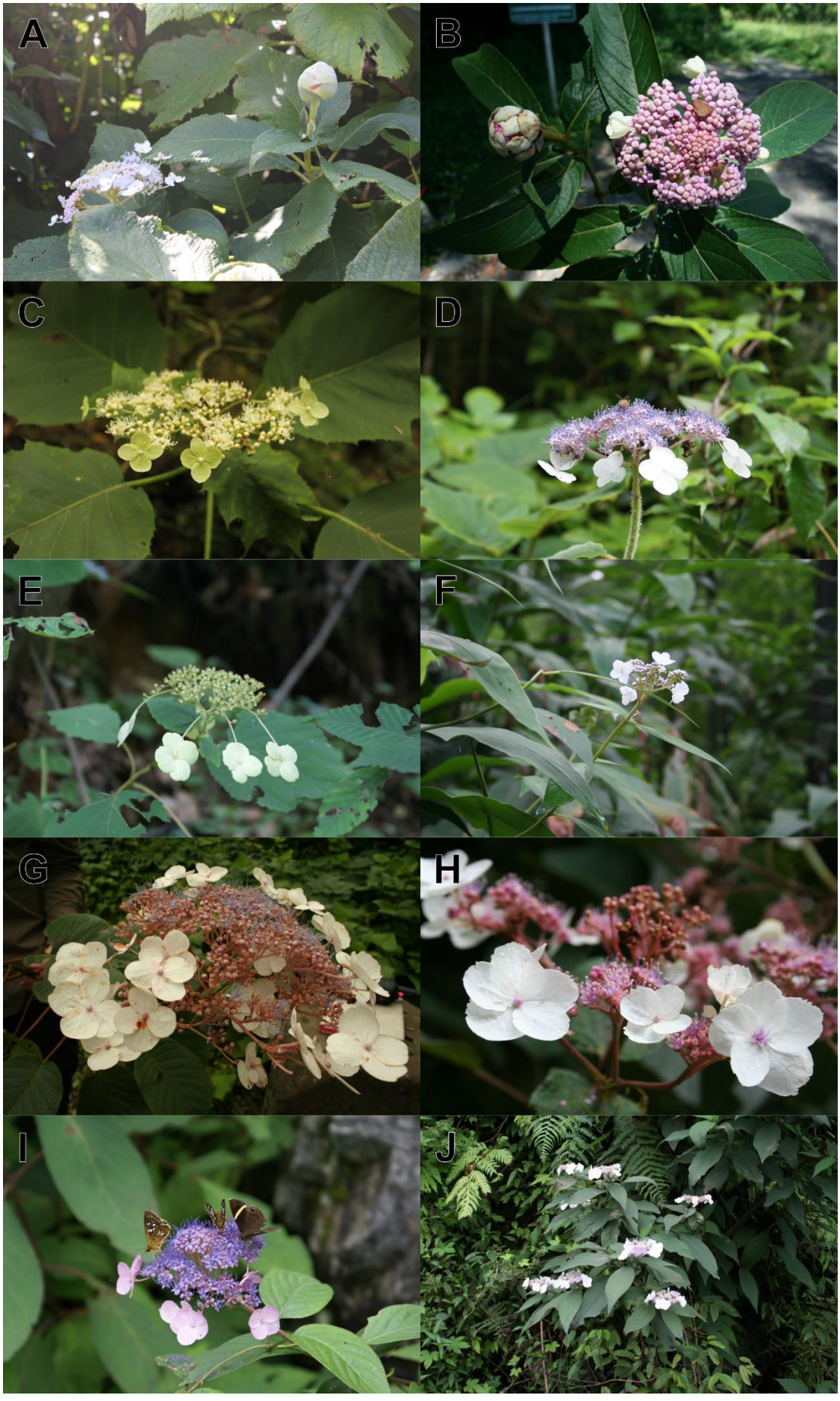
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**Figure 3.1 (previous page): General morphology for representatives of sect. *Asperae*.** A: *Hydrangea involucrata* (Japan), B: *H. longifolia* (Taiwan), C: H. *sikokiana* (Japan), D: *H. sargentiana* (Hubei, China), E: *H. longipes* (Hubei, China), F: *H. strigosa* (Hubei, China), G: *H. robusta* (Sichuan, China), H: *H. kawakamii* (Taiwan), I: *H. villosa* (Hubei, China), J: *H. aspera* (Sichuan, China).

Species circumscription and identification is notoriously difficult in the genus *Hydrangea* L., with widely varying numbers of species recognized by different authors (e.g. McClintock, 1957: 24 worldwide; Wei & Bartholomew, 2001: 33 only in China). The previously paraphyletic *Hydrangea* (Samain et al., 2010; Granados Mendoza et al., 2013) was recently rendered monophyletic by expanding its circumscription to include eight closely related genera (De Smet et al., 2015a). Furthermore, a new infrageneric classification, supported by morphological and molecular data was proposed, consisting of 16 monophyletic sections. The focal group of this study, *Hydrangea* section *Asperae* (Rehder) Y.De Smet & Samain (hereafter named sect. *Asperae*), is distributed throughout eastern and southeastern Asia, with the highest diversity in central China. Most revisions addressing the genus *Hydrangea* agree on the recognition of the Japanese and Taiwanese representatives of sect. *Asperae* as separate species, owing to their distinct morphology (Figure 3.1 1: A, B & C). The remaining nominal taxa constitute the *H. aspera* Buch.-Ham. ex D.Don species complex, within which species boundaries have been unclear. According to McClintock (1957), this complex represents a single, wide-spread species, *H. aspera*. Moreover, she proposed four subspecies, based on the pubescence of the abaxial leaf surface, and the shape of petioles and leaves: *H. aspera* subsp. *aspera*, *H. aspera* subsp. *strigosa*, *H. aspera* subsp. *robusta* and *H. aspera* subsp. *sargentiana*. In contrast, other classifications (e.g. Wei & Bartholomew, 2001) recognize these subspecies and several other nominal taxa as distinct species, splitting the *H. aspera* complex into eight (Wei in: Wei & Bartholomew, 2001) or nine (Bartholomew in: Wei & Bartholomew, 2001) species. These nominal taxa (morphospecies) differ greatly in their ecology and geographic distribution. Some can be found across a wide geographic area (*H. strigosa* Rehder) while others are only known from a single location (*H. sargentiana* Rehder). Furthermore, several members of sect. *Asperae* occur in sympatry while others remain strongly geographically isolated, such as *H. kawakamii* Hayata, endemic to the island of Taiwan. As is often the case for purely morphology-based classifications, the difference in number of recognized species in the *H. aspera* complex hinges on differential emphasis on certain morphological characters for species identification. This uncertainty regarding species boundaries is exacerbated by the

lack of knowledge regarding molecular variation and therefore evolutionary relationships within sect. *Asperae*. However, two cytogenetic studies (Cerbah et al., 2001; Mortreau et al., 2010) have demonstrated variation in the genomic organization among members of sect. *Asperae*. While *Hydrangea* species typically present a chromosome number of 2n=36, most members of sect. *Asperae* have 2n=34, with the exception of *H. involucrata* Siebold (2n=30). Furthermore, studying the chromosomal organization of the subspecies recognized by McClintock, Mortreau et al. (2010) found that a subset of specimens in *H. aspera* subsp. *aspera* to which they refer as the "kawakamii-group" shows a chromosome number 2n=36. The authors therefore suggest that *H. aspera* subsp. *aspera* can be split into two taxa, coinciding with the described species *H. villosa* Rehder and *H. kawakamii*, based on differing chromosome organization.

The unclear taxonomic status of distinct morphotypes, showing different geographic distributions and genomic organization render sect. *Asperae* an ideal candidate to evaluate the capability of coalescent-based species delimitation to stabilize taxonomy in difficult groups. To this end, this study compares the evolutionary lineages proposed by a multilocus coalescent-based species delimitation algorithm (Yang & Rannala, 2010) with species boundaries proposed by strict monophyly and the most recent morphological species delimitation in sect. *Asperae* (Wei & Bartholomew, 2001). Furthermore, the potential of leaf pubescence to discriminate between evolutionary lineages in this section will be evaluated, as this is one of the main morphological characters both traditionally and recently employed to distinguish between sect. *Asperae* morphotypes.

**Material and methods**

Taxon sampling and initial morphological identification

This study included 29 specimens identified as representatives of sect. *Asperae* and one species from its sister clade *Hydrangea* sect. *Cornidia* as outgroup. Most of these specimens were collected in China (provinces of Sichuan and Hubei) and Japan in 2011 and 2012. Other samples were obtained from herbarium material (Table S3.1). Initial identification of specimens followed the identification key in the Flora of China (Wei & Bartholomew, 2001), using Wei's more restrictive species boundaries. However, this key excludes the two Japanese species *H. involucrata* and *H. sikokiana* Maxim., which were identified using their original

description and comparing to type specimens. Furthermore, during field work in Hubei, specimens closely resembling the type of *H. villosa* were found. This taxon is not included in Wei's key, as this author considers this taxon to be synonymous with *H. aspera*. However, its distinct morphology and indications of aberrant genomic organization (Mortreau et al., 2010) warrant the inclusion of these specimens under the name *H. villosa*. This resulted in the recognition of ten putative species as a starting point for the coalescent based species delimitation. This approach is beneficial, since the algorithm applied here is unable to split taxa containing two or more related species. Furthermore, each identified specimen was morphologically compared to type material and original descriptions. All published taxa belonging to sect. *Asperae* are included in this study, with the exception of *H. coacta* C.F. Wei which is morphologically indistinguishable from *H. aspera*, as described in the Flora of China (Wei & Bartholomew, 2001).

Extraction, amplification and sequencing

A modified CTAB method (Doyle & Doyle, 1987) was used to extract total genomic DNA from silica gel dried leaf tissue or herbarium material. Two chloroplast intergenic sequences (IGS) and one chloroplast intron sequence were obtained for each specimen (*trnV-ndhC* IGS, *rpl32-ndhF* IGS, *trnL-rpl32* IGS and *ndhA* intron), apart from sequencing four nuclear regions (*TIF3H1*, *SMC1*-44, *SMC1*-22 and ITS). Primers and PCR amplification conditions for the chloroplast regions followed Granados Mendoza et al. (2013), except for the *ndhA* intron, for which primers published by De Smet et al. (2015a) were used. The ITS region was amplified using primers ITS1 and ITS4, following PCR conditions as described by White et al. (1990). Primers for amplifying both regions of the *SMC1* gene and the *TIF3H1* gene were designed based on the sequences of *Cornus wisoniana*, *C. officinalis*, and *Philadelphus incanus* generated by Zhang et al. (2012), and are specific for sect. *Asperae*. For a list of primer sequences see Table S3.2 in Appendix 2. Loci *SMC1*-44 and *SMC1*-22 are two regions of the same *SMC1* gene, but as the connecting region could not be amplified, both regions are analyzed separately to avoid creating chimeric sequences by combining PCR fragments from different alleles. For the chloroplast as well as the ITS regions, PCR products were cleaned using EXO-FASTAP (Thermo scientific, Pittsburgh, PA, USA). PCR products for *TIF3H1*, *SMC1*-44 and *SMC1*-22 were cloned using the Pgem T-easy Cloning Kit (Promega, Fitchburg, WI, USA). A minimum of 5 clones per accession were PCR-amplified directly from plated cultures according to

manufacturer's instructions. Sequencing used the SP6 and T7 primers for cloned copies, and the primers applied in the PCR cycles for other regions. All sequencing was performed at Macrogen Europe. Raw sequences were edited and combined into contigs with Sequencher v5.0.1 (Gene Codes Corporation , Ann Arbor, MI, USA ). Alignments were generated with Prank v120712 (Löytnyoja & Goldman, 2005). All newly generated sequences were deposited in the European Nucleotide Archive (ENA, Table S3.1 in Appendix 2).

Single gene trees and concatenated analysis

Phylogenetic analyses were conducted on each locus individually, using Bayesian methods. Models of sequence evolution were selected using the Akaike information criterion implemented in jModeltest v2.3.1 (Darriba et al., 2012). When models unavailable in MrBayes 3.2.1 (Huelsenbeck & Ronquist, 2001) were selected, the next most parameterized model available was used. Each analysis was run for 20 million generations, using four chains in each of four independent runs with a sample frequency of 1000. Convergence of the Markov chains was assessed using the standard deviation of split frequencies, assuming convergence when this parameter drops below 0.01. Furthermore, convergence for each run was assessed in Tracer v1.6 (Rambaut & Drummond, 2013), as were effective sample sizes for all parameters.

Species tree estimation

All single gene alignments were used in Bayesian species tree estimation with *BEAST (Heled & Drummond, 2010). Best substitution models recovered by jModeltest or the next most general model were used. We ran *BEAST with five independent runs of 200 million generations each, sampling every 10000 generations, using uncorrelated relaxed clock models. LogCombiner v1.6.2 (Drummond & Rambaut, 2007) was used to combine the logs for the five independent runs, checking the resulting log in Tracer to verify if the effective sample size for all parameters exceeded 200. Tree files were combined using LogCombiner, discarding the first 5000 sampled trees as burn-in for each separate run. TreeAnnotator v1.6.2 (Drummond & Rambaut, 2007) was applied to calculate the Maximum clade credibility (MCC) tree from the combined dataset of trees.

Since *BEAST requires the taxa to be a priori assigned to species, taxa were identified as mentioned above. Furthermore, since single gene trees showed diversification between two

groups of *Hydrangea strigosa*, these two clusters were assigned to different taxa. As the species tree generated by *BEAST would be used for species delimitation with BP&P v.3.0 (Yang & Rannala, 2010), it is better to erroneously split a true species than to lump two non-sister taxa (Reid et al., 2012), since this method can lump taxa in the input tree, but not split them.

Bayesian species delimitation

Bayesian species delimitation was conducted using BP&P for all eight sequenced loci. This method requires an a priori defined species tree, and thus an initial allocation of all specimens to potential species. We used the species tree resulting from the *BEAST analysis as guide tree for the BP&P runs, but since the position of *Hydrangea villosa* was only weakly supported in this phylogram, we ran independent analyses for each possible resolution for the position of *H. villosa* as suggested by Leaché & Fujita (2010). Furthermore, BP&P runs can use one of two possible algorithms (1 or 0), and different combinations for prior distribution on the ancestral population size ($\theta$) and root age ($\tau_0$). Since these priors have been shown (Zhang et al., 2011) to influence the outcome of species delimitation, we ran BP&P for three different combinations of priors as suggested by Leaché & Fujita (2010). Both priors are assigned a gamma distribution: $G(\alpha,\beta)$, with a prior mean $\alpha/\beta$ and variance $\alpha/\beta^2$. The first combination of priors assumed small population sizes and relatively shallow divergences: $\theta \sim G(2,2000)$ and $\tau_0 \sim G(2,2000)$. The second set of priors assumed large population sizes and deep divergences: $\theta \sim G(1,10)$ and $\tau_0 \sim G(1,10)$. The final combination of priors is a mixture of priors that assumes large ancestral population sizes and relatively shallow divergence among species: $\theta \sim G(1,10)$ and $\tau_0 \sim G(2,2000)$, which is a conservative combination of priors favoring models containing fewer species. Each of these three prior combinations were run with both possible algorithms (1 and 0), and for each of three possible species trees, for a total of 18 combinations of parameters. Each BP&P run consisted of 100000 generations, sampling every second generation, with a burn-in of 4000 generations. Each combination of parameters was first run for a limited amount of generations to select the fine tuning parameters for the MCMC moves which resulted in acceptance proportions between 0.15 and 0.7. Furthermore, each analysis was run twice to ensure proper mixing of the transmodel algorithm.

Scanning electron microscopy

Pubescence of the abaxial leaf surface was documented with scanning electron microscopy for each sampled morphotype. Dried leaves of similar age were sampled. The area documented was the same for all leaves, being the location where the main vein meets a secondary vein close to the middle of the leaf blade. Microscopic examination was performed with a Supra 40 VP SEM (Carl Zeiss, Germany) equipped with a cryopreparation unit (Emitech K1250X, Quorum Technologies Ltd, Ashford, Kent, UK) to obtain high-resolution images of abaxial leaf surfaces. Samples were glued to metal holders using TissueTek® O.C.T.™ conducting fluid (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands), frozen in liquid nitrogen, and transferred into the cryochamber (-130°C). After sublimation at -70°C for 25 minutes, samples were sputter-coated with approximately 10 nm of gold-palladium prior to examination in the SEM at an accelerating voltage of 5 kV while kept at -100 °C. At least three images were taken per leaf including close-up images of the surface and trichomes structures.

**Results**

Single gene trees

Our data matrix of 240 sequences shows 8 missing sequences. Despite several attempts, we were unable to generate sequences for these combinations of markers and specimens. Single gene trees for chloroplast and nuclear markers agree on topology of the deeper branches. Specimens identified as *Hydrangea longifolia* Hayata and *H. involucrata* form a well-supported clade in all gene trees (Figures S3.1-S3.8 in Appendix 2), which is sister to a larger clade containing all other representatives of sect. *Asperae*. In the latter clade, *H. sikokiana* is recovered as monophyletic and sister to the *H. aspera* species complex. However, this sister position is not always strongly supported, and even absent in the gene trees recovered from *trnV-ndhC* IGS and ITS, where *H. sikokiana* is recovered as a sister clade to the *H. longipes* Franch. – *H. involucrata* clade, or *H. involucrata* respectively. Within the *H. aspera* complex, gene trees reveal widespread topological discordance and varying resolution. However, some well-supported clades are shared among gene trees. Specimens identified as *H. villosa* are consistently recovered in a supported monophyletic clade (with the exception of *SMC1*-44). A clade consisting of specimens identified as *H. longipes* and *H. sargentiana* is recovered in all regions with very high support. Specimens ascribed to *H. kawakamii* are recovered in a supported clade, or are part of an unresolved polytomy. The taxon designated as *H. strigosa*

is recovered as polyphyletic in all gene trees. In the nuclear gene trees, representatives of this species are distributed across two well-supported clades, coinciding with their geographic distribution; one clade contains specimens collected in Hubei (China), while the other specimens originated from Sichuan (China). Chloroplast gene trees recover a similar split for specimens ascribed to *H. strigosa*, but lack the resolution to support each clade as monophyletic. The remaining taxa *H. robusta* and *H. aspera* are not recovered as monophyletic groups, but specimens identified as these taxa cluster together in all chloroplast gene trees. However, although most specimens identified in the field as *H. aspera*, *H. robusta* or *H. strigosa* (Sichuan collections) are recovered as a highly supported clade in plastid gene trees, two specimens are repeatedly recovered outside this clade. These specimens (*H. aspera* 1349 and *H. robusta* 1351) were collected in Nepal and India respectively, at locations near the type locality for these taxa. Specimens of these three nominal taxa are not consistently grouped together in the nuclear gene trees.

Species tree

The MCC tree obtained from the five independent *BEAST analyses provides better resolution for the evolutionary relationships within sect. *Asperae* compared to the single gene trees (Figure 3.2). The topological placement for the Japanese species (*H. involucrata*, *H. sikoniana*) and *H. longifolia* concurs with that found in the single gene trees. Within the *H. aspera* complex, the *BEAST analysis provides improved resolution and nodal support over the single gene analyses. A split between *H. sargentiana* and *H. longipes* is well supported, and these two morphospecies form a clade sister to the rest of the complex, which is split into two clades. A first clade consists of *H. robusta*, *H. aspera* and *H. strigosa* (Sichuan population). This clade is recovered with posterior probability (PP) of 1; however, relationships within this clade remain unsupported (PP: 0.84). The second clade contains *H. villosa*, *H. kawakamii* and *H. strigos*a. The sister relationship between *H. strigosa* (Hubei population) and *H. kawakamii* received high support (PP: 1), whereas the position of *H. villosa* as sister to these two putative species remains unsupported (PP: 0.54).

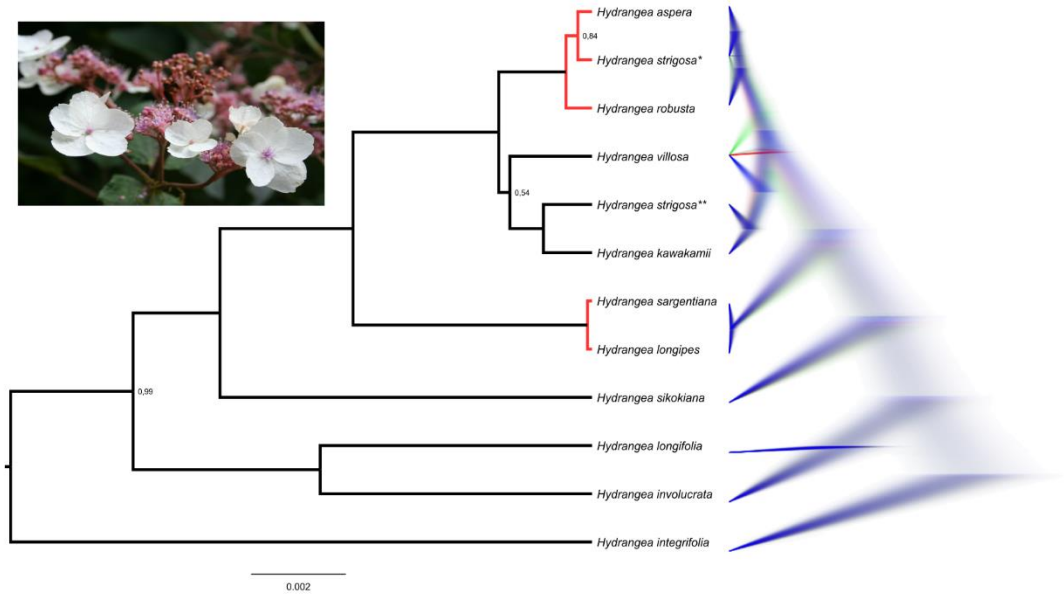



**Figure 3.2: Maximum clade credibility species tree inferred from *BEAST (left) and claudogram (right).** In this tree, only posterior probabilities lower than 1 are displayed, and nodes with speciation probabilities lower than 1 (as inferred in BP&P) in any of the prior combinations are shown in red. Therefore, nominal taxa connected to these red branches are considered conspecific in a conservative interpretation of the BP&P results. The claudogram represents the posterior distribution of species trees inferred in the five independent *BEAST runs. Different colors represent different topologies, in which the blue topology coincides with the MCC tree. Higher color density is indicative of areas in the species tree with higher topological agreement.

### Bayesian species delimitation

Bayesian species delimitation results for sect. *Asperae* are summarized in Figure 3.3. Only three nodes in the guide tree received speciation probabilities below 1 for all analyses: the node splitting *H. sargentiana* and *H. longipes*, and the two nodes separating *H. aspera*, *H. strigosa* (Sichuan population) and *H. robusta*. Placement of *H. villosa* in the guide tree and the choice of algorithm 0 or 1 did not affect the number of species recognized, only resulting in minor changes in the posterior probabilities for the three unsupported nodes. Prior distribution for $\tau$ and $\theta$ had a minor impact on the speciation probabilities for the nodes splitting *H. sargentiana* from *H. longipes* and *H. aspera* from *H. strigosa* (Sichuan population). However, speciation probability associated with the node splitting *H. robusta* from the *H. aspera* – *H. strigosa* clade varies strongly in response to changes in the prior distribution for $\tau$ and $\theta$. Despite this variation, PP for this node never exceeds 0.95; consequently *H. robusta* is not supported as a separate species by BP&P. Remarkably, in only one of the 18 possible parameter combinations,

the node splitting *H. sargentiana* and *H. longipes* receives a PP of 1 (Figure 3.3), while other combinations of parameters never result in a PP higher than 0.22. This PP remains constant after re-running BP&P for this combination of parameters.

| Algorithm | Guide tree | Theta | Tau | Node 1 | Node 2 | Node 3 |
|---|---|---|---|---|---|---|
| 0 | Topology 1 | 2, 2000 | 2, 2000 | 0,08 | 0,01 | 0,00 |
| | | 1, 10 | 1, 10 | 0,15 | 0,79 | 0,18 |
| | | 1, 10 | 2, 2000 | 0,22 | 0,92 | 0,32 |
| 1 | Topology 1 | 2, 2000 | 2, 2000 | 0,08 | 0,02 | 0,00 |
| | | 1, 10 | 1, 10 | 0,00 | 0,79 | 0,20 |
| | | 1, 10 | 2, 2000 | 0,00 | 0,84 | 0,26 |
| 0 | Topology 2 | 2, 2000 | 2, 2000 | 0,08 | 0,01 | 0,00 |
| | | 1, 10 | 1, 10 | 0,05 | 0,49 | 0,06 |
| | | 1, 10 | 2, 2000 | 0,00 | 0,61 | 0,10 |
| 1 | Topology 2 | 2, 2000 | 2, 2000 | 0,09 | 0,01 | 0,00 |
| | | 1, 10 | 1, 10 | 0,06 | 0,47 | 0,05 |
| | | 1, 10 | 2, 2000 | 0,07 | 0,72 | 0,16 |
| 0 | Topology 3 | 2, 2000 | 2, 2000 | 0,09 | 0,01 | 0,00 |
| | | 1, 10 | 1, 10 | 0,00 | 0,82 | 0,20 |
| | | 1, 10 | 2, 2000 | 0,00 | 0,81 | 0,22 |
| 1 | Topology 3 | 2, 2000 | 2, 2000 | 0,07 | 0,01 | 0,00 |
| | | 1, 10 | 1, 10 | 0,02 | 0,79 | 0,21 |
| | | 1, 10 | 2, 2000 | 1,00 | 0,90 | 0,29 |

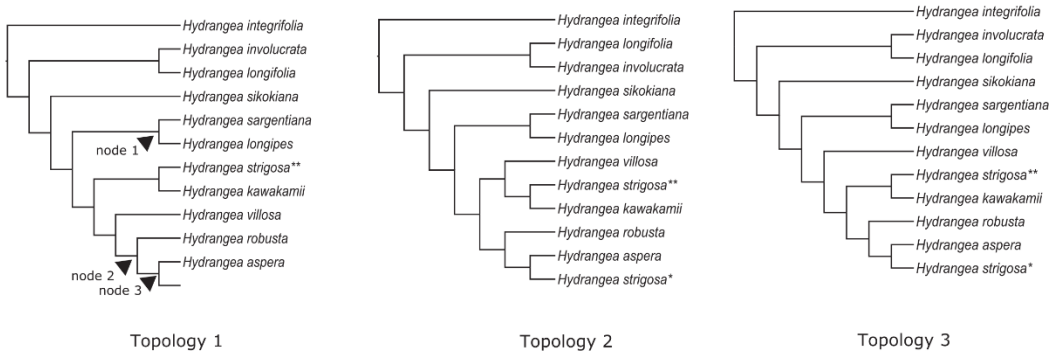



**Figure 3.3: Summary of posterior speciation probabilities calculated by BP&P.** The figure shows posterior probabilities for different combinations of: priors for tau and theta, guide tree topology, algorithm 0 or 1. All other nodes in the tree consistently scored a posterior probability of 1 for every combination of parameters.

### Abaxial leaf surface pubescence

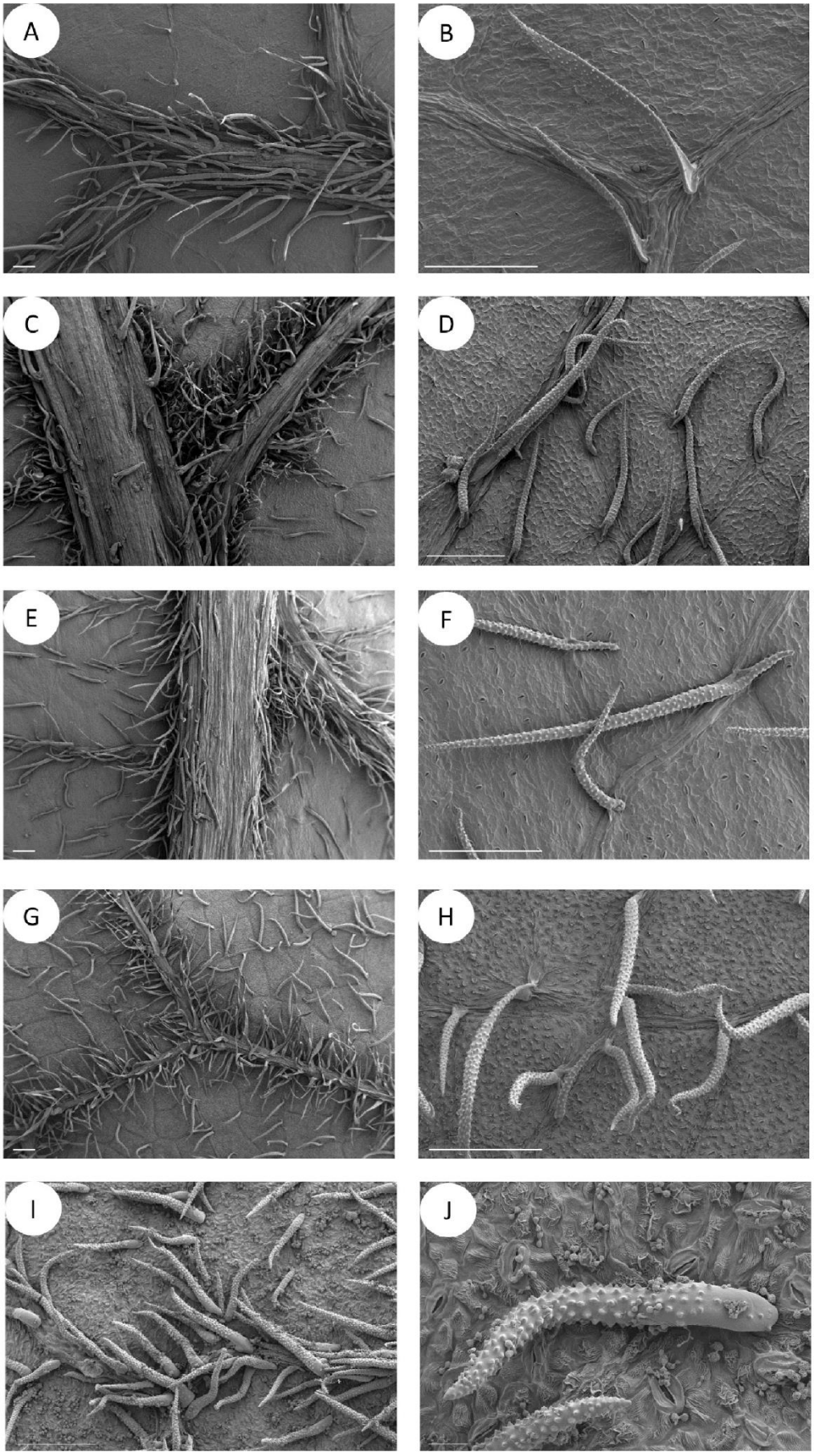
The different nominal taxa included in this study were morphologically heterogeneous with respect to the pubescence of their abaxial leaf surface (Figure 3.4). Most observed trichome types coincide with the types described in protologues and previous revisions of sect. *Asperae*. Besides variation in the morphology of the trichomes, differences in the ornamentation of the leaf surface were observed, more specifically, in the presence or absence of white papillae.

For nominal taxa *H. sikokiana* (Figure 3.4: A & B), *H. involucrata* (Figure 3.4: C & D), *H. kawakamii* (Figure 3.4: G & H) and *H. aspera* (Figure 3.4: K & L), trichomes on the lower leaf surface can be described as long and erect, with conspicuous tubercles on their surface. Differences in appearance between these taxa is mainly due to variation in the density of the pubescence, and length of trichomes. A similar type of trichome is found in specimens morphologically ascribed to *H. villosa* (Figure 3.4: O & P), where they are supplemented with longer, stiff hairs on the larger veins of the leaves. A similar situation occurs in *H. longipes* (Figure 3.4: M & N), but here dense groups of these hairs can be found in the axils formed by the main and secondary veins, visible as white tufts to the naked eye.

Two nominal taxa, *H. strigosa* (Figure 3.4: Q & R) and *H. robusta* (Figure 3.4: I & J) exhibit small appressed hairs on their lower leaf surface. The surface of these trichomes is adorned with small tubercles. Both taxa differ in the girth of these hairs, with those present in *H. strigosa* being much narrower than those of *H. robusta*.

Of the putative species examined in this study, two exhibited an autapomorphous type of trichomes. In *H. longifolia* (Figure 3.4: E & F), the lower leaf surface shows appressed hairs similar to those of *H. strigosa* and *H. robusta* interspersed with two-branched appressed hairs (Figure 3.4: F), which are especially dominant on larger veins and petioles. Petioles, flowering stems and main veins of the abaxial leaf surface show trichomes exhibiting a conspicuous fleshy base (Figure 3.4: S & T) in *H. sargentiana*, which are not observed in any other species of *Hydrangea*. These fleshy trichomes lend the petioles and inflorescences of this putative species its distinctive habit (Figure 3.1: D).

Apart from variation in pubescence type, two examined taxa differ from the others in the presence of papillae on the abaxial leaf surface. These are white and very prominent in *H. strigosa* (both Sichuan and Hubei populations) (Figure 3.4: Q & R), but less conspicuous in *H. kawakamii* (Figure 3.4: G & H).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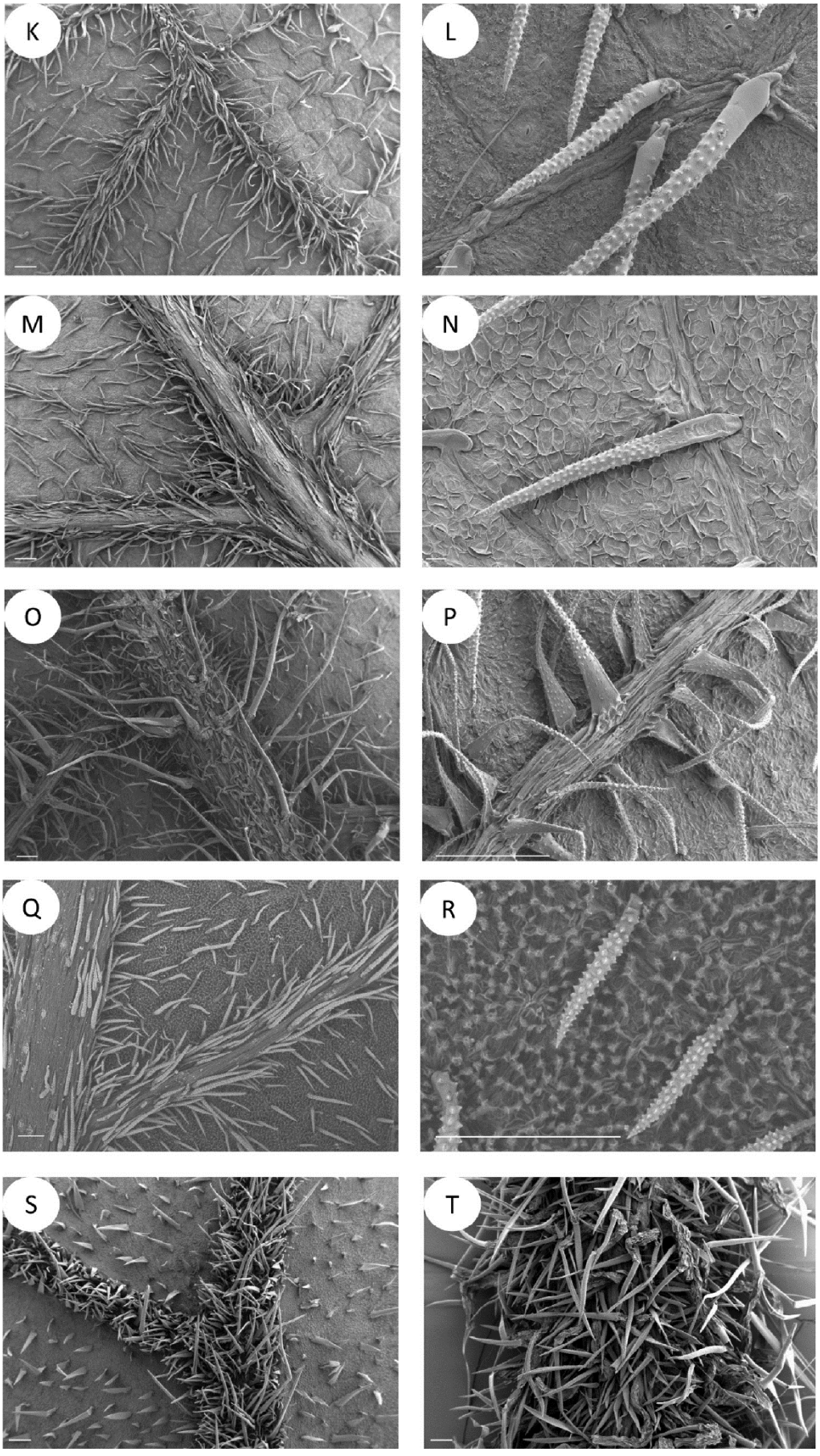
K
L
M
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O
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T

**Figure 3.4 (two previous pages): Scanning electron micrographs for the abaxial leaf surface of sect. *Asperae* representatives.** The left column displays general overviews, while the right column presents details of the typical trichomes for each nominal taxon under study: A & B: *H. sikokiana*, C & D: *H. involucrata*, E & F: *H. longifolia*, G & H: *H. kawakamii*, I & J: *H. robusta*, K & L: *H. aspera*, M & N: *H. longipes*, O & P: *H. villosa*, Q & R: *H. strigosa*, S & T: *H. sargentiana*. Scale bars represent 200μm in A, B, C, D, E, F, G, H, I, K, M, O, P, Q, R, and 20μm in J, L, N.

## Discussion

### Reciprocal monophyly versus coalescent-based species delimitation

Our analyses support the recognition of several independent evolutionary lines within *H.* sect. *Asperae*, and is the first study to offer molecular evidence for the presence of separate lineages within the *H. aspera* complex. Furthermore, our results highlight an advantage of employing multilocus, coalescent-based species delimitation over reciprocal monophyly in single gene trees. Utilizing these coalescent-based methods provided better resolution for both evolutionary relationships and species boundaries within the focal section. Nevertheless, the operational criterion of reciprocal monophyly in gene trees is a valid way of discerning independent evolutionary lineages, albeit a very strict one. Indeed, a substantial amount of generations can be required for two lineages to reach reciprocal monophyly (Hudson & Coyne, 2002; Knowles & Carstens, 2007). This criterion will therefore be unable to identify recently diverged lineages, as these have a high chance of harboring ancestral polymorphisms, rendering them polyphyletic for certain loci. In contrast, species delimitation methods based on coalescent theory represent a probabilistic approach to recognizing separate evolutionary lineages, not requiring reciprocal monophyly or fixed differences. Rather, these methods utilize information from multiple molecular markers to test alternative hypotheses of species delimitation, while allowing for gene tree discordance caused by genetic drift (ILS in the case of BP&P) (Rannala & Yang, 2003; Knowles & Carstens, 2007; Yang & Rannala, 2010). Although these coalescent-based methods are more sensitive in recognizing recently diverged lineages, most contemporary methods fail to discern lineages in the face of strong gene flow. Although the BP&P algorithm has been shown to be robust against a limited amount of gene flow (Zhang et al., 2011), this might limit its utility in sympatric species, where hybridization and introgression are more likely. Furthermore, the analysis has been shown to be sensitive to choice of the priors on ancestral population size and species divergence times (Leaché & Fujita, 2010; Zhang et al. 2011).

Species delimitation in *Hydrangea* section *Asperae*

Application of multi-locus coalescent-based species delimitation to our dataset of ten nominal taxa currently recognized in sect. *Asperae* resulted in the recognition of eight separate lineages. A number of these correspond to a single nominal taxon, whereas others show less straightforward correspondence to named morphotypes. These lineages include: 1) *H. involucrata* from Japan, 2) *H. longifolia* endemic to Taiwan, 3) the Japanese *H. sikokiana*, 4) specimens identified *as H. sargentiana* and *H. longipes*, 5) *H. kawakamii* endemic to Taiwan, 6) specimens identified as *H. strigosa* collected in Hubei, China, 7) *H. villosa* from China, and 8) specimens morphologically ascribed to the nominal taxa *H. robusta*, *H. aspera* and *H. strigosa* collected in Sichuan, China. A subset of these lineages correspond to highly supported monophyletic groups in all (1,2,3,4) or a substantial subset (5,7) of the gene trees. Furthermore, they are morphologically clearly identifiable based on clear-cut diagnostic characters, such as abaxial leaf pubescence (e.g. Figure 3.4).

Results from the coalescent analyses and gene trees suggest *H. involucrata*, *H. longifolia* and *H. sikokiana* to be separate evolutionary lineages. All gene trees recovered these lineages as monophyletic, which combined with their distinct morphology advocates their recognition as clearly diverged species. Geographic isolation from the other members of sect. *Asperae* is possibly the driving factor behind this pronounced divergence.

Within the *H. aspera* complex, two lineages identified in the coalescent analyses coincide with named morphospecies (*H. kawakamii*, *H. villosa*). Although they are only recovered as monophyletic in a subset of the gene trees, high speciation probabilities in all coalescent analyses and a distinctive morphology provide ample evidence to support these nominal taxa as separate evolutionary lineages. The lack of support for monophyly of these taxa in some, but not all, gene trees illustrates the shortcomings of using strict monophyly as the sole criterion for species recognition. Both taxa can represent separate evolutionary lineages, but some loci might experience ILS, or low sequence divergence, obscuring the evolutionary relationships of specimens belonging to *H. villosa* and *H. kawakamii*. The lack of resolution in most gene trees concerning the placement of these two species could represent an indication of the presence of these confounding factors.

The remaining three lineages recognized by the coalescent analyses present two opposing conflicts between nominal (morphology-based) taxonomy and sequence-based species delimitation. In a first case, two morphologically very distinct taxa are strongly supported to constitute a single species based on molecular data. In the second case, a morphologically homogenous group of specimens is split up into two evolutionary distinct lineages.

The operational criteria of strict monophyly and Bayesian species delimitation suggest morphospecies *H. sargentiana* and *H. longipes* to constitute a single species. Moreover, sequences recovered for all eight loci are nearly identical across specimens identified as these taxa. Morphologically however, both putative species are distinct. Petioles and stems of *H. sargentiana* are covered with conspicuous fleshy trichomes (Figures 3.1D, 3.4T) while this type of indument is completely absent from *H. longipes*. Both putative species differ greatly in general appearance: *H. sargentiana* forming large leaves and inflorescences with purple central flowers, while *H. longipes* develops white central flowers and smaller leaves with distinct long and slender petioles. Furthermore, *H. sargentiana* is unique within section *Asperae* in being known from a single wild population in Hubei, China (De Smet et al., 2015b). While *H. longipes* does occur in the same region, its geographic distribution is far wider, covering the Chinese provinces of Hubei and Sichuan. Phenotypic divergence preceding molecular divergence can indicate a recent speciation event, caused by variation in a limited subset of loci. Such speciation would be difficult to detect using a limited subset of neutral markers, as these might not carry any record of the speciation event (Fujita et al., 2012). An alternative explanation for the lack of molecular divergence is strong and ongoing gene flow between both morphospecies. The lack of specimens with intermediate morphology, and the perseverance of the typical *H. sargentiana* morphology amidst a larger population of *H. longipes* morphotypes argue against strong intermixing of both forms. Since *H. sargentiana* can maintain its distinct morphology within the larger geographic distribution of *H. longipes*, we suggest that both morphotypes represent separate evolutionary lineages. Discordance between genetic and morphological divergence between *H. sargentiana* and *H. longipes* could suggest a recent divergence of *H. sargentiana* from the geographically more widespread morphotype. In this case sequence divergence between the two morphotypes would be expected to remain low, insufficient variation having accumulated, and ancestral polymorphisms not having sorted.

*Hydrangea strigosa* is reported to be a widespread species, distributed from Western Sichuan to Eastern Hubei. Our molecular data suggests two different lineages within this morphospecies; one situated in Hubei, and one from Sichuan. The Hubei lineage is supported as distinct by all coalescent-based analyses, as well as the monophyly criterion (for a subset of the sampled loci). The Sichuan lineage is supported as monophyletic by the ITS gene tree, whereas the remaining sequenced regions and all coalescent analyses failed to support this lineage as distinct. Instead, this Sichuan lineage of *H. strigosa* is closely related to *H. robusta* and *H. aspera*, which also occur in Sichuan. All coalescent-based analyses support the recognition of these three morphotypes as a single evolutionary lineage. Our data therefore suggest that *H. strigosa* forms a distinct evolutionary lineage only when occurring in allopatry from the closely related nominal taxa *H. aspera* and *H. robusta*. Indeed, in Sichuan, where these putative species co-occur, a gradual transition can be found between populations of these species, along an altitudinal gradient (McClintock, personal observation on mt. Emei), strongly suggesting gene flow between these entities. In Hubei, on the other hand, no specimens morphologically identifiable as *H. aspera* or *H. robusta* were found in sympatry with the sampled *H. strigosa* specimens (personal observation). Similar patterns have been observed in fucoid brown algae, with species constituting separate evolutionary lines in allopatry, but exhibiting extensive gene flow in sympatry with closely related taxa (Zardi et al., 2011). Therefore, with the current knowledge, we consider the Hubei lineage of *H. strigosa* strongly supported as an independent evolutionary lineage. This lineage furthermore contains specimens collected at the type location of *H. strigosa*, ensuring the connection of this evolutionary lineage to the nominal taxon. Species boundaries between *H. strigosa*, *H. aspera* and *H. robusta* in the Chinese province of Sichuan are less straightforward. With the sampling of specimens and markers achieved in this study, it is unclear whether these named taxa represent a single evolutionary lineage, or if their lumping in our analyses is caused by the sensitivity of the utilized methods to gene flow. Future studies should explore the population level diversity of these taxa in Sichuan, addressing the possibility of extensive gene flow along altitudinal gradients.

**Conclusions**

Our analyses were able to unravel part of the difficult *H. aspera* species complex. Following our coalescent based species delimitation and the operational criterion of reciprocal monophyly, at least three morphotypes warrant recognition as species. These morphotypes are: *H. villosa*, *H. kawakamii* and *H. strigosa* (Hubei lineage). Despite the lack of molecular divergence, we propose the recognition of *H. sargentiana* and *H. longipes* as separate species, owing to their differing morphology and geographical isolation. Finally, this study was unable to provide evidence for the divergence of *H. strigosa* (Sichuan), *H. aspera* and *H. robusta*, suggesting them to represent a single, morphologically variable species, or a species complex experiencing heavy gene flow. However, since these morphotypes were not sampled at their type location, the connection to these published names is uncertain. A similar study including specimens with a clear connection to these published names could provide further insight into their species status.

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**Chapter IV**

# Genome-wide RADseq data resolves phylogeny and species boundaries in the *Hydrangea aspera* species complex

*"There is a long history of how DNA sequencing can bring certainty to people's lives."*
Craig Venter (1946 - …)

**Abstract**

The genus *Hydrangea*, well-known for its highly ornamental representatives, is plagued by taxonomical difficulties. One of these is the lack of clearly defined species boundaries, which is highly apparent in the Asian *H.* section *Asperae*. This group contains a wide variety of morphotypes, distinguished by subtle morphological features, connected by intermediate forms. The latter is the driving factor behind unclear species boundaries in the group, manifested as widely fluctuating numbers of species recognized between and even within subsequent revisions of *Hydrangea*. The explicit adoption of a species concept as rigorous framework integrating molecular and morphological data will aid in stabilizing species boundaries and will require access to sufficiently polymorphic molecular markers. Here the utility of RAD sequencing markers for resolving plant species complexes is evaluated. Based on a sampling of 26 specimens identified as ten nominal taxa, different datasets generated by ipyrad and a combination of Stacks and SiliX were used to conduct a variety of species delimitation algorithms. Additionally, since the dataset utilized in this study largely coincides with a previous study utilizing low copy nuclear markers for the same goals, both methods can be compared. Despite low and uneven sequencing coverage, the RAD data could be used to gain additional evidence for the recognition of *H. involucrata*, *H. longifolia*, *H. sikokiana*, *H. sargentiana*, *H. longipes*, *H. kawakamii* and *H. villosa* as independently evolving metapopulation lineages (species). Nominal taxon *H. strigosa* contained two lineages, of which only one can be recognized as a species, while *H. aspera* and *H. robusta* could not be split up based on the available data.

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## Introduction

The decreasing cost of high-throughput sequencing has seen a rise in its adoption for phylogenetic and ecological studies (Harrison & Kidner, 2011; Clugston et al., 2019). Typically, these fields in biology require high amounts of polymorphic markers, available for a large dataset of non-model organisms. Restriction site associated DNA sequencing (RADseq) allows to target the massive sequencing potential of high-throughput sequencing towards a subset of sites scattered throughout the genome, by generating sequence data adjacent to specific restriction sites (Baird et al., 2008). Targeting a subset of the genome allows for greater depth of coverage per locus, for a larger set of samples for a given budget. Furthermore, RADseq does not require prior genomic information for the taxa under study, such as a reference genome. Consequently, RADseq has been employed in population genetics (Roda et al., 2013; Vandepitte et al., 2013; Wang et al., 2013; Guo et al., 2014; Twyford & Friedman, 2015; Massatti et al., 2016; Nazareno et al., 2018; Warschefsky & von Wettberg, 2019) as well as phylogenetics (Eaton & Ree, 2013; Paun et al., 2015; Eaton et al., 2016; Ahrens et al., 2017; Clugston et al., 2019) for both model and non-model plant groups. Lagging behind in this development is the field of molecular-based species delimitation, with only a limited number of studies utilizing RADseq for plant species delimitation (e.g. Wagner et al., 2018), compared to other organisms (e.g. snails: Razkin et al., 2016; lizards: Nieto-Montes de Oca et al., 2017; insects: Dincă et al., 2019; corals: Quattrini et al., 2019). Nevertheless, adoption of this technique has the potential to provide insights into species limits between closely related taxa for which traditionally obtained markers were hard to obtain, or showed limited divergence.

In an attempt to unify novel molecular insights with traditional morphology-based classification, the genus *Hydrangea* L. has seen several systematic and taxonomic changes at both the genus and species level in recent years. The genus itself was expanded to include eight formerly recognized, closely related satellite genera (Samain et al., 2010; De Smet et al., 2017), rendering the genus monophyletic. As a consequence, several well-known and morphologically distinct taxa were merged into *Hydrangea*, with the current circumscription of the genus emphasizing the evolutionary proximity of the contained taxa. Other authors, focusing on the morphological recognizability of previously published taxa, proposed to split up *Hydrangea*, with the generation of several new, morphologically heterogenous genera

(Ohba & Akiyama, 2016). The counter-intuitive taxa generated by this splitting approach have been criticized (Samain et al., 2019), and will not be followed in the current study.

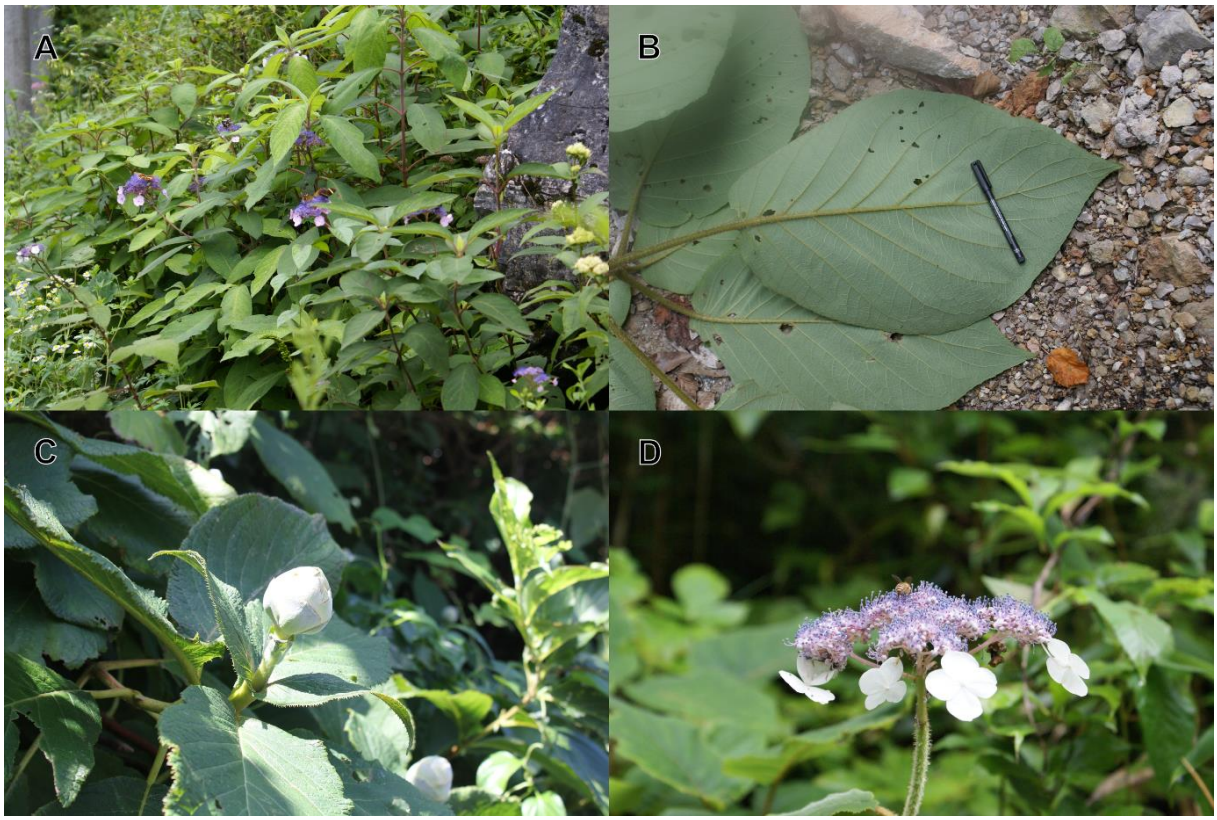


**Figure 4.1: *Hydrangea*. sect. *Asperae* morphology.** A: General habitus of H. aspera. B: Leaf size of *H. sargentiana*. C: young inflorescence of *H. longifolia*, with enveloping involucral bracts. D: Inflorescence of *H. sargentiana*, showing the characteristic fleshy hairs on the peduncle.

The predominately east and southeast Asian *Hydrangea* section *Asperae* (Rehder) Y.DeSmet & Samain (hereinafter referred to as sect. *Asperae*) has been plagued by uncertain species boundaries. Traditionally, as is the case in many taxa, new species were described based on differing morphology, compared to described species. In doing this, authors of novel species implicitly adopt a morphology-based criterion for species delimitation (similar to the phenetic species criterion, Sokall & Crovello, 1970), without objectively quantifying the evidence for the inferred species boundary. As a consequence, subsequent revisions might interpret the morphological differences as insufficient to warrant species status, instead preferring to allocate novel morphotypes to subspecies (McClintock, 1957) or merge them with other species. A striking example of this within sect. *Asperae* is the *H. aspera* species complex (Figure 4.1). This group consists of several nominal taxa, excluding the Japanese and some Taiwanese members of the section. Different revisions have allocated these taxa to a wide range of

recognized species (Rehder, 1911; McClintock, 1957; Wei & Bartholomew) depending on the weight assigned to certain morphological characters. In one of the most recent revisions of the complete genus, McClintock (1957) organized the morphological variation in this species complex into four main forms, recognized as subspecies of *H. aspera*. Two morphotypes, exhibiting lanceolate to ovate leaves, are distinguished by their leaf pubescence being strigose (*H. aspera* subsp. *strigosa*) or velutinous (*H. aspera* subsp. *aspera*). A third morphotype shows ovate to broadly ovate leaves adorned with strigose pubescence. The fourth and final morphotype is distinguished by the presence of thick, fleshy hairs on stems, petioles and abaxial leaf surface (*H. aspera* subsp. *sargentiana*). According to McClintock, all other variation can be ascribed to intermediate forms, which do not warrant recognition as species. Subsequent authors have interpreted these morphological differences as sufficient to recognize separate species, in some cases rescuing nominal taxa from synonymy based on particular morphological apomorphies. An example of the latter is found in *Hydrangea longipes*, which is differentiated from other sect. *Asperae* morphotypes by the long, slender petioles (Wei & Bartholomew, 2002). This taxonomical confusion serves to illustrate the need for clear and motivated definitions of species boundaries, within the framework of an explicit species concept. In this regard, the general lineage concept of species (de Queiroz, 2007) was adopted by De Smet et al. (2017) in order to stabilize taxonomy in the genus *Hydrangea*. Under this framework (de Queiroz, 2005a), rivaling and often contradicting species "concepts" (e.g. biological, phenetic, phylogenetic species concept) are regarded as contingent properties of independently evolving metapopulation lineages (species). Therefore, each species delimitation method or algorithm can be regarded as an operational criterion for deciding whether the entities under study have diverged sufficiently to be regarded as independently evolving. One of the advantages of this approach is that each of these criteria represent an explicitly documented line of evidence towards the interpretation of species boundaries. Even contradictions between species delimitation algorithms can contribute to the understanding of the biological processes driving speciation. The first adoption of this approach for sect. *Asperae* identified three groups of nominal taxa (De Smet et al., 2017). The first group displays general agreement among all lines of evidence examined, and consists of nominal taxa *H. involucrata*, *H. longifolia*, *H. sikokiana*, *H. kawakamii* and *H. villosa*. Specimens in the second group could clearly be identified as separate nominal taxa, due to diverging morphology, but

were genetically uniform, based on the sampled markers. For this group, containing *H. sargentiana* and *H. longipes*, De Smet et al. (2017) interpreted divergence in morphology as sufficient to recognize independently evolving lineages. The final group of specimens showed limited morphological and molecular divergence, falling into McClintock's subspecies *aspera*, *strigosa* and *robusta*. Interestingly, one of the morphotypes, *H. strigosa*, constitutes a morphologically coherent entity, but is identified as consisting of two separate lineages, occurring in two geographically separate regions (De Smet et al., 2017). One of these lineages (situated in the Chinese province of Hubei) forms a separate lineage according to the multispecies coalescent, which was interpreted as sufficient evidence for recognition at the species level. The other lineage (centered in the Chinese province of Sichuan) however, is recovered as conspecific with individuals ascribed to the nominal taxa *H. aspera* and *H. robusta* by the multispecies coalescent. This was interpreted as the consequence of recurring gene flow and introgression taking place when the three nominal taxa occur in sympatry, as is the case in the Sichuan region. Interestingly, all three morphotypes generally occur along an altitudinal gradient, with intermediate forms connecting the morphologically typical populations (McClintock 1957, personal observation on Erlang Shan, Wawu Shan, Hailuogou Glacier Park). However, the molecular markers utilized in De Smet et al. (2017) were unable to confirm the nature of this species complex, and a call was made for testing more variable markers. Testing of additional polymorphic markers, such as can be provided by RADseq, could verify whether the observed low genetic divergence is inherent to the selected markers, or a consequence of biological processes such as gene flow.

Starting with a plastid-based phylogenetic hypothesis (Samain et al., 2010; De Smet et al., 2015a) and continuing on towards the coalescent based species trees generated from several nuclear and plastid markers (De Smet et al., 2017), evolutionary relationships within sect. *Asperae* are currently well-known. The exact position of *H. villosa* within the *H. aspera* species complex, however, remains unresolved, as well as the phylogenetic positioning within the *H. aspera - H. robusta - H. strigosa* group.

In the current study, the potential of RAD sequencing to generate high amounts of informative markers is employed to improve insights into sect. *Asperae* phylogenetics and species boundaries. This section, and the rest of the genus *Hydrangea*, could greatly benefit from increased availability of informative molecular markers. First, such markers could shed light

on the genetic diversity and phylogenetic relationships within the recalcitrant *H. aspera-H. strigosa-H. robusta* complex. Secondly, the uncertain genealogy of *H. villosa* could be resolved by additional molecular evidence. Finally, since the present study utilizes the same specimens as the Sanger-sequencing based approach (De Smet et al., 2017) tackling the same questions, a comparison between both techniques can be drawn.

**Material and methods**

Sampling, sequencing and preprocessing

The 26 accessions used in this study are identical to those used in De Smet et al. (2017), with the exception of samples 1164 (*H. aspera*) and 1074 (*H. strigosa*), which were included in this study to substitute for two samples of the same nominal taxa for which insufficient DNA could be recovered. This sampling represents ten nominal taxa belonging to sect. *Asperae*, and one accession of the related genus *Philadelphus* (Table S4.1 in Appendix 3) as outgroup, collected at type locations where possible (Figure 4.2). Allocation of specimens to nominal taxa follows the aforementioned authors. Total genomic DNA was extracted using a modified CTAB method (Doyle & Doyle, 1987) from silica gel dried leaf material. The extracted genomic DNA of each specimen was individually barcoded and processed into a reduced complexity library according to the Restriction site associated DNA sequencing protocol described in Baird et al. (2008), using the Illumina TruSeq library preparation kit. In short, each DNA extract was digested using the sbfI restriction enzyme, after which Illumina P1 adaptors containing unique barcodes were ligated. After pooling across samples and shearing, fragments between 500-750bp were selected on an electrophoresis gel. Size selected fragments were ligated to the P2 adapter, and selectively PCR amplified. The resulting library was run on an Illumina MiSeq at the Nucleomics core, VIB, Leuven, Belgium, generating 250bp paired end reads. Raw reads were preprocessed using a five-step procedure. First, low quality ends were trimmed employing FastX v. 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). Next, adapters were trimmed with cutadapt v. 1.2.1 (Martin, 2011). In a third step, FastX v. 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/) and ShortRead v. 1.18.0 (Morgan et al., 2009) were applied to remove small reads (< 15bp), polyA-reads (when more than 90% of the bases are A's), ambiguous reads (containing N's), low quality reads (> 50% of bases < Q25) and artifact reads (all but one bases in the read equal one base type). Next, reads of which the paired read

was removed were discarded. In the final preprocessing step, bowtie 2.1.0 (Langmead & Salzberg, 2012) was used to remove reads aligning with Phix or the human genome (hg19).


**Figure 4.2: Distribution map.** Geographic distribution of the samples used in the present study.

Ustacks-SiLiX analyses

The resulting preprocessed dataset was demultiplexed using the process-radtags script distributed with the Stacks pipeline (Catchen et al., 2013); only reads containing unambiguous barcode and restriction cut site sequences were retained. Next, the Ustacks algorithm (Catchen et al., 2013) was used to build stacks (groups of strictly identical reads within individuals), which then could be clustered to form putative loci. The algorithm allows the setting of multiple parameters in this clustering process, and multiple combinations were tested for their potential to recover informative loci. Finally, the combination of parameters proposed by Cariou et al. (2013) was used: the maximum number of differences between stacks within a locus (M:13), number of allowed mismatches to cluster secondary reads to putative loci (N: 9) and the minimum depth of stacks (m: 2). Employing custom scripts, the consensus

sequences generated by Ustacks were extracted, generating a fasta file with putative loci for each studied individual. To identify homologous sequences among the studies specimens, the method proposed by Cariou et al. (2013) was adopted. These authors analyzed the results from an all-against-all BLASTN (Altschul et al., 1990) comparison with the SiLiX algorithm (Miele et al., 2011), clustering sequences with a minimum level of sequence similarity over a minimal length. The same authors remarked on the detrimental effects of restriction sites located in repeated regions, arguing for the removal of clusters containing more than one locus from at least one of the species. Here, SiLiX clusters containing more than one sequence of any of the sampled individuals were removed from further analysis. Through further filtering of these clusters (based on the presence of nominal taxa), three datasets were acquired. A first contained only the clusters in which all nine putative species under study were represented by at least one sequence. This dataset will be referred to as dataset RADa. A second dataset consisted of clusters in which the putative species *H. aspera*, *H. strigosa* (Sichuan population), *H. strigosa* (Hubei population), *H. villosa*, *H. kawakamii*, *H. robusta*, *H. longipes* and *H. sargentiana* were represented by at least a single sequence (dataset RADc). This approach was based on the finding that the proportion of orthologous RAD tag pairs retrieved by BLASTN and SiLiX decreases with divergence time (Cariou et al., 2013). Divergence times used in creating this subset were based on phylogenetic distances recovered in De Smet et al. (2017). A final dataset (RADscn) was generated by combining dataset RADa with the alignments used in species tree estimation by De Smet et al. (2017) (with the exclusion of sequences belonging to the outgroup *H. integrifolia*). Multiple sequence alignments were generated from each cluster using muscle v. 3.8.31 (Edgar, 2004), keeping all parameters at their default setting. For the dataset RADc, the 100 phylogenetically most informative alignments, as determined by number of phylogenetically informative positions, were used in species tree estimation. For the other datasets, all identified clusters were used.

Ipyrad analyses

As an alternative to the Ustacks and SiLiX approach, the demultiplexed, preprocessed FASTQ files were used to run ipyrad v.0.9.42 (Eaton & Overcast, 2020). For a first analysis, all ipyrad parameters were kept to their default value, and two samples for which sequencing depth was low (samples 1459 and 1689) were removed. The resulting single nucleotide polymorphisms (SNPs) were used in phylogenetic reconstruction using BEAST v. 2.6.0

(Drummond & Rambaut, 2007). Models of sequence evolution were selected using the Akaike information criterion implemented in jModeltest v. 2.3.1 (Darriba et al., 2012). One run of 20 million generations was used, sampling every 1000 generations, and discarding the first 25% as burn-in. The SNPs output of the same ipyrad pipeline was utilized in a STRUCTURE 2.3.4 (Pritchard et al., 2000) analysis. This encompassed 1475 SNPs, for 23 individuals. Two of the original 26 samples were removed from this analysis, due to low read number after filtering. The outgroup included in the RADseq analysis (*Philadelphus*) was also removed for lack of overlapping RAD loci with the rest of the samples. A burn-in of 5.000 was used prior to a MCMC of 10.000 generations, with three replicates per value of K (number of inferred genotypic groups or populations), ranging from 4 to 12. The delta K method (Evanno et al., 2005), as available in Structure Harvester (Earl & vonHoldt, 2012), was employed to determine the optimal K value.

Phylogenetic inference: concatenated tree

A single concatenated alignment was generated from the seven alignments of dataset RADa for Bayesian phylogenetic inference. Models of sequence evolution were selected using the Akaike information criterion implemented in jModeltest v. 2.3.1 (Darriba et al., 2012). The analysis was run in BEAST v.2.6.0 (Drummond & Rambaut, 2007), as five independent runs of 50 million generations each. Convergence was checked in Tracer v. 1.7.1 (Rambaut & Drummond, 2013), and all runs combined using logcombiner (Drummond & Rambaut, 2007). The resulting trees were summarized as a Maximum Clade Credibility Tree in TreeAnnotator (Drummond & Rambaut, 2007).

Phylogenetic inference: species tree

Species trees were estimated with *BEAST (Heled & Drummond, 2010). For datasets RADa and RADscn, five independent *BEAST runs were performed for 50 million generations each. For dataset RADc, the analysis was run for 90 million generations. All *BEAST analyses used uncorrelated relaxed clock models, sampling every 1000 generations. For each of the runs, Tracer v. 1.7.1 (Rambaut & Drummond, 2013) was employed to assess whether the effective sample size for all parameters in the combined analyses exceeded 200. The logs of independent runs for each respective dataset were combined in LogCombiner v1.6.2 (Drummond & Rambaut, 2007), removing the first 25% of samples in the MCMC chain as

burn-in. Tree files were combined with LogCombiner, discarding the first 25% of sampled trees in each run as burn-in. From each combined dataset of trees (per alignment dataset), a Maximum clade credibility (MCC) tree was calculated using TreeAnnotator v. 1.6.2 (Drummond & Rambaut, 2007). Taxa were a priori assigned to species as described in De Smet et al. (2017). Samples identified as the nominal taxon *H. strigosa* were assigned to two different taxa (based on their geographical location). As the species tree generated by *BEAST would be used for species delimitation with BPP (Yang & Rannala, 2010), it is better to erroneously split a true species than to lump two non-sister taxa (Reid et al., 2012), since this method can lump taxa in the input tree, but will not split them.

### Bayesian species delimitation

Bayesian species delimitation was conducted using the program BPP v. 4.1.4 (Yang, 2015; Flouri et al., 2018). Algorithm A10 (species delimitation without estimation of species tree) requires an a priori defined species tree, and initial allocation of all specimens to potential species. Therefore, we used the species tree resulting from the *BEAST analysis as guide tree for the BPP runs. However, since the position of *Hydrangea villosa* was only weakly supported in this phylogram, we ran independent analyses for each possible resolution of the *H. villosa* position as suggested by Leaché & Fujita (2010). Furthermore, BBP was run for datasets RADa and RADscn. This method uses the multispecies coalescent model to compare different models of species delimitation (Yang & Rannala, 2010; Rannala & Yang, 2013) in a Bayesian framework, accounting for incomplete lineage sorting due to ancestral polymorphism and gene tree-species tree discordance. The population size parameters ($\theta$s) are assigned the inverse-gamma prior IG(3, 0.002). The divergence time at the root of the species tree ($\tau$0) is assigned the inverse-gamma prior IG(3, 0.004). The algorithm was repeated for three different species trees, conforming to these used in De Smet et al. (2017). Each analysis was run at least twice to confirm consistency between runs resulting in a total of 12 independent analyses (two datasets, three species tree topologies, two reruns for each combination).

## Results

### Sequencing run and data processing

The raw dataset consisted of 7.802.205 fragments, 250bp×2 read length for a total of 3,9Gb. Following the five-step preprocessing procedure, the preprocessed dataset consisted of 5.723.679 fragments of mean read length 244.87bp×2 for a total of 2.803.136.888 bases. Demultiplexing the dataset with the process-radtags script (Catchen et al., 2013), with retention of reads containing unambiguous barcode and restriction site sequences, reduced the total fragments in the dataset to 3.803.241. These retained reads are distributed very unevenly across the 26 sampled individuals, ranging from 640 (*H. sikokiana* 1689) to 486.655 (*H. sargentiana* 1468) retained fragments per sample (Table S4.2 in Appendix 3).

Running Ustacks and extracting the consensus sequence for each supported stack by custom scripts resulted in a varied number of retained loci per specimen. Applying the approach proposed by Cariou et al. (2013), a total of 5762 alignments were generated. Of these, 2141 contained more than one sequence for at least one of the specimens. Since these sequences might represent non-orthologous loci, alignments containing such potential problematic sequences were removed. Of the remaining 3621 alignments, only seven contained at least one representative of all sampled nominal taxa, outgroup excluded (dataset RADa). Dataset RADc, consisting of loci present in at least one specimen representing the nominal taxa *H. aspera*, *H. strigosa* (Sichuan population), *H. strigosa* (Hubei population), *H. villosa*, *H. kawakamii*, *H. robusta*, *H. longipes* and *H. sargentiana*, contained 298 alignments. None of the loci recovered were shared by all 26 samples, or the 25 ingroup samples.

Running the ipyrad pipeline with the aforementioned demultiplexed fragments resulted in the retention of 1.505 filtered loci. Distribution of these loci across samples is summarized in Appendix 3, Table S4.2, and ranges from 6 *(H. sikokiana* 1674) to 914 (*H. longipes* 1400). None of the loci were shared by more than 20 samples.

Phylogenetic reconstruction, concatenated dataset, SNP dataset

Running Bayesian phylogenetic reconstruction based on the concatenated dataset (RADa) resulted in a phylogenetic hypothesis with low support throughout the *H. aspera* complex (Figure 4.3). The rest of the topology is highly similar to the one inferred from the SNP data recovered by the ipyrad pipeline. The latter provides more resolution for the *H. aspera* species complex, recovering most nominal taxa as monophyletic clades. Both phylogenetic hypotheses recover *H. involucrata* and *H. longifolia* as monophyletic, forming a clade sister (PP:

1) to the rest of sect. *Asperae*. This larger clade consists of *H. sikokiana*, which is recovered (PP: 1) as sister to all other taxa in the Ustacks dataset, but with unresolved position in the SNP dataset. Both datasets recover *H. sargentiana* and *H. longipes* as sister to the rest of the *H. aspera* species complex, with varying support; PP: 1 in Ustacks dataset, PP: 0.76 in SNP dataset. The rest of the *H. aspera* species complex consists of two main clades. One (PP: 1 in Ustacks dataset, PP: 0.96 in SNP dataset) joins *H. strigosa* (Hubei lineage) and *H. kawakamii* with one of two samples identified as the nominal taxon *H. strigosa* (Sichuan lineage). The other sample of the latter is recovered in a clade (PP: 0.93 in Ustacks dataset, PP: 0.81 in SNP dataset) with nominal taxa *H. robusta*, *H. aspera* and *H. villosa*. For the concatenated dataset, however, one of the samples identified as *H. robusta* is recovered outside the two large *H. aspera* species complex clades.

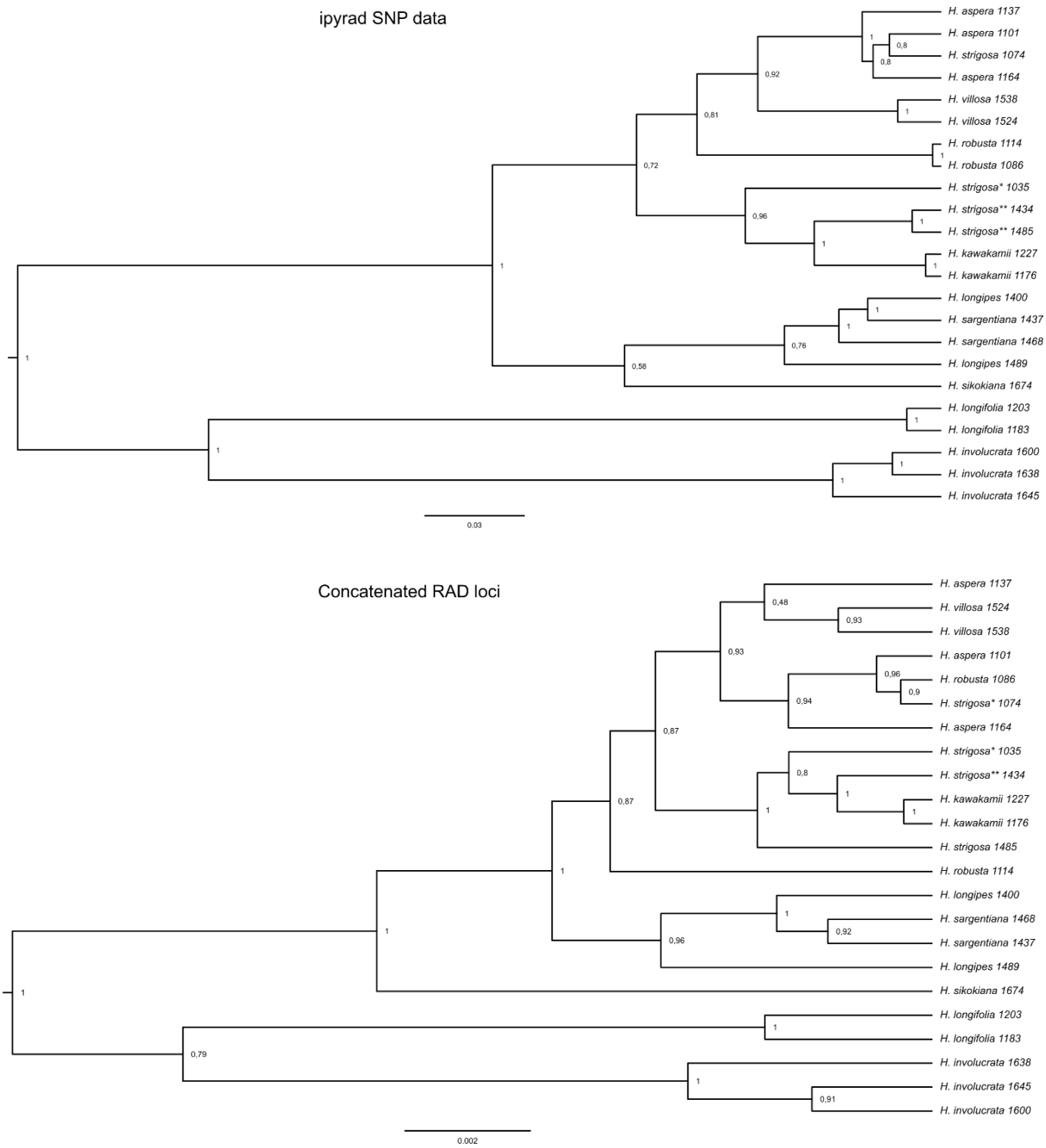


**Figure 4.3: Concatenated gene trees.** Maximum clade credibility trees generated for the SNP dataset resulting from the ipyrad analysis, and the concatenated dataset generated from the seven alignments containing all sampled species resulting from the Ustacks/SiliX analysis. Values indicated at the nodes are posterior probabilities. *Hydrangea strigosa* is split up into the Sichuan lineage (*) and Hubei lineage (**).

### Bayesian species tree reconstruction

No supported topological discordances between the species trees inferred from the three multilocus datasets were recovered (Figure 4.4). The highest resolution was acquired for dataset RADscn (Figure 4.4). The topology recovered for this dataset is congruent with the species tree published in De Smet et al. (2017). However, the current species tree recovered

higher support for two of the three nodes not receiving a PP of 1 in the study by De Smet et al. (2017). The current species tree joins *H. aspera* and *H. strigosa* (Sichuan) in a clade with PP: 0.96, while the previous study recovered a PP of 0.84. The node placing the *H. longipes* – *H. sargentiana* clade sister to the rest of the *H. aspera* species complex receives minimally higher support by including the RAD loci (PP increases from 0.99 to 1). The only node not receiving significant support (PP: 0.72) is related to the placement of *H. villosa* among a group consisting of nominal taxa *H. aspera*, *H. strigosa* (Sichuan population), *H. robusta* and a group joining *H. villosa*, *H. strigosa* (Hubei population) and *H. kawakamii*.

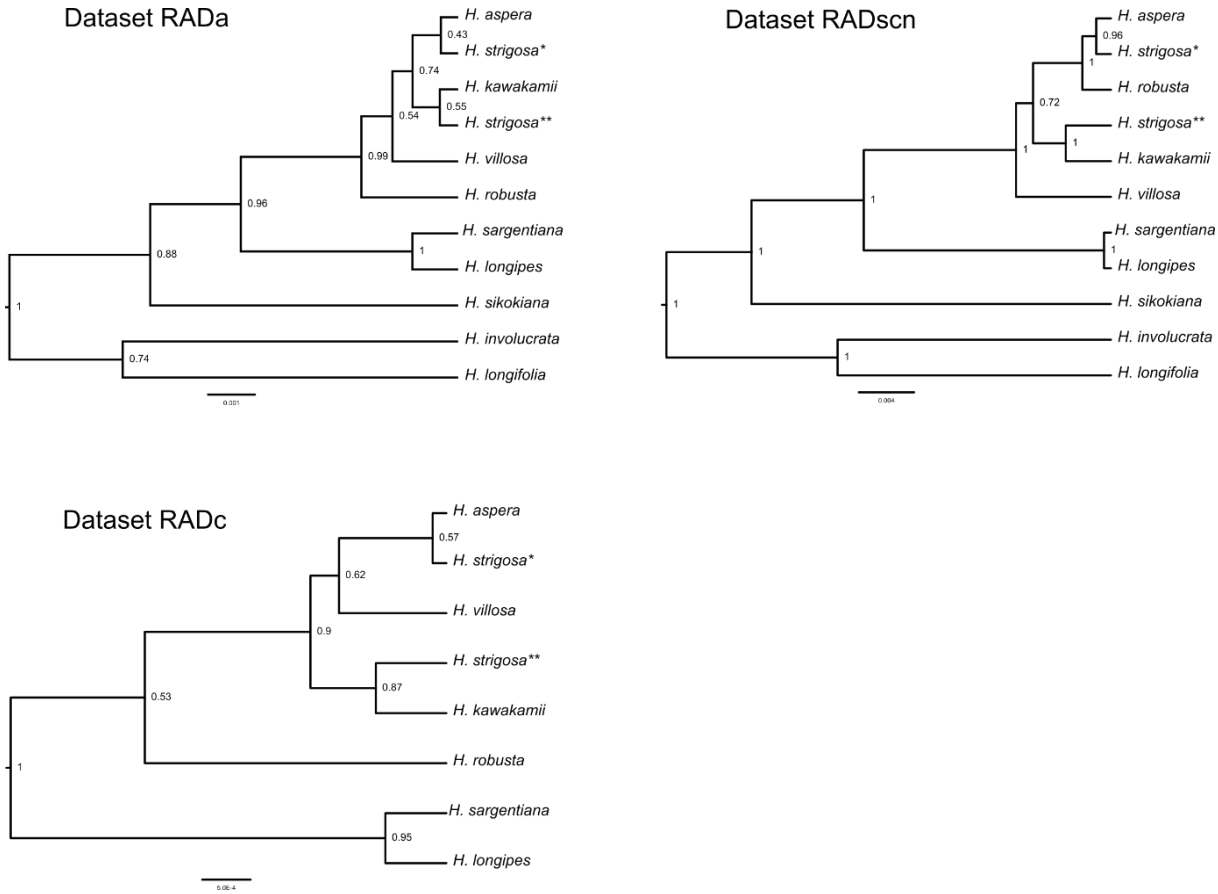


**Figure 4.4: Species trees.** Maximum clade credibility species trees inferred from *BEAST runs for datasets RADa, RADscn and RADc. Posterior probabilities are depicted along the respective nodes. *Hydrangea strigosa* is split up into the Sichuan lineage (*) and Hubei lineage (**).

Bayesian species delimitation in BPP

The results for Bayesian species delimitation with BPP using datasets RADa and RADscn are summarized in Figure 4.5. For the RADscn dataset, all of the nominal taxa in the analysis are supported with a PP of 1, with the exception of *H. sargentiana*, *H. longipes*, and the *H. aspera* - *H. robusta* – *H. strigosa* (Sichuan lineage) group. The first two are recovered as a single species

with speciation probability 1. For the second group, all BPP analyses using the RADscn dataset propose a single evolutionary lineage consisting of the nominal taxa *H. aspera*, *H. strigosa* (Sichuan lineage) and *H. robusta*. Remarkably, for one of the prior combinations (topology 1, Figure 4.5), *H. robusta* receives significant (PP: 0.95) support as a separate lineage.

For the RADa dataset, posterior speciation probabilities above 0.95 are only recovered for nominal taxa *H. longipes*, *H. involucrata* and *H. sikokiana* (Figure 4.5). The entire *H. aspera* species complex is divided into two putative species, one containing nominal taxa *H. sargentiana* and *H. longipes*, and one containing all other sampled taxa. The alternative topologies tested in the BPP analyses had minimal impact on the estimated speciation probabilities, although the node connecting *H. robusta* to the *H. aspera* – *H. strigosa* clade reached significant support in only one topological configuration.

| Dataset | Topology | Replicate run | node 1 | node 2 | node 3 | node 4 | node 5 | node 6 |
|---|---|---|---|---|---|---|---|---|
| RADscn | Topology 1 | 1 | 0,01 | 0,95 | 0,24 | 1 | 1 | 1 |
| | | 2 | 0,02 | 0,93 | 0,2 | 1 | 1 | 1 |
| | Topology 2 | 1 | 0,02 | 0,82 | 0,12 | 1 | 1 | 1 |
| | | 2 | 0,02 | 0,83 | 0,11 | 1 | 1 | 1 |
| | Topology 3 | 1 | 0,01 | 0,89 | 0,16 | 1 | 1 | 1 |
| | | 2 | 0,01 | 0,89 | 0,16 | 1 | 1 | 1 |
| RADa | Topology 1 | 1 | 0,21 | 0,02 | 0,01 | 0,05 | 0,49 | 0,08 |
| | | 2 | 0,21 | 0,02 | 0,01 | 0,05 | 0,49 | 0,08 |
| | Topology 2 | 1 | 0,23 | 0,06 | 0,01 | 0,01 | 0,21 | 0,07 |
| | | 2 | 0,22 | 0,06 | 0,01 | 0,01 | 0,21 | 0,08 |
| | Topology 3 | 1 | 0,21 | 0,04 | 0,01 | 0,01 | 0,13 | 0,49 |
| | | 2 | 0,21 | 0,05 | 0,01 | 0,02 | 0,15 | 0,54 |

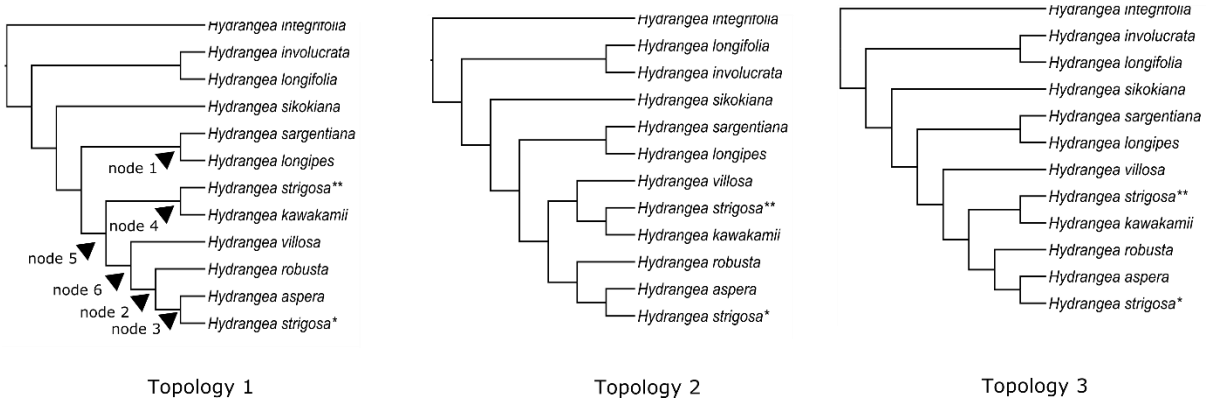


**Figure 4.5: BPP results.** Summary of posterior speciation probabilities calculated by BPP for different combinations of guide tree topology (as depicted below) and dataset used in the analysis. Posterior speciation probabilities are given for each of the nodes who did not consistently garner a PP of 1 across topologies and datasets. All other nodes were recovered with a PP: 1 for all combinations.

Structure analysis

A STRUCTURE analysis was performed using 1475 SNPs detected in the ipyad pipeline, for 23 sampled individuals. The barplot for K=11, which is the optimal value for K according to the delta K approach (Evanno et al., 2005), is displayed in Figure 4.6. At this value for K, admixture is limited to several representatives of the *H. aspera* species complex and *H. sikokiana*. The mixed heritage of the *H. sikokiana* specimen in the study is most likely an artifact created by the high amounts of missing data (low number of shared SNPs, owing to low sequencing coverage) for this sample. Other clusters recovered by the algorithm are highly congruent with the clades observed in the phylogenetic hypotheses. A uniform genetic structure is inferred for nominal taxa *H. longifolia* and *H. involucrata*, congruent with their position as separate clades sister to the rest of sect. *Asperae*. A cluster containing specimens identified as *H. sargentiana* and *H. longipes* shows little admixture and coincides with a supported clade joining both taxa. A cluster joining the Taiwanese *H. kawakamii* and *H. strigosa* coincides with the recovery of these taxa as a supported clade in the phylogenetic hypotheses based on the concatenated, SNP and RADscn (species tree) datasets.

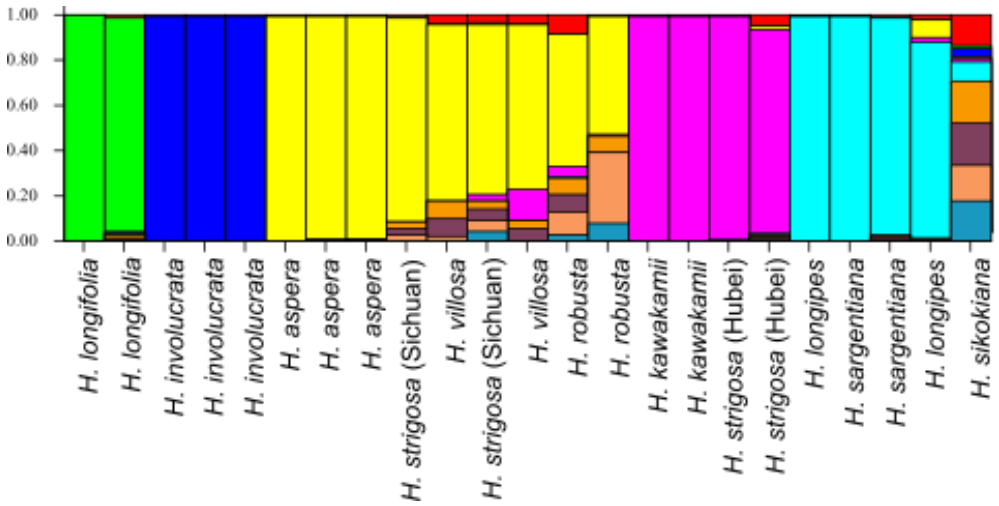


**Figure 4.6: Structure bar plot.** Results of the STRUCTURE analysis for the SNP dataset generated by the ipyrad pipeline. Depicted here is the distribution for the most likely K value (K = 11), as determined by the delta K approach (Evanno et al., 2005). Each bar represents a single individual, for which the identification as one of the nominal taxa under study is indicated below the barplot. Genetic clusters are shown in the same

## Discussion

Species delimitation in sect. *Asperae*

The current study represents a first attempt to harness the potential of high-throughput sequencing for species delimitation in the genus *Hydrangea*. Despite low and uneven sequencing coverage across samples, sufficient data were gathered to explore species boundaries in sect. *Asperae* using various methods: strict monophyly, Bayesian species delimitation and population structure analysis. Each of these operational criteria produces a hypothesis regarding species boundaries in sect. *Asperae*, based on their implicit or explicit definition of species. Adhering to the general lineage concept of species (de Queiroz, 1999, 2007, 2011), each of these newly acquired lines of evidence can be added to those already available through previous molecular studies (De Smet et al., 2017) and morphology-based revisions (McClintock, 1947; Wei & Bartholomew, 2001),therefore creating evidence-based, stable species hypotheses within sect. *Asperae*.

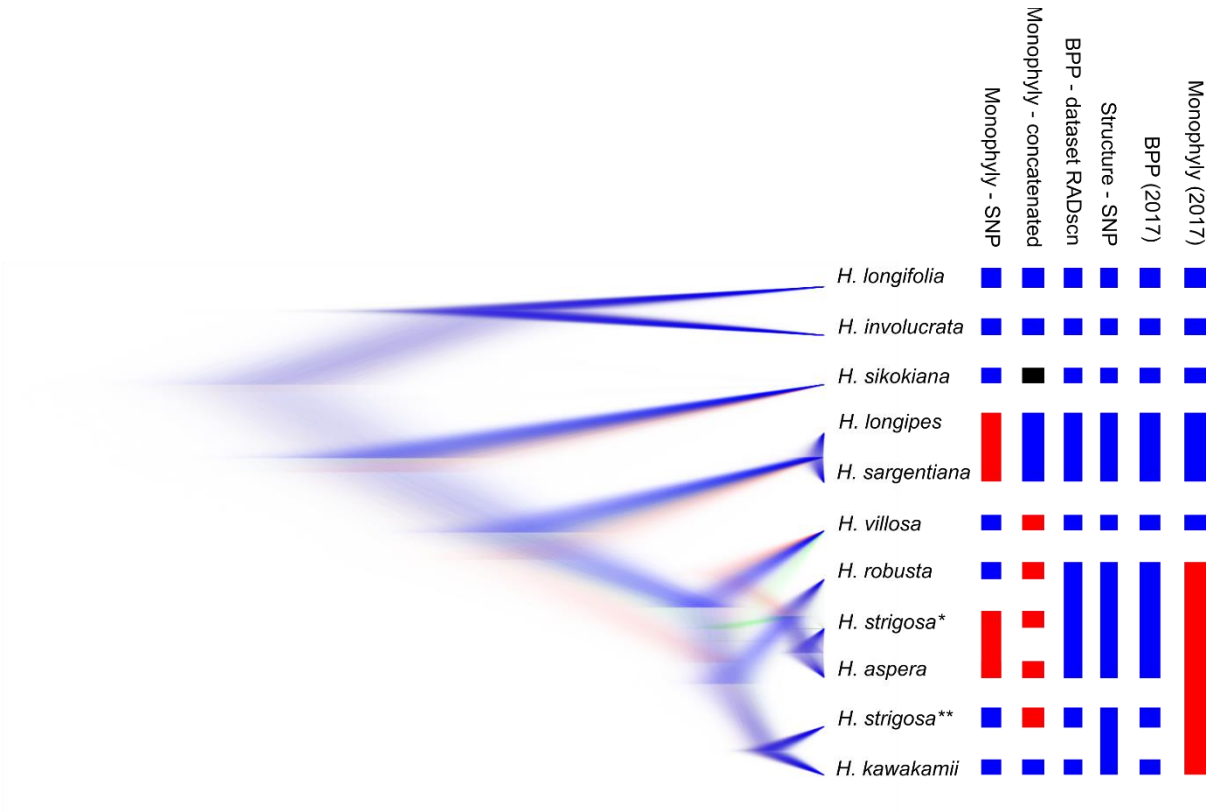


**Figure 4.7: Comparison of different operational criteria.** The claudogram (left) represents the posterior distribution of species trees inferred in the five independent *BEAST runs. Different colors represent different topologies, in which the blue topology coincides with the MCC tree. Higher color density is indicative of areas in the species tree with higher topological agreement. The colored bars (right) represent species delimitations inferred by the different operational criteria obtained in this study, and De Smet et al. (2017). The latter are indicated as (2017). Blue bars represent supported groups of nominal taxa, while red bars represent inferred clusters of nominal taxa which did not receive significant

support in their respective operational criterium. The black bar represents the fact that insufficient samples were present in the study to infer monophyly for *H. sikokiana*.

For three of the nominal taxa included in the study, all operational criteria presented here agree on their recognition as separate evolutionary lineages (Figure 4.7). These taxa are the Japanese *H. involucrata* and *H. sikokiana*, and the Taiwanese endemic *H. longifolia*. Young inflorescences of *H. involucrata* and *H. longifolia* are unique within sect. *Asperae* for being covered in ovate, involucrate bracts, while *H. sikokiana* is the only representative in the group showing lobed leaves. These morphological features, combined with their geographical isolation from most other representatives of sect. *Asperae* have contributed to the widespread acceptance of species status for these taxa (McClintock, 1957; Wei & Bartholomew, 2001). The current study provides additional evidence for the molecular divergence of these taxa from each other and the rest of the section, in agreement with previous studies (De Smet et al., 2017). This growing body of evidence rejects earlier morphology-based interpretations of *H. longifolia* as a variety of *H. involucrata* (McClintock, 1957).

The remaining seven nominal taxa sampled here represent the central *H. aspera* species complex. Disagreements in placement of species boundaries among varying operational criteria render this group an interesting case study for the application of the general lineage concept of species.

Nominal taxa *H. sargentiana* and *H. longipes* are recovered as a single species by all molecular marker-based operational criteria previously presented (De Smet et al., 2017). In this case, the adoption of RADseq (albeit at very low coverage) was unsuccessful in recovering higher amounts of genetic divergence compared to traditional nuclear or plastid markers (Figure 4.7). Moreover, SNP data recovered from the ipyrad analysis were unable to recover population structure within both taxa (Figure 4.6), and fail to recover either taxon as monophyletic (Figure 4.3). Two other lines of evidence, however, geographic location and diagnostic morphological characters, contradict these molecular findings. *Hydrangea longipes* has a wide distribution on stream banks, valleys and mountain slopes of the Chinese provinces Gansu, Guizhou, Hebei, Henan, Hubei, Hunan, Shaanxi, Sichuan and Yunnan. Occurring within the same type of habitat, only one wild population of *H. sargentiana* is known. This population occurs in sympatry with the more widespread *H. longipes*, and was only recently rediscovered (De Smet

et al., 2015b). Furthermore, both nominal taxa are easily distinguished based on pubescence of stems, petioles and abaxial leaf veins, as well as leaf size. In *H. sargentiana* petioles, braches and abaxial leaf veins are covered in thick, branched, fleshy trichomes, that are unique within the genus *Hydrangea*. Petioles are long and slender in *H. longipes*, while *H. sargentiana* exhibits thicker petioles. Based on these morphological differences and the fact that *H. sargentiana* seems to uphold a separate population with distinct morphology in sympatry with *H. longipes*, it is argued here that both taxa represent independently evolving metapopulation lineages, and thus should be recognized as species. The lack of genetic divergence between this taxa could be due to their recent divergence, or ongoing gene flow. This case perfectly exemplifies the advantages of comparing multiple lines of evidence. Simple adherence to one of the molecular-based species delimitation algorithms (for example the STRUCTURE analysis) would have seen both taxa merged into a single species. By analyzing different types of data, however, a more nuanced picture is revealed. Additionally, assigning species status to the rare *H. sargentiana* morphospecies can contribute in the conservation of this unique pool of variation within the genus *Hydrangea*, currently only known from a single population (De Smet et al., 2015b).

The inverse situation of limited morphological divergence, but clearly identified molecular divergence supported by multiple algorithms (monophyly in SNP-based Bayesian phylogeny, posterior speciation probability in BPP) can be observed for nominal taxa *H. villosa* and *H. kawakamii*. Subtle morphological differences with the widespread *H. aspera* has led several authors to merge these morphospecies into a single taxon (McClintock, 1957; Wei & Bartholomew, 2001). However, the RADseq data presented here (Figure 4.7) presents ample evidence for the recognition of these nominal taxa as separate species, in agreement with previous molecular-based studies (De Smet et al., 2017). Such disagreement among lines of evidence could be explained as morphological differentiation lagging behind genetic differentiation in diverging taxa. Or, as is suggested to be more prominent here, emphasis on certain traditionally employed morphological characters might obscure divergence. Indeed, *H. kawakamii* can be differentiated from *H. aspera* by seed coat morphology, as well as geographical isolation, being endemic to Taiwan. For *H. villosa*, the abaxial leaf indument differs from that described for *H. aspera*, as shown in De Smet et al. (2017), rendering both nominal taxa readily distinguishable from *H. aspera*. Characters employed traditionally for

diagnosing species in sect. *Asperae* include shape and size of the leaf and petiole. Since these have not diverged sufficiently for *H. villosa* and *H. kawakamii* to be differentiated from *H. aspera*, focusing on this character exclusively (operational criterion morphological divergence) will obscure the evolutionary history of these taxa.

The nominal taxon *Hydrangea strigosa* has been suggested to represent an independent evolutionary lineage when in allopatry with the closely related *H. aspera* and *H. robusta* (De Smet et al., 2017). However, when occurring in sympatry, previous studies were unable to find genetic divergence between these nominal taxa. Furthermore, sympatric populations easily ascribed to each of these morphotypes are interconnected by populations of intermediate forms (intermediate leaf shape and abaxial pubescence). These observations have led previous revisions (McClintock, 1957) to allocate these three nominal taxa to a single species, *H. aspera*, while molecular-based studies emphasized the need for more polymorphic markers to rule out artefacts in the choice of markers as driving force behind this interpretation. The polymorphic markers generated in this study were unable to distinguish between *H. robusta*, *H. aspera* and *H. strigosa* when occurring in close geographic proximity. Indeed, most molecular methods propose the recognition of two lineages in this group of morphotypes. One containing the individuals identifiable as *H. strigosa*, occurring in the Chinese province of Hubei, in allopatry from *H. robusta* and *H. aspera*. A second consisting of all specimens identifiable as these latter two nominal taxa and the Sichuan-based representatives of *H. strigosa*. Comparing all lines of evidence available to date, the current study confirms the earlier findings of De Smet et al. (2017), proposing to recognize *H. strigosa* as a separate species, since the type location for this taxon is situated in Hubei. For *H. aspera* and *H. robusta*, currently only limited morphological differences and the recovery of a monophyletic clade in the SNP phylogenetic hypothesis support recognition of two separate species. It is therefore proposed to recognize one species, *H. aspera*, with two subspecies *H. aspera* subsp. *aspera* and H. *aspera* subsp. *robusta*, to represent the morphological and altitudinal divergence between these nominal taxa. It is believed that sampling of both nominal taxa at their type locations will further unravel this species complex. The present and previous (De Smet et al., 2017) results have provide sufficient tools to evaluate this complex in future studies.

Phylogenetic relationships within sect. *Asperae*

The combined analyses of RADseq data and previously sequenced traditional markers improved support for two nodes in the sect. *Asperae* species tree (Figure 4.4). One of these nodes, defining the sister relationship between *H. aspera* and *H. strigosa* (Sichuan population) is recovered with significant support (PP: 0.96) in the present study. The same relation was inferred, albeit unsupported (PP: 0.84), in the phylogenetic analysis based solely on traditional molecular markers (De Smet et al., 2017). The only unsupported node remaining in the phylogenetic hypothesis denotes the position of *H. villosa* among two clades making up the remainder of the *H. aspera* complex. Likewise, the phylogenetic analysis based on 1475 SNPs could not recover the relationship between these three clades (Figure 4.3). Owing to the limited sequencing depth across the individual samples, causing a high proportion of missing data, drawing conclusions regarding the general utility of RADseq for phylogenetic reconstruction in this group is partially hampered. The phylogenetic hypothesis which can be inferred, however, is in line with expectations based on geographical isolation and morphological characteristics of sect. *Asperae* representatives. Moreover, no supported incongruences with previously inferred phylogenetic hypotheses (Samain et al., 2010; De Smet et al., 2015a; 2017) were detected. The two species exhibiting ball-shaped young inflorescences (Japanese *H. involucrata* and Taiwanese *H. longifolia*) covered by involucral bracts are highly supported as sister taxa in a clade sister to the rest of the section. Within this larger clade, the other Japanese representative of the group is recovered as sister to the remaining (mostly Chinese) representatives of the group. The sister relationships between nominal taxa *H. sargentiana* and *H. longipes* are concordant with their overlapping geographical ranges. The close relationship between *H. strigosa* (Hubei population) and *H. kawakamii* (Taiwanese endemic) seems contra-intuitive based on geography, while their morphology is exceedingly similar. The relationship between this clade, *H. villosa* and a clade containing morphologically similar *H. robusta*, *H. aspera* and *H. strigosa* remains unresolved. Availability of more variable markers, and samples from the type locations of each of these published names, could alleviate their evolutionary relationships.

Low and uneven read depth across samples

Following the approach proposed by Cariou et al. (2013), using SiLiX to build alignments from consensus sequences produced by Ustacks, the *Hydrangea* dataset generated 5762 potential alignments of orthologous sequences. However, further filtering revealed that 2141 of these alignments contained more than one sequence for at least one of the sampled individuals. Because of the high potential for being non-orthologous (Cariou et al., 2013), they were removed from further analysis, reducing the number of alignments with 37%. Furthermore, only seven of the remaining alignments contained at least one sequence for each of the sampled nominal taxa, while none of them represented all of the sampled individuals. The independent analysis through the ipyrad pipeline encountered the same limitations. The presence of such problematic loci is to be expected in taxa with a history of genome rearrangements such as whole-genome or gene duplications, or presence of large amounts of repeat sequences (Andrews et al., 2016). Genome reorganizations are prevalent within kingdom Plantae (Adams & Wendel, 2005; Soltis et al., 2015), and have been detected in sect. *Asperae*. Indeed, variations in chromosome number have been described (Funamoto & Tanaka, 1988; Mortreau et al., 2010; Cerbah et al., 2011) among taxa within the section, which could be the driving factor behind the large amount of problematic reads detected in this study. Moreover, this phenomenon might influence the earlier steps in the analysis of the raw RADseq data. As shown in Table S4.2 (Appendix 3), the number of reads produced per sampled individual are highly variable, effectively eliminating several individuals from further analyses for lack of shared loci. This variation across individuals can largely be ascribed to uneven quality and quantity of the input DNA (Davey et al., 2013; Xu et al., 2014), or is the consequence of specific characteristics of the RADseq protocol. Davey et al. (2013) proposed several mechanisms through which these biases in read depth can occur, such as PCR GC bias and restriction fragment length bias. In this study, however, the variation in chromosome number and the underlying genomic reorganization can additionally be invoked to explain part of the variation in read depth among samples.

**Conclusion**

The current study presents the first attempt to apply RADseq to species delimitation and phylogenetic reconstruction in the genus *Hydrangea*. Despite low and highly uneven sequencing coverage across the individual samples, these new lines of evidence were able to

solidify several insights in sect. *Asperae* species boundaries. A combination of different operational criteria provided sufficient support for the recognition of nominal taxa *H. involucrata*, *H. longifolia*, *H. sikokiana*, *H. sargentiana*, *H. longipes*, *H. kawakamii* and *H. villosa* as independently evolving metapopulation lineages (species). Nominal taxon *H. strigosa* can be recognized as an independent species, but experiences heavy gene flow when in sympatry with *H. robusta* and *H. aspera*. The results available at this time suggest the merging of the latter two nominal taxa into a single species (*H. aspera*), until other lines of evidence, including samples from the respective type locations of these taxa become available. Although a higher sequencing depth could provide more resolution in both species delimitation and phylogenetic hypotheses, this study pinpoints fields in which improvements can be made within *Hydrangea* systematics and evolutionary studies.

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**Chapter V**

# Taxonomic treatment of *Hydrangea* sect. *Asperae*

*"If the names are unknown knowledge of the things also perishes."*

Carl Linnaeus (1707-1778)

**Abstract**

Traditional, morphology-based species delimitation in *Hydrangea* sect. *Asperae* has been difficult. This difficulty can, in part, be ascribed to the presence of subtle morphological variations in leaf shape and size, in some cases creating morphological continua connecting previously described morphotypes. In previous studies, new emphasis on discrete characters such as the pubescence of the abaxial leaf surface, and insights from molecular data has shed light on the evolutionary history of the section. Combining these new data under the framework of an explicitly cited species concept (general lineage concept of species) allows for the postulation of species boundaries within the section. The resulting delimited taxa can be understood as hypotheses, corroborated by a set of lines of evidence. In this way, ten independently evolving metapopulation lineages (species) can be identified within *H.* sect. *Asperae*, of which each is linked to a previously published name. In this way, there was no need to describe new species in this section, although the circumscription of several nominal taxa changed to include other previously published names.

An adapted version of this chapter will be submitted for formal publication.

## Introduction

Taxonomy and systematics of the Hydrangeaceae tribe Hydrangeeae (Cornales) have seen a recurrent integration of molecular data over the last decades (Soltis et al., 1995; Hufford et al., 2001; Jacobs, 2010; Samain et al., 2010; Granados Mendoza et al., 2013; De Smet et al., 2015a). First suggestions of the polyphyletic nature of the genus *Hydrangea* (Samain et al., 2010) sparked researchers to corroborate these findings using additional molecular markers (Granados Mendoza et al., 2013), culminating in a drastic classification change for the tribe (De Smet et al., 2015a). This new classification merged eight morphologically divergent satellite genera into *Hydrangea*, in order to create a stable classification, reflecting evolutionary history. Although this classification was accepted by several contemporary authors (Lin & Chung, 2017; Samain et al., 2019; Sodusta, 2019), others proposed morphology-based, evolutionary non-informative alternatives (Ohba & Akiyama, 2016, 2017; Huang et al., 2018). Within the classification proposed by De Smet et al. (2015a), the larger genus *Hydrangea* is split into several sections, congruent with supported clades retrieved in the nuclear and chloroplast based phylogenetic hypotheses. One of these sections, *H.* sect. *Asperae*, contains small to larger deciduous shrubs, with the largest diversity centered in mainland China and Japan (Figure 5.1). Several taxa exhibit wide distributions, being collected from Nepal or India until northern China (*H. aspera*), while others are highly localized in their distribution (*H. sargentiana*, *H. villosa*). Inflorescences in this section are corymbose cymes consisting of a dense cluster of smaller central flowers, encircled by relatively few large showy flowers, referred to here as marginal flowers. This arrangement has been suggested to increase attractiveness to pollinators, occasionally acting as landing sites in certain taxa (Sato & Kato, 2019). Since the merging of several genera into *Hydrangea*, *H.* sect. *Asperae* contains one species exhibiting an aberrant morphology, *H. platyarguta*. This taxon develops larger flowers compared to the other representatives, showing a multitude (>25) of yellow stamens, and developing apically poricidal capsules. The remaining taxa in the section have denser inflorescences of smaller flowers showing 8-12 (mostly 10) generally purplish stamens and developing into capsules dehiscing between the styles. With the exclusion of *H. platyarguta*, the remaining taxa described within *H.* sect. *Asperae* are morphologically close, with the number of recognized taxa varying widely between (Rehder, 1911; Chun, 1954; McClintock, 1957; Wei, 1991) and even within (Wei & Bartholomew, 2001) previous revisions of the genus. Nevertheless,

authors tend to agree on the species status for the two Japanese representatives of the section (*H. involucrata* and *H. sikokiana*). Indeed, the main discrepancy between revisions is situated in the interpretation of morphological divergence centered around the *H. aspera* species complex (De Smet et al., 2017). Depending on the diagnostic value attached to certain morphological characters such as leaf shape (Figure 5.2) and pubescence, authors traditionally recognized between one (McClintock, 1957) and nine (Rehder, 1911) separate species. Remarkably, morphological variation in the group seems sufficiently complex as to instigate differences in interpretations even within revisions (Wei & Bartholomew, 2001). In order to stabilize species boundaries in the section, De Smet et al. (2017) gathered different lines of evidence from both molecular and morphological sources of data. To this end, abaxial pubescence of leaves was documented objectively using scanning electron microscopy (SEM). Exploring the utility of several low copy nuclear markers (chapter 3) as well as RADseq (chapter 4), the speciation history of *H.* sect *Aspera* was investigated. Both types of molecular data were used in coalescent-based species delimitation algorithms to test alternative interpretations of species boundaries, as well as more traditional operational criteria such as reciprocal monophyly and shared genetic variation. Integrating all these lines of evidence within the framework of the general lineage concept of species (de Queiroz, 1999), a well-supported species hypothesis was proposed recognizing nine genetically distinct lineages (in addition to *H. platyarguta*). In order to link these lineages with formally published names, the current chapter proposes a revised taxonomy for *Hydrangea* sect. *Asperae*. In keeping with the philosophical framework of the general lineage concept of species, all operational criteria supporting the recognition of the formally described species are mentioned explicitly. This approach should promote stability in species boundaries, presenting each recognized species as a hypothesis garnering support from different lines of evidence.

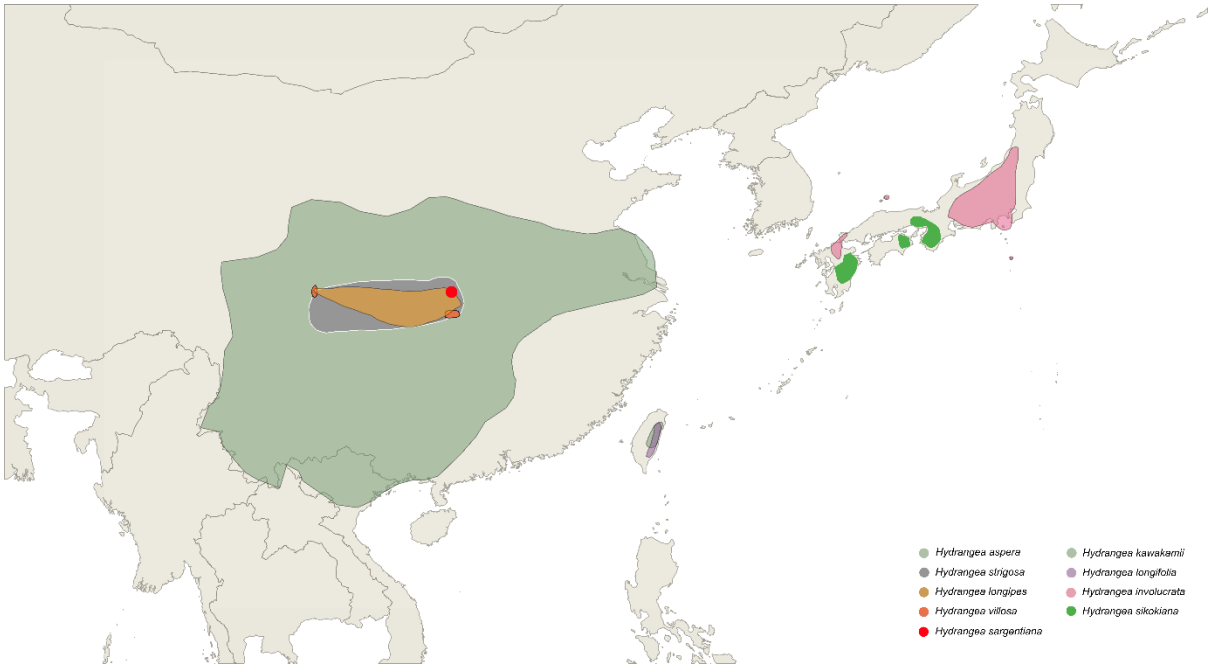


**Figure 5.1: Distribution of *Hydrangea* sect. *Asperae* in Mainland China, Japan and Taiwan.** Distribution of the specimens used in this study and previous chapters. Personal collections, herbarium material and material grown in garden (wild collected) are included. Specimens labeled *H. aspera* and *H. robusta* collected in Nepal and India are not depicted.

## Materials and methods

Morphological variability of published taxa was studied in herbarium specimens (including type material) loaned from herbaria (AAU, CAS, E, G, GB, GENT, K, MICH, S, US, WU) (abbreviations according to Thiers, continuously updated), as well as living collections in the Ghent Botanical Garden, Arboretum Wespelaar (Haacht, Belgium), White House Farm (Sevenoaks, UK) and Crûg Farm (Caernarfon, UK). Herbarium specimens studied are listed in Appendix 4 (Table S5.1). Initial distribution data for all nominal taxa were acquired from different sources: labels on living plants and herbarium material, original descriptions and revisions of the genus *Hydrangea* (Rehder, 1911; McClintock, 1957; Wei & Bartholomew, 2001). Since some geographical data were unclear, either because the name of the locality changed, was lost in translation or was not mentioned in sufficient detail, online gazetteers and travel accounts of plant collector E.H. Wilson (Ferguson, 1983; Flanagan & Kirkham, 2009) were consulted. Summarizing these locations, areas of interest were identified based on species diversity and presence of type locations. In order to assess *in situ* population status, and collect fresh specimens for molecular and morphological study, field work was executed in the Chinese provinces of Sichuan and Hubei, the island of Taiwan and Japan. During fieldwork,

each collection consisted of fresh leaves collected in silica gel, herbarium specimens reflecting leaf diversity and inflorescence (if present), observation of the conservation status of the population and detailed GPS coordinates. Herbarium specimens and silica gel dried samples were provided with a collection number and preserved in the Ghent University Herbarium (GENT). Details on collected specimens used in this study are summarized in Appendix 4 (Table S5.2).

Identification of the collected specimens occurred through morphological comparison to type material, species descriptions and revisions. Additional diagnostic morphological characters were assessed using a stereomicroscope, of which abaxial leaf pubescence was documented using a Supra 40 VP SEM (Carl Zeiss, Germany) equipped with a cryopreparation unit (Emitech K1250X, Quorum Technologies Ltd, Ashford, Kent, UK). Results of scanning electron microscopy were presented in chapter 3 (De Smet et al., 2017). Identified specimens were utilized to study morphological variability in published taxa, and in molecular studies inferring phylogenetic relationships and species boundaries in the section (De Smet et al., 2017; chapters 3 and 4). Lineages identified in these studies were morphologically compared to the morphological descriptions available in the most recent revision of the section (Wei & Bartholomew, 2001). When observed lineages did not coincide with taxa described by these authors, new diagnostic descriptions were assembled (*H. villosa*, *H. involucrata*, *H. sikokiana*). On the other hand, when identified lineages concur with the descriptions in Wei & Bartholomew (2001) these descriptions were adopted, and amended where necessary (*H. aspera*, *H. strigosa*, *H. longipes*, *H. kawakamii*, *H. longifolia*).

**Taxonomic treatment**

***Hydrangea* section *Asperae*** (Rehder) Y. De Smet & Samain

*Platycrater* Siebold & Zucc., Fl. Jap. 1: 64, t. 27 (1835); in Abh. Nath.-Phys. C. Königl. Bayer. Akad. Wiss. 4(2): 192 (1845). Ohba & Akiyama include the entire section *Asperae* in the genus *Platycrater*.

*Hydrangea* ser. *Asperae* (Rehder) H. Ohba in K. Iwats. & al., Fl. Jap. 2b: 85. 2001.

*Hydrangea* sect. *Euhydrangea* subsect. *Asperae* Rehder in Sargent, Pl. Wilson. 1: 39. 1911.

*Hydrangea* sect. *Euhydrangea* subsect. *Piptopetalae* Maximowicz, Mémoires Académie Imperiale des Sciences de St. Pétersbourg, ser. 7, 10(16): 8 (Revisio *Hydrangearum* Asiae

Orientalis). 1867. In part.

*Hydrangea* subgenus *Euhydrangea* sect. *Japonico-sinensis*, subsect. *Piptopetalae* Schnieder, Handbuch der Laubholzkunde, 388, 1905. In part.

Type species: *H. aspera* D.Don.

General morphology of *H.* sect. *Asperae*

Morphologically this section can be recognized by the completely inferior ovary, with the capsular fruits being hemispherical with a truncate apex. Ripe fruits open with a fissure between the styles (only *H. platyarguta* develops poricidal capsules). Petals of the fertile flowers fall separately, or sometimes slightly cohering at the apex. Styles usually two, seeds winged at both ends.

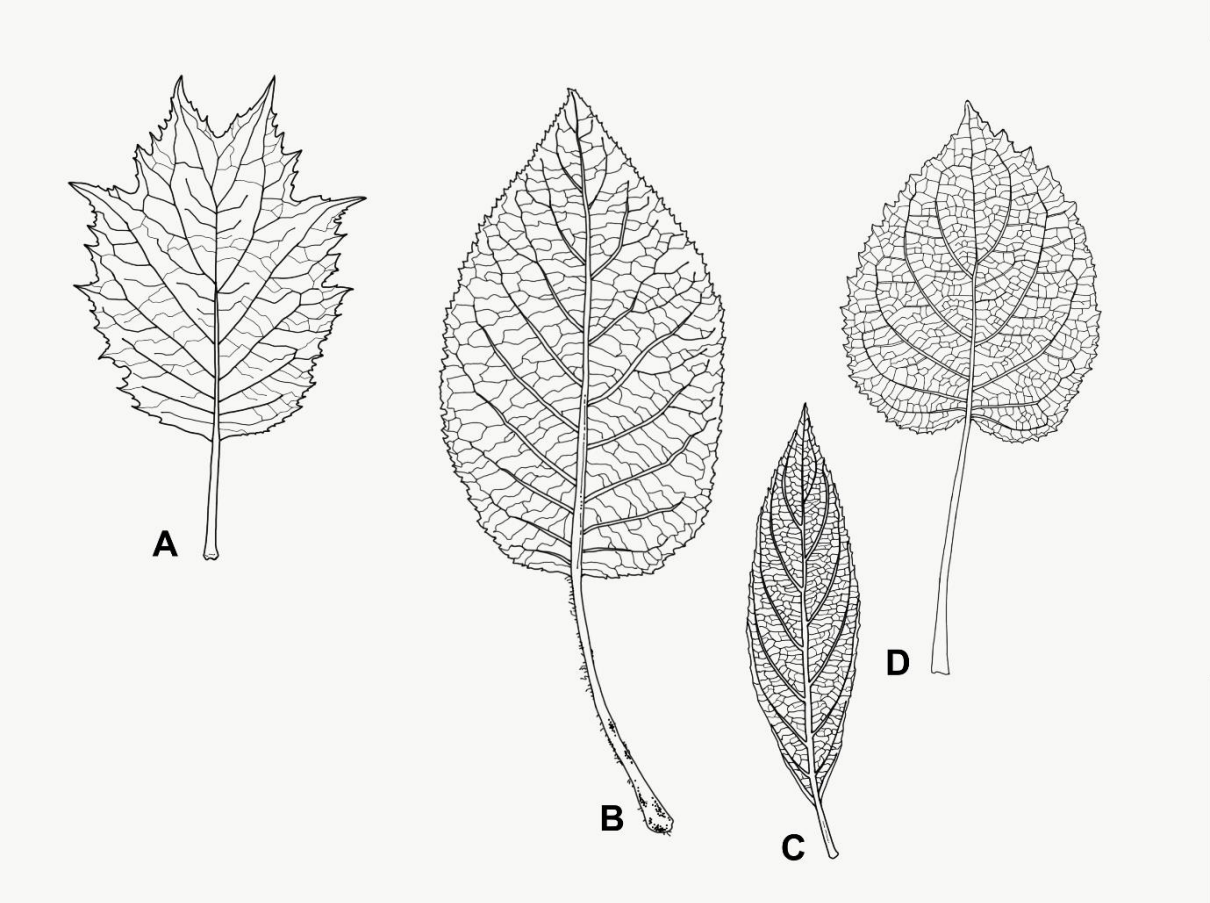


**Figure 5.2: Leaf shape diversity in *Hydrangea* sect. *Asperae*.** Several distinct leaf shapes exist in the section, with the pinnately lobed leaves of *H. sikokiana* (A) being easily recognizable. Other taxa, however, can show a continuous variation between several shapes. B: Oblong-ovate leaf with long, thick petiole covered in fleshy hairs, typical of *H. sargentiana.* C: Lanceolate leaf with short thick petiole, typical for *H. strigosa*. D: Ovate leaf with long slender petiole, typical for *H. longipes*. Figure created by the author and V. Henau.

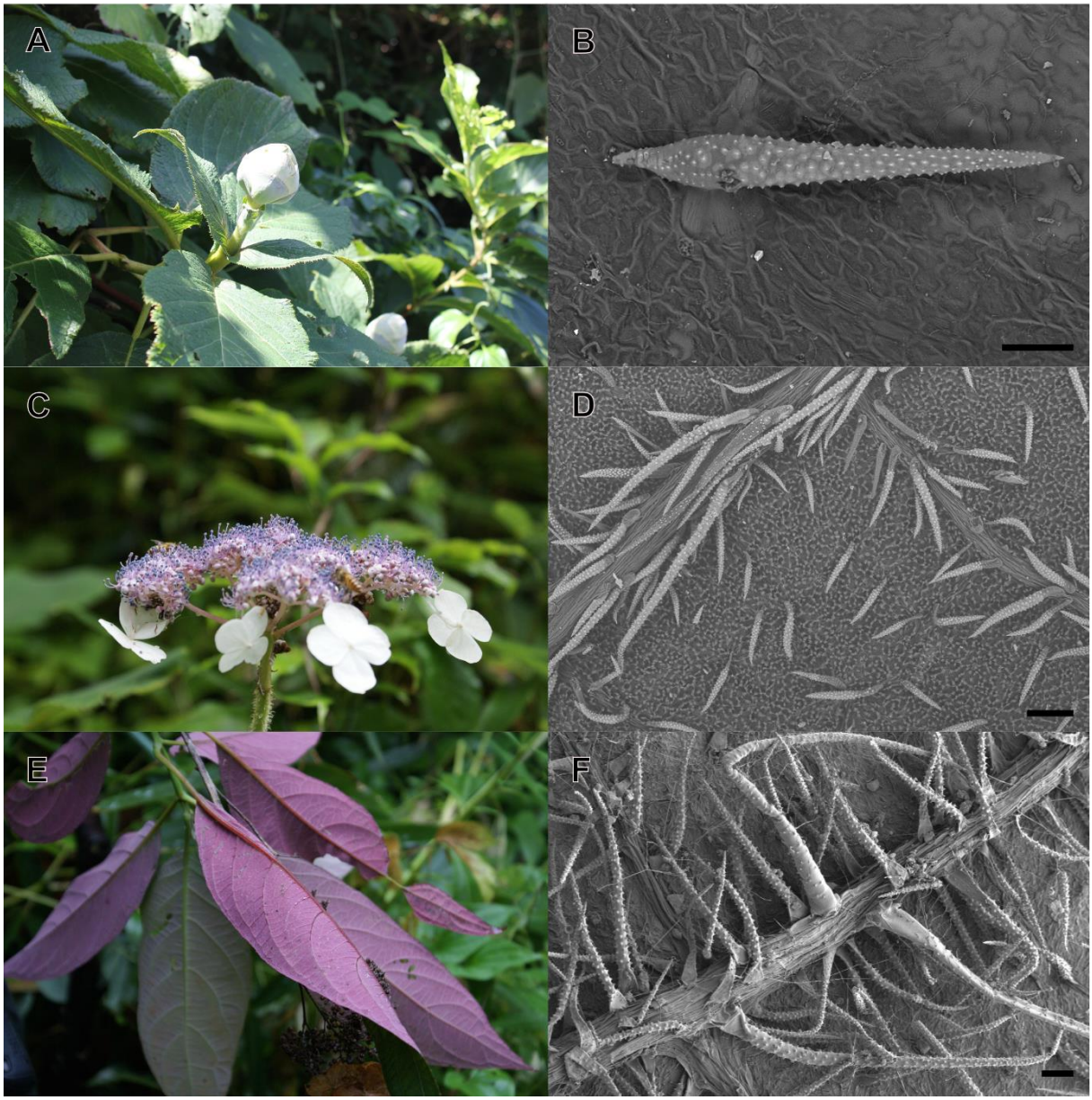


**Figure 5.3: Diagnostic characters for several taxa of *Hydrangea* sect. *Asperae*.** A: Young inflorescence of *H. involucrata*, showing the involucral bracts enveloping the young inflorescence. B: Scanning Electron Micrograph of branching hairs on the abaxial leaf surface of *H. longifolia*. C: Inflorescence of *H. sargentiana*. D: Scanning Electron Micrograph of the abaxial leaf surface in *H. strigosa*, showing appressed hairs, and white papillae. E: The purple colour of abaxial leaf surface, present in some *H. strigosa* specimens. F: Scanning Electron Micrograph of the abaxial leaf surface in *H. villosa*, showing the long, erect, villous hairs on the main veins.

Morphological identification key for the species of *Hydrangea* section *Asperae*:

*Hydrangea platyarguta* is not included in this identification key, as its aberrant morphology makes it easily recognizable.

1a. Leaves pinnately lobed (Figure 5.2A), endemic to Japan. ..... *H. sikokiana*
1b. Leaves not lobed, occurring in China, Japan, Taiwan, Nepal, India, Sikkim, Vietnam..... 2

2a. Young inflorescence enveloped in broadly ovate to rounded involucral bracts (Figure 5.3A), leaving scars at the base of the inflorescence when fully grown ..... 3
2b. Young inflorescence with lanceolate bracts, not completely enveloping the young inflorescence, leaving no notable scars at the base of fully developed inflorescences..... 4

3a. Petioles, branchlets and abaxial leaf surface exhibiting appressed, two-branched hairs (Figure 5.3B). Leaves lanceolate. Endemic to Taiwan..... *H. longifolia*
3b. Petioles, branchlets and abaxial leaf surface with appressed, simple hairs. Leaves ovate. Endemic to Japan ..... *H. involucrata*

4a. Petioles, branchlets and abaxial midveins of leaves covered in thick fleshy hairs (Figure 5.3C), the tips of which are split into two thin ends. Hairs greenish translucent with dark brown apexes when fresh, brownish yellow in dried specimens ..... *H. sargentiana*
4b. Petioles, branchlets and abaxial midveins with simple, non-branching hairs or glabrous. 5

5a. Petioles long and thin (Figure 5.2). Leaf lamina membranous, with tufts of white hairs visible to the naked eye present in the axils of lateral veins on abaxial surface ..... *H. longipes*
5b. Petioles thick and short or thick and long. Leaf lamina papery, no such white tufts present ..... 6

6a. Abaxial leaf surface covered with appressed hairs and white papillae, granting a whitish aspect to the leaf (Figure 5.3D). In some cases, the abaxial surface presents a purplish color when fresh (Figure 5.3E), fading to dull green or brown in herbarium specimens ... *H. strigosa*
6b. No such papillae present, abaxial leaf surface with villous erect or appressed hairs. Abaxial leaf surface light to darker green, retaining color or darkening to brown in dried specimens 7

7a. Petioles with erect hairs, either long and conspicuous or short, densely pubescent. Younger branches terete..... 8

7b. Petioles with short white to grayish appressed hairs or glabrous. Younger branches obscurely to notably 4-angled. Seeds with longitudinal veins only. ..... ..... *H. aspera*

8a. Long villous, erect hairs on petioles, branchlets, peduncles and abaxial leaf surface (Figure 5.3F). Hairs on abaxial midveins thicker, brownish translucent when fresh. Leaves lanceolate, younger branches terete. Endemic to China. ..... *H. villosa*

8b. Petioles and peduncles densely short pubescent, erect hairs yellowish to white in fresh specimens, darkening in dried specimens. Branchlets glabrescent, sometimes short erect hairs on young branchlets, never with long and villous hairs. Seeds with smaller, transverse veins between longitudinal veins. Endemic to Taiwan ..... *H. kawakamii*

1. ***Hydrangea sikokiana*** Maxim., Bulletin de l'Academie Imperiale des Sciences de St-Petersbourg, sér. 3 31: 42. 1887.

*Type information*. Tanaka s.n. collected in Japan, Honshu Island, Wakayama and Mie prefectures.

*Cytological data*. 2n = 36 (Funamoto & Tanaka, 1988).

*Morphological description*. Shrubs small, 1-2 m high. Branchlets, petioles and peduncles covered with appressed hairs. Petiole 2-18 cm long, leaf blade pinnately lobed, showing 4-6 lobes, 8-21 cm long, 8-20 cm wide. Leaves adaxially with scattered hairs along veins, abaxially with long and erect hairs exhibiting tubercles only visible under high magnification (above 50X). Inflorescences corymbose cymes, 12-30 cm wide. Young inflorescences with lanceolate to slightly ovate bracts 10-30 mm long, covering but not enveloping the inflorescence. Fully developed inflorescence with lanceolate bracts in axils of peduncles throughout inflorescence. Marginal flowers total diameter 1-3 cm, few and conspicuous, white, sepals 4, rounded. Central flowers small, white. Hypanthium 1-1,6 mm in length, calyx lobes 5, broadly deltoid. Petals 5, white, 2-4 mm long, truncate at base. Stamens mostly 10, but in some cases 8 or 9, filaments 3,5-5,5 mm long. Capsule apex truncate, 2-3 mm long. Styles 2,1-1,5 mm long. Seeds brown, ellipsoid, winged at both ends. Seed coat striate veined.

*Relationships*. Recovered as sister to all continental taxa of the section (excluding the Japanese *H. involucrata* and Taiwanese *H. longifolia*).

*Operational criteria*. Supported as a separate evolutionary lineage based on divergent morphology (lobed leaves), multilocus coalescent species delimitation based on plastid and low copy nuclear markers (De Smet et al., 2017) as well as RADseq data (chapter 4), reciprocal monophyly based on RADseq data (chapter 4), shared genetic variation (chapter 4).

*Discussion.* The first of only two *H.* sect. *Asperae* representatives endemic to Japan, *H. sikokiana* is easily distinguished from other species based on several morphological characters. It is most readily differentiated from *H. involucrata*, another Japanese *H.* sect. *Asperae* species, by the presence of pinnately lobed leaves and the absence of involucral bracts. Since lobed leaves only occur in one other taxon of the genus, the American species *H. quercifolia*, *H. sikokiana* is one of the most easily identifiable species within this section. Apart from this morphological

differentiation, *H. sikokiana* is supported to be molecularly divergent from its closest relative, *H. involucrata*, based on several molecular markers and delimitation algorithms (De Smet et al., 2017; chapters 3 and 4).

*Distribution in literature*. Japan; Honshu: Wakayama (Mt. Koya), Mie and Nara Prefecture (Tonomine, Mt. Odaigahara).

*Wild populations sampled in this study*. Japan; Shikoku: Tokushima Prefecture (Kamikatsu-cho)

2. ***Hydrangea involucrata*** Siebold. Novorum Actorum Academiae Caesareae Leopoldino-Carolinae Germanicae Naturae Curiosorum 14(2): 691. 1829.

*Type information*. Von Siebold collected in Japan. (L).

*Cytological data*. 2n = 30 (Mortreau et al., 2010; Cerbah et al., 2001; Funamoto & Tanaka, 1988).

*Morphological description*. Shrubs small, 1-2 m high. Branchlets, peduncles and petioles covered in appressed hairs. Branchlets terete. Petioles 1,5-8 cm long, leaf blade ovate, 10-26 cm long, 5-17 cm wide, adaxially with scattered appressed hairs along veins, abaxially with long and erect hairs exhibiting tubercles only visible under high magnification (above 50x). Inflorescences corymbose cymes, 10-15cm wide, bracts ovate, enveloping immature inflorescence before falling, leaving conspicuous scars at the bottom of the inflorescence. A stark contrast in color for peduncles and branch below these scars occurs. Marginal flowers few and conspicuous, purple, sepals 4, rounded, total diameter 1-3 cm. Central flowers small, purple. Hypanthium 1,2-1,5 mm in length, 5 calyx lobes, deltoid in shape, 0,2-0,6 mm. Petals 5, purple, 2-3 mm long, truncate at base. Ovary with 2 or 3 styles, 1-2 mm long. Capsule apex truncate, 3-4 mm long and with small erect translucent hairs. Seeds brown, ellipsoid, winged at both ends; seed coat striate veined.

*Relationships*. Recovered as closely related to *H. longifolia*, forming a clade sister to the rest of *H.* sect. *Asperae*.

*Operational criteria.* Supported as a separate evolutionary lineage based on divergent morphology (absence of branched hairs, involucral bracts), multilocus coalescent species delimitation using plastid and low copy nuclear markers (De Smet et al., 2017), as well as

RADseq data (chapter 4), reciprocal monophyly based on RADseq data (chapter 4), shared genetic variation (chapter 4).

*Discussion.* Endemic to Japan, *H. involucrata* is one of two *H.* sect. *Asperae* species showing involucral bracts, the other being *H. longifolia*. Prior to anthesis, the inflorescence is enveloped by tightly clustered, long, almost ovate bracts, giving the young inflorescence a ball-like appearance (Figure 5.3A). When these involucral bracts dehisce, they leave a row of clearly visible scars below the secondary branches of the inflorescence. These scars present an important way of differentiating the inflorescences from those present in the *H. aspera* species complex. This species is distributed throughout the Japanese island of Honshu, as well as the volcanic islands in the Philippine sea to the south of Tokyo (e.g. Oshima, Toshima, Niijima, Kozushima). Populations of *H. involucrata* on the more remote islands have sometimes been ascribed varietal status (e.g. *H. involucrata* var. *idzuensis*), but none of the analyses in this work have been able to corroborate this divergence. All molecular studies presented in previous chapters (De Smet et al., 2017; chapter 3 and 4) show a clear divergence between *H. involucrata* and the morphologically similar *H. longifolia*. Additionally, the latter taxon exhibits a unique type of pubescence on stems, petioles and leaves. This, together with their geographically distinct habitat, provides strong evidence for recognizing both taxa at the species level.

*Distribution in literature*. Japan; Honshu: Kanagawa Prefecture (Yokohama, Kawasaki, Kamakura, Miyanoshita, Hakone Park, Mt. Takao), Chiba Prefecture (Owari, Kiyozumi-yama), Fukushima Prefecture (Mt. Haguro), Gifu Prefecture (Norikura, Washiga-take), Gumma Prefecture (Ikaho), Nagano Prefecture (Usui-toge, Mt. Izuna, Mt. Tsubakura, Kuramoto, Kiso near On-take-san, Asamayama), Shiba Prefecture (Shinano), Tochigi Prefecture (Nikko), Tokyo Prefecture (Tokyo, Mt. Takao, Hachijo), Yamanashi Prefecture (Motsuko) and Shikoku

*Wild populations sampled in this study*. Japan; Honshu: Tokyo Prefecture (Hinohara, Hakone Park), Oshima island, Shiga prefecture (Gero city), Nagano Prefecture (Takamori-cho).

3. ***Hydrangea longifolia*** Hayata. Journal of the College of Agriculture, Imperial University of Tokyo 25(19): 91-92. 1908.

*Type information*. T. Kawakami & G. Nakahara 690 collected in Taiwan, Taitō Prefecture, Torokusha. (CAS!)

*Synonyms*. *Hydrangea involucrata* Siebold var. *longifolia* (Hayata) Y. C. Liu; *H. strigosa* Rehder var. *longifolia* (Hayata) Chun.

*Cytological data*. No data available.

*Description*. Shrubs 1-3 m high. Branchlets, petioles, leaf blades, and inflorescences densely appressed hairy with both simple and 2-branched hairs. Branchlets dark brown-red, terete or slightly obtusely angled near apex. Petiole thin, 1.5-2 cm long; leaf blade lanceolate, 10-20 × 3-4.5 cm, papery, adaxially with more 2-branched hairs than simple hairs, abaxially with fewer 2-branched hairs than simple hairs, secondary veins 8-10 on both sides of midvein, slender, abaxially prominent, base obtuse to cuneate, margin aristate serrulate, apex caudate-acuminate. Inflorescences corymbose cymes, ca. 9 × 11-14 cm; bracts ovate, ca. 2 × 1.5 cm, densely puberulous and enveloping immature inflorescence before falling, leaving conspicuous scars at the bottom of the inflorescence. A stark contrast in color for peduncles and branch below these scars occurs. Marginal flowers few, with sepals 4, elliptic to broadly ovate, 1.5-1.8 × 1.1-1.5 cm in fruit. Central flowers small, purple. Hypanthium 1-1,5 mm in length, 5 calyx lobes, deltoid in shape, 0,2-0,6 mm, white to whitish purple in color. Petals 5, purple, 2-3 mm long, truncate at base. Stamens 10, purple filament, globose, greenish to purple anther. Capsule apex truncate, ca. 3 × 3.5-4 mm, with simple hairs and a few 2-branched hairs, apex truncate; persistent calyx teeth triangular, ca. 0.5 mm; styles 2, persistent, erect to recurved, 1.5-2 mm, distally enlarged. Seeds brown, ellipsoid, compressed, ca. 0.5 mm, winged at both ends; wings 0.2-0.4 mm; seed coat striate veined.

*Relationships*. Recovered as closely related to *H. involucrata*, forming a clade sister to the rest of *H.* sect. *Asperae*.

*Operational criteria*. Supported as a separate evolutionary lineage based on divergent morphology (presence branched hairs on petioles and leaves, involucral bracts), multilocus coalescent species delimitation using plastid and low copy nuclear markers (De Smet et al., 2017) as well as RADseq data (chapter 4), reciprocal monophyly based on RADseq data (chapter 4), shared genetic variation (chapter 4).

*Discussion.* This species is morphologically similar to *H. involucrata*, but differing in the presence of branching, appressed hairs on the stems, peduncles and abaxial leaf surface (see chapter 2; De Smet et al., 2017). This taxon was often synonymized with *H. involucrata* (Chun, 1954; McClintock, 1957; Liu; 1976), based on the shared appearance of the young inflorescences. However, sufficient lines of evidence are available to recognize *H. longifolia* at the species level (De Smet et al., 2017; Chapter 3 and 4). One of two *H*. sect. *Asperae* representatives endemic to Taiwan, *H. longifolia* is easily identifiable in the field by the presence of the ball-shaped young inflorescence, which is lacking in *H. kawakamii*, the other Taiwanese endemic.

*Distribution in literature.* Taiwan; Taitou (Torokusha).

*Wild populations sampled in this study.* Taiwan; Yilan County (Kefang, Taiping Shan), Taichung municipality (Taroko National Park).

4. ***Hydrangea sargentiana*** Rehder. Plantae Wilsonianae an enumeration of the woody plants collected in Western China for the Arnold Arboretum of Harvard University during the years 1907, 1908 and 1910 by E.H. Wilson edited by Charles Sprague Sargent 1(1): 29. 1911.

*Type information*. E.H. Wilson 722 collected in China, Hubei Province, Xingshan Xian. (BM!)

*Synonyms. Hydrangea aspera* D. Don subsp. *sargentiana* (Rehder) E. M. McClintock.

*Cytological data*. 2n = 34 (Mortreau et al., 2010; Cerbah et al., 2001).

*Description*. Shrubs 2-3 m high. Branchlets, petioles, and peduncles with dense, brownish, semitranslucent, long, apically forked and acute hairs. Branchlets thick. Petiole thick, 3-9 cm; leaf blade abaxially gray-green to slightly purple when fresh, adaxially dark green, elliptic, oblong-ovate, or broadly ovate, 9–52 × 6–32 cm, submembranous to thinly papery, abaxially densely slightly curved villous, adaxially densely translucent strigose, secondary veins 8-11 on both sides of midvein, abaxially prominent, base rounded to shallowly cordate, margin irregularly triangular dentate to denticulate, apex acuminate. Inflorescences corymbose cymes, 10-16 cm wide, apex arcuate; branches numerous, crowded together at apex of peduncle. Marginal flowers few, with sepals 4, white, obovate-orbicular to broadly orbicular,

0.9-1.4 × 0.8–1.7 cm in fruit, margin entire. Central flowers with calyx tube campanulate, ca. 1 mm; teeth triangular, ca. 0.5 mm. Petals white to purplish blue, ovate, ca. 2 mm. Stamens unequal, some of shorter ones equaling petals, longer ones ca. 4 mm. Anthers purplish blue. Ovary inferior. Styles 2, ca. 1.5 mm in fruit; stigmas capitate, small. Capsule hemispheric, 3-4 mm in diam., apex truncate. Seeds brown, ellipsoid, slightly compressed, winged at both ends; seed coat striate veined.

*Relationships*. No genetic divergence detected from the closely related *H. longipes*. Forming a clade with the latter, sister to the rest of the continental species of *H.* sect. *Asperae*.

*Operational criteria*. No genetic support was recovered for separation from *H. longipes*. With respect to morphology and geographic distribution, both taxa are highly dissimilar.

*Discussion.* The presence of distinct fleshy trichomes with branched tips on stems, inflorescence and the larger veins of the abaxial leaf surfaces render this species easily recognizable. These morphological features are found to be autapomorphic and provide ample grounds for recognizing the taxon as a distinct species, when coupled with the molecular divergence from the rest of the group as found in chapters 3 and 4 (De Smet et al., 2017). Molecular data infer *Hydrangea sargentiana* as closely related to the morphologically divergent *H. longipes*. The latter nevertheless lacks the distinct fleshy trichomes, exhibits much thinner petioles and peduncles, among other distinguishing characteristics. Furthermore, *H. sargentiana* has a very limited geographical distribution, with the only known population being located in the Chinese province of Hubei, Xingshan Xian, in sympatry with the closely related *H. longipes*. Before the collections made within the framework of this thesis, only one collection of *H. sargentiana* was described, attributed to E.H. Wilson. Plants grown from this original collection can still be found in the Royal Botanic Garden Edinburgh. Subsequent collections of plants labeled *H. sargentiana* (or *H. aspera* subsp. *sargentiana*) are often attributable to *H. robusta*, lacking the characteristic fleshy trichomes (e.g. the specimen labeled *H. sargentiana*, collected by Kirkham, Flanagan, Howick & McNamara as SICH1801, see Appendix 4 (Table S5.1). Several herbarium specimens collected at the type location, and clearly attributable to *H. sargentiana* were collected during the course of the present study (Box 5.1), and are deposited in the herbarium GENT.

*Distribution in literature*. China; Hubei Province (Xingshan Xian).

*Wild populations sampled in this study.* China; Hubei Province (Xingshan Xian, Shennongjia)

**Box 5.1: Re-discovering *Hydrangea sargentiana*, a taxon in need of conservation action.**

This box is adapted from De Smet, Y., Larridon, I., Bauters, K., Goetghebeur, P., Wanke, S., Samain, M.S., 2015. Rediscovering *Hydrangea sargentiana*, a taxon in need of conservation action. *Acta Horticulturae* 1087: 221-224.

**Introduction**

Despite containing several popular garden ornamental shrubs, the genus *Hydrangea* (Hydrangeaceae, Cornales) still faces a plethora of taxonomical and systematic difficulties. Past studies have shown the eight other genera in tribe Hydrangeeae (*Broussaisia*, *Cardiandra*, *Decumaria*, *Deinanthe*, *Dichroa*, *Pileostegia*, *Platycrater* and *Schizophragma*) to be nested within *Hydrangea*, rendering the latter polyphyletic (Samain et al., 2010; Granados Mendoza et al., 2013). To alleviate this undesirable situation, De Smet et al. (2015a) proposed a novel classification, merging these satellite genera into *Hydrangea*. This new view on *Hydrangea* taxonomy succeeds in reflecting the close evolutionary relationships between some *Hydrangea* s.s. species and taxa previously classified in different genera. It is believed that this change of view will urge plant breeders to explore new combinations of closely related species for interspecific crossing experiments.

On a lower taxonomical level, the genus *Hydrangea* is riddled with uncertainty regarding species boundaries. For example, the last worldwide revision of the genus (McClintock, 1957) recognized three separate species in *Hydrangea* section *Asperae*; *H. involucrata*, *H. sikokiana* and *H. aspera*. The latter, predominantly Chinese, taxon was subdivided into four subspecies: subsp. *aspera*, subsp. *strigosa*, subsp. *robusta* and subsp. *sargentiana*. However, in the latest version of the Flora of China (Wei & Bartholomew, 2001), these subspecies are recognized as species, along with several other taxa that are not recognized at species level by McClintock (1957). Remarkably, in a footnote one of the authors suggests the treatment of several of these recognized species as one widespread, variable entity, merging several taxa recognized as species in the same manuscript (Figure 1.8). We believe this confusion regarding species boundaries in *Hydrangea* to be partially caused by the unknown morphological variability present in some taxa. In one particular taxon of section *Asperae*, studying this variability has been problematic because of the low number of available wild-collected specimens, and unrealiable identifications of herbarium material.

*Hydrangea sargentiana* was first collected by E.H. Wilson in Hubei province, China during his expeditions for the Arnold arboretum of Harvard University in 1907. The presence of conspicuous fleshy trichomes on stems and leaves (Figure 5.3C) prompted C.S. Sargent to recognize it as a distinct species in his Plantae Wilsonianae (Sargent, 1913). Living specimens grown from this first collection can still be found in the Royal Botanic Garden Edinburgh, as the voucher specimen in the herbarium clearly states its connection to Wilson's specimen (picture available at http://elmer.rbge.org.uk/bgbase/vherb/bgbasevherb.php?cfg=bgbase/vherb/zoom.cfg&filename=E00112994.zip&queryRow=2). However, in order to study the variability in this taxon, more wild collections should be available for study. Herbaria around the world hold specimens labelled *H. sargentiana*, which might indicate

other localities for this taxon, and a larger sample of individuals to grasp the variability inherent in this entity. The goals of this study were to verify the identity of the specimens labelled *H. sargentiana* in various herbaria, focussing on the presence of the fleshy trichomes as a diagnostic character. Furthermore, the geographic data present on the type material collected by Wilson was used to explore Hubei, China for any extant populations of this taxon, thus aiming to collect more wild-origin material for *H. sargentiana*. A molecular study, comparing the wild collected specimens to the type specimen and other related taxa has also been undertaken, and results from this study will be published elsewhere.

**Materials and methods**

Herbarium specimens labelled *H. sargentiana* were acquired from different herbaria (CAS, WU, P, K, US) and compared to the type specimen (Wilson, nr. 772), as well as the living individual at Royal Botanic Gardens Edinburgh. For this, the morphology and pubescence of leaves and stems was documented, as well as the shape of fertile flowers and fruits.

In order to find an extant population of *H. sargentiana*, the area described in Wilson's work, Hsing-shan Hsien, Hubei, China, was explored, as well as the surrounding areas.

**Results and discussion**

All herbarium specimens labelled *H. sargentiana* collected subsequent to Wilson's expedition lack the conspicuous fleshy trichomes characteristic for this taxon. These specimens are probably referable to *H. robusta*, an allied species of section *Asperae*. Unravelling the relation between these two taxa, as well as their species status will require a more in depth morphological and molecular study. This work has been undertaken at the Research Group Spermatophytes and will be published elsewhere. Specimens labelled *H. sargentiana* which do exhibit the fleshy trichomes are always labelled "cultivated plant", and often a reference to the individuals grown at Edinburgh is made.

Exploration of the locality mentioned on the label of the type specimen for *Hydrangea sargentiana* resulted in the re-discovery of a moderately sized, diffuse population strongly resembling the type. This morphotype seems limited to a very small area in Shennongjia, Hubei province, China at an altitude between 1300-1700m. The wide surroundings of this area were explored, but no other populations of this taxon were found. In total, herbarium material and silica-gel dried leaves were collected from 25 specimens spread across the putative *H. sargentiana* population. These specimens are stored in the Ghent University Herbarium (De Smet & Bauters 1437-1440, 1443, 1445-1454, 1468-1472, 1474-1475)

**Conclusion**

As no other localities or earlier collections for *Hydrangea sargentiana* were discovered in this study, this taxon is believed to exhibit a very narrow geographic distribution. Therefore, actions ensuring the conservation of this unique taxon are highly desirable. Cooperation with Botanic Gardens Conservation International (BGCI) has been established in order to take the necessary steps for the conservation of wild populations of *H. sargentiana*. Studies regarding the species status of *H. sargentiana* are being undertaken, using this newly collected material to study the morphological and molecular variability of the taxon.

5. ***Hydrangea longipes*** Franch. Nouvelles archives du muséum d'histoire naturelle, sér. 2 8: 227-228. 1885.

*Type information*. David s.n. collected in China, South-east Xizang province, Mupin. (HT & IT: P!)

*Synonyms*. *H. longipes* var. *longipes* Wei & Bartholomew; *Hydrangea aspera* D. Don var. *longipes* (Franchet) Diels; *H. discocarpa* C. F. Wei; *H. hemsleyana* Diels; *H. hemsleyana* var. *pavonliniana* Pampanini; *Hydrangea* longipes var. fulvescens (Rehder) W.T. Wang ex. C.F. Wei; *Hydrangea fulvescens* Rehder; *H. fulvescens* var. *rehderiana* (C. K. Schneider) Chun; *H. rehderiana* C. K. Schneider; *H. longipes* var. *lanceolata* Hemsley.

*Cytological data*. No cytological data available.

*Description*. Shrubs 1-3 m tall. Branchlets yellowish to brown, terete, pubescent. Petiole 3-15 cm, thin, sparsely pilose to subglabrous; leaf blade usually greenish on both surfaces when dry, lanceolate, oblong-ovate or -obovate, broadly ovate, or broadly obovate, 4-22 × 3-12 cm, membranous to papery, abaxially sparsely appressed pubescent, or densely tomentose-villous with hairs spreading, brown, longer, and thicker along secondary veins and especially midvein. Long erect, white hairs forming tufts in the axils of midvein and secondary veins. Adaxial leaf surface sparsely strigose, secondary veins 6-8 on both sides of midvein, abaxially elevated, base broadly cuneate, truncate, or shallowly cordate, margin irregularly roughly serrate, apex acute to acuminate. Inflorescences corymbose cymes, 7-20 cm wide, apex truncate to slightly arcuate; branches short, densely shortly hairy, hairs thick. Marginal flowers few, with sepals 4, white, obovate, broadly so, or suborbicular, 0.8-2.2 × 0.9-2.2 cm, margin entire or few denticulate. Central flowers with calyx tube cupular; teeth triangular, ca. 0.5 mm long. Petals white, oblong-ovate. Stamens 10, unequal; anthers broadly oblong to subglobose. Ovary inferior. Styles 2, usually recurved, 0.5-1.5 mm in fruit. Capsule cupular, 2.5-3.5 mm in diam., apex truncate. Seeds brownish, narrowly ellipsoid to oblong-obovoid, rarely subglobose, compressed, shortly winged at both ends; seed coat striate veined.

*Relationships*. No genetic divergence detected from the closely related *H. sargentiana*. Forming a clade with the latter, sister to the rest of the continental species of *H.* sect. *Asperae*.

*Operational criteria*. No genetic support was recovered for separation from *H. sargentiana*. Morphologically and in geographic distribution both taxa are highly dissimilar.

*Discussion.* This species is easily recognizable by the length and appearance of the petioles. The lower leaf surface shows white tufts of hair in the axils between the midvein and secondary veins, a character unique within *H.* sect. *Asperae*. As depicted in chapters 3 and 4 (De Smet et al., 2017), this morphotype exhibits low molecular divergence from *H. sargentiana*, from which it differs in the absence of the distinct fleshy trichomes, smaller leaves, thinner petioles and generally smaller inflorescences. These morphological differences, along with differences in geographic distribution (*H. longipes* occurs throughout Hubei, while only a single population of *H. sargentiana* is known) represent enough evidence to consider both morphotypes as independent evolutionary lines, and therefore species (De Smet et al., 2017; chapters 3 and 4). The name *H. longipes* was described independently by Franchet (1885) and Hemsley (1887). In studying the type specimens, no morphological distinction between the two was found that would warrant recognition of two different species. The small differences in leaf shape and pubescence fall within the phenotypic variation found in *H. longipes*.

*Subdivisions*. Several varieties have been described to accommodate the variability in pubescence of the abaxial leaf surface. However, in studying wild populations and herbarium specimens, it is obvious that multiple intermediate forms exist, connecting these clear-cut variabilities. In order to avoid confusion in the placement of these intermediate forms, no varieties are formally described here. The diagnostic characters provided here for *H. longipes* are sufficient to recognize the independently evolving metapopulation lineage linked to this published name. Therefore, no further subdivisions possibly confounding this link to evolutionary history are necessary.

*Distribution in literature*. China; Sichuan Province (Mupin, Wa-ssu Xian, Wan-chuan Xian, Sungpan, Lungan Fu, Nanchuan); Hubei Province (Chan-lo Xian, North and South of Yichang, Patung, Xingshan Xian).

*Wild populations sampled in this study*. China; Hubei Province (Dalaoling, Shennongjia, Xingshan Xian, Langping)

6. ***Hydrangea villosa*** Rehder. Plantae Wilsonianae an enumeration of the woody plants collected in Western China for the Arnold Arboretum of Harvard University during the years 1907, 1908 and 1910 by E.H. Wilson edited by Charles Sprague Sargent 1(1): 29-30. 1911.

*Type information*. E.H. Wilson 1227 collected in China, Western Hubei, Fang Xian, 1200-1800 m. (A!)

*Synonyms. H. villosa* Rehder; *H. villosa* var. *delicatula* Chun; *H. villosa* f. *sterilis* Rehder; *H. villosa* var. *strigosior* (Diels) Rehder; *H. villosa* var. *velutina* (Rehder) Chun.

*Cytological data*. 2n = 34 (Mortreau et al., 2010).

*Description*. Shrubs 1-3m high. Branchlets, petioles and leaf blades covered in long villous hairs, light translucent to brownish red in fresh specimens, darker in dried specimens. Branchlets reddish-brown, terete. Petioles thick, 1-4 cm long, leaf blade elliptical to obovate-lanceolate, 10-20 cm long and 3,5-6,5 cm wide. Adaxially with appressed to erect tapering long hairs, base swollen. Abaxial surface with long villous hairs, those on midvein thicker, brownish translucent when fresh, darker in dried specimens. Leaf margins denticulate, not lobed. Marginal flowers 3-4 cm diameter and purplish, petals 4, obovate, with denticulate margin. Central flowers purple. Hypanthium 1-2 mm long, 5 calyx lobes oblong-ovate in shape, 2 mm long, purplish in color. Petals 5, purple, about 2mm long, truncate at base. Stamens 10, globose purplish anther. Styles 2, capsule 2,5-3 mm diameter, with apex truncate. Seeds brown, ellipsoid, winged at both ends; seed coat striate veined.

*Relationships*. All *H.* sect. *Asperae* topologies inferred to data are unable to completely resolve the position of *H. villosa*. It is however supported to be closely related to two other clades, one containing *H. strigosa* (Hubei lineage) and *H. kawakamii*, and another containing *H. aspera*, *H. robusta* and *H. strigosa* (Sichuan lineage).

*Operational criteria*. Supported as a separate evolutionary lineage based on divergent morphology (long erect hairs on peduncles, petioles and branchlets) multilocus coalescent species delimitation using plastid and low copy nuclear markers (De Smet et al., 2017) as well as RADseq data (chapter 4) and reciprocal monophyly based on RADseq data (chapter 4).

*Discussion.* This Chinese species was synonymized with *H. aspera* in several revisions (McClintock, 1957; Wei & Bartholomew, 2001), owing to the limited morphological differences between both taxa. However, variation in both chloroplast and nuclear markers (De Smet et al., 2017; chapter 3 and 4) is sufficient to warrant recognition of *H. villosa* as independent species. Furthermore, pubescence of lower leaf surface in *H. villosa* is different from close relatives such as *H. aspera* and *H. strigosa* (chapter 2; De Smet et al., 2017). Indeed, *H. villosa* is the only species in *H*. sect. *Asperae* showing long erect hairs on the main veins of the abaxial leaf surface as well as the petioles and peduncles.

*Distribution in literature.* China; Sichuan Province (Wa-ssu Xian, Wen-chuan Xian, Pan-lan-shan, West of Kuan Xian).

*Wild populations sampled in this study*. China; Hubei Province (Wufeng Xian).

7. ***Hydrangea kawakamii*** Hayata. Journal of the College of Agriculture, Imperial University of Tokyo 25(19): 90–91, pl. 8. 1908.

*Type information*. Kawakami & U. Mori nr. 1875 collected in Taiwan, mt. Morrison. (CAS !)

*Cytological data*. 2n = 36 (Mortreau et al., 2010).

*Description*. Shrubs 1-3 m high. Young branchlets, petioles, and inflorescences densely yellow-brown pubescent. Branchlets dark gray, terete, glabrescent. Petiole 2-9 cm; leaf blade oblong-ovate to elliptic, 9-12 × 4.5-10 cm, papery, abaxially densely covered in erect hairs showing conspicuous tubercles, adaxially sparsely strigose, secondary veins 6 or 7 on both sides of midvein, abaxially prominent, base broadly cuneate to rounded, margin irregularly doubly serrate, apex acute to shortly acuminate. Inflorescences corymbose cymes, lax, 10-14 cm wide, apex truncate to slightly arcuate. Marginal flowers with sepals 3 or 4, suborbicular, 1-2 cm long, margin acutely dentate. Central flowers with calyx tube cupular, ca. 1.5 mm long; teeth broadly triangular, ca. 1 mm long. Petals oblong-ovate, ca. 2 mm. Stamens 10, unequal, longer ones ca. 5 mm; anthers subglobose, ca. 0.5 mm long. Ovary inferior. Styles 2 (or 3), ca. 1.5 mm long in fruit. Capsule hemispheric, 2-3 × 3-4 mm, apex truncate. Seeds fusiform, shortly winged at both ends; seed coat striate veined with thin, transverse veins in-between.

*Relationships*. Recovered as sister to *H. strigosa* (Hubei lineage).

*Operational criteria*. Supported as a separate evolutionary lineage based on divergent morphology (long erect hairs on peduncles, petioles and branchlets) multilocus coalescent species delimitation using plastid and low copy nuclear markers (De Smet et al., 2017), as well as RADseq data (chapter 4) and reciprocal monophyly based on RADseq data (chapter 4).

*Discussion.* An endemic shrub found at higher altitudes (above 2000m) in Taiwan, morphologically similar to *H. aspera* with large, ovate leaves and erect hairs on lower leaf surface. This taxon was synonymized with *H. aspera* by both McClintock (1957) and Bartholomew (Wei & Bartholomew, 2001) based on these, and other (highly similar inflorescence) similarities. However, molecular data suggest significant divergence between the Taiwanese and mainland taxa (De Smet et al., 2017; chapters 3 and 4). This divergence is mirrored by a subtle morphological difference: the seed coat in *H. kawakamii* is striate with smaller transverse veins in between the larger ones, creating a reticulate pattern, which is absent in *H. aspera*. The pattern is best observed in young seeds, at a minimal magnification of 50x. Although morphological differences are minute, the molecular divergence, together with the geographic isolation of *H. kawakamii*, provide sufficient evidence for its recognition as separate species.

*Distribution in literature.* Taiwan; Nantou County (Yu Shan).

*Wild populations sampled in this study*. Taiwan; Yilan County, Taichung Municipality.

8. ***Hydrangea strigosa*** Rehder. Plantae Wilsonianae an enumeration of the woody plants collected in Western China for the Arnold Arboretum of Harvard University during the years 1907, 1908 and 1910 by E.H. Wilson edited by Charles Sprague Sargent 1(1): 31-32. 1911.

*Type information*. E.H. Wilson nr. 765 collected in Western Hubei, north and south of Yichang. (HT: A!, IT: E!, US!, W!)

*Synonyms*. *Hydrangea aspera* D. Don var. *angustifolia* Hemsley; *H. aspera* var. *macrophylla* Hemsley; *H. aspera* var. *sinica* Diels; *H. aspera* subsp. *strigosa* (Rehder) E. M. McClintock; *H. strigosa* var. *angustifolia* (Hemsley) Rehder; *H. strigosa* var. *macrophylla* (Hemsley) Rehder; *H.*

*strigosa* var. *purpurea* C. C. Yang; *H. strigosa* var. *sinica* (Diels) Rehder; *H. strigosa* f. *sterilis* Rehder; *Premna merinoi* Léveillé.

*Cytological data*. 2n = 34 (Cerbah et al., 2001).

*Description*. Shrubs 1-3 m tall. Branchlets gray-brown, terete or obscurely 4-angled, densely strigose; bark usually peeled off into fragments. Petiole 1-7 cm, strigose; leaf blade abaxially whitish green or sometimes purplish red to reddish when fresh but gray-brown to gray-green in dried specimens, adaxially black-brown, oblong, ovate-lanceolate, or obovate-lanceolate, 8-28 × 2-10 cm, papery, abaxially densely covered in white papillae and gray-white strigose, adaxially sparsely strigose to subglabrous, secondary veins 7-10 on both sides of midvein, abaxially prominent, base obtuse, cuneate, or rounded, margin serrulate, apex acuminate. Inflorescences corymbose cymes, to 28 cm wide, apex slightly arcuate; branches spreading, gray-white strigose. Marginal flowers with sepals 4 or 5, white to purplish red, broadly ovate, broadly elliptic, suborbicular, or broadly orbicular, margin entire to denticulate. Central flowers with calyx tube campanulate, ca. 2 mm long; teeth triangular, ca. 0.5 mm. Petals purplish red, oblong-ovate, 2-2.5 mm. Stamens 10, unequal, 3-6 mm; anthers oblong, ca. 0.5 mm. Ovary inferior. Styles 2, erect to recurved, slightly clavate, ca. 2 mm in fruit. Capsule urn-shaped, 3-3.5 mm in diameter, apex truncate. Seeds brown, broadly ellipsoid, 0.3-0.5 mm long, winged at both ends; wings 0.2-0.3 mm long; seed coat striate veined.

*Relationships*. The Hubei lineage, which is linked to the type location, and therefore carries the published name (De Smet et al., 2017; Chapter 3 and 4), is recovered as sister to *H. kawakamii*. A second lineage coinciding with the *H. strigosa* morphotype is recovered in Sichuan, where it is closely related to *H. aspera* and *H. robusta*.

*Operational criteria*. Supported as a separate evolutionary lineage based on divergent morphology (abaxial leaf surface exhibiting white papillae and strigose hairs) multilocus coalescent species delimitation using plastid and low copy nuclear markers (De Smet et al., 2017), as well as RADseq data (chapter 4) and reciprocal monophyly based on RADseq data (chapter 4).

*Discussion.* When this species occurs in allopatry from the closely related *H. aspera* and *H. robusta*, it is clearly identifiable as an independent evolutionary lineage based on both

morphological and molecular data (De Smet et al., 2017; chapter 3 and 4). However, when occurring in sympatry, gene flow has been suggested to occur between these morphotypes, obscuring their genetic divergence. In these areas (e.g. Sichuan) morphology of specimens varies along an altitudinal gradient. Specimens referable to *H. strigosa* occur at lower altitudes (500-1200 m), while *H. aspera* and *H. robusta* populations grow at higher altitudes (900-4000 m and 1800-2800 m, respectively). Populations of morphotypes clearly ascribable to each of these nominal taxa can be observed, nevertheless a plethora of intermediate forms can be found in between these "pure" morphs. These intermediate populations can exhibit a pubescence on their abaxial leaf surface somewhat in between the typical appressed strigose hairs and more erect, villous hairs, leaf shapes between lanceolate and obovate, or combine the absence of white papillae on the lower leaf surface with typical *H. strigosa* pubescence and leaf shape. The presence of these papillae seems to be a reliable character to identify specimens belonging to the independent *H. strigosa* lineage. Indeed, they do not occur in intermediate populations, and are clearly observable in all *H. strigosa* specimens occurring in allopatry from the closely related species.

*Distribution*. China; Hubei Province (North and South of Yichang Xingshan Xian, Patung Xian, Fang Xian, Packang, South of Wushan), Sichuan Province (Omei Shan, Nanch'uan, Shan-tzu-p'ing)

*Wild populations sampled in this study.* China; Hubei Province (Shennongjia, Nanyang, Muyuping, Wufeng, Langping)

9. ***Hydrangea aspera*** D. Don. Prodromus florae Nepalensis, 211. 1825.

*Type information*. Buchanan, s.n. collected in Nepal: Narainhetty. (BM!)

*Cytological data*. 2n = 36 or 34 (Cerbah et al., 2001).

*Synonyms*. *Hydrangea aspera* f. *emasculata* Chun; *H. aspera* var. *strigosior* Diels; *H. aspera* var. *velutina* Rehder; *H. glabripes* Rehder; *H. coacta* C.F.Wei; *H. robusta* J. D. Hooker & Thomson, J. *Hydrangea aspera* D. Don subsp. *robusta* (J. D. Hooker & Thomson) E. M. McClintock; *H. longialata* C. F. Wei; *H. maximowiczii* H. Léveillé; *H. rosthornii* Diels; *H. rotundifolia* C. F. Wei.

*Hydrangea robusta* var. *griffithii* C.B. Clarke ; *Hydrangea oblongifolia* Blume ; *Hydrangea aspera* var. *scabra* Rehder.

*Description*. Shrubs or small trees, usually 1-4 m, but can be up to 10m in height. Young branches and peduncles with yellow-brown, short, erect hairs, or grayish-white appressed hairs. Petioles with appressed hairs or glabrous. Branchlets with brown bark, terete to conspicuously 4-angled. Petioles can be thick, short or long, ranging from 1 to 15 cm. Leaf lamina lanceolate, elliptic, oblong or any intermediate shape, 5-35 cm long, 2-22 cm wide, papery to the touch. Adaxial leaf surface sparsely or densely strigose, abaxial surface with either gray-white appressed hairs, or yellowish-brown erect hairs. Leaf margin irregularly serrate or doubly so, apex acute to acuminate. Inflorescence corymbose cymes, ranging from 8 to 30 cm in fruit, peduncles can be 4-angled and very thick to terete and less thick. Marginal flowers greenish white to pinkish, purple or reddish-purple, 4 or 5 lobes which are broadly ovate, 1-3,8 cm long, 0,9-3,5 cm wide, dentate, serrate, crenulate or entire. Central flowers with calyx tube cupular, 1-1,5 mm long, lobes triangular to ovate, 0,5-1 mm long. Petals purple to purple-red, 1,5-2,5mm long, ovate-lanceolate to ovate. Stamens 10-14, usually unequal, anthers purple to purple-red. Ovary completely inferior, 2 or 3 styles spreading to recurved, 1-2 mm. Capsules with apex truncate, 3-5mm. seeds fusiform, winged at both ends, 0,4-0,5 mm; striate veined.

*Relationships*. Closely related to *H. strigosa* (Sichuan lineage), part of an unresolved relationship between with two other clades; one containing *H. kawakamii* and *H. strigosa* (Hubei lineage) and another consisting of *H. villosa* (De Smet et al., 2017).

*Operational criteria*. The merging of morphotypes *H. robusta*, *H. strigosa* and *H. aspera* into a single lineage is supported by multilocus coalescent species delimitation based on plastid and low copy nuclear markers (De Smet et al., 2017), as well as RADseq data (chapter 4) and shared genetic variation (chapter 4). Morphologically intermediate forms connecting populations of each of the abovementioned morphotypes suggest heavy gene flow or introgression.

*Discussion.* The remaining nominal taxa classified in *H.* sect. *Asperae* can be seen as forming an intricate species complex. Several distinct morphotypes have been described, mainly differing in the shape and pubescence of leaves and petioles. However, various intermediate

phenotypes can be identified, connecting described nominal taxa. Further population level studies are required in order to determine the nature of these intermediate populations. One interpretation could be that they represent contact zones between previously diverged entities, experiencing heavy gene flow and introgression which obscures species boundaries. Another interpretation invokes phenotypic plasticity of a single evolutionary lineage, caused by differing conditions at increasing altitude. For the lack of biological explanation for this variation, previous authors have differed in their interpretation of species boundaries in the complex, arbitrarily assigning morphotypes the status of species, subspecies or variation (e.g. Rehder, 1911; McClintock, 1957; Wei & Bartholomew, 2001). The interpretation of the boundaries between the constituting species represents one of the main reasons for taxonomic confusion within the section, a situation exacerbated by the lack of insight into molecular variation within the group. Employing several nuclear and plastid markers (De Smet et al., 2017; chapter 3), as well as RADseq data (chapter 4), several morphotypes previously placed in the *H. aspera* species complex could be identified as constituting separate evolutionary lineages. However, for specimens identifiable as morphospecies *H. aspera* and *H. robusta*, no genetic divergence could be inferred. As populations of these nominal taxa often occur in sympatry, and populations exhibiting an intermediate morphology are widespread in their contact zone, recognizing both as independent evolutionary lineages is impossible given the presently available data. Nevertheless, it needs to be noted that morphologically distinct populations can be found, exhibiting the "typical" phenotypic characters as described for each of the morphospecies. Furthermore, in a study by Cerbah et al (2001), individuals identified as *H. aspera* and *H. robusta* showed differing chromosome numbers (2n=36 and 2n=34, respectively). Further investigation into population genetics in contact zones, and species delimitation studies including the type locations for both taxa could further elucidate this apparent species complex. With the currently available data, however, a morphologically variable species *H. aspera* is recognized, including both typical *H. aspera* and *H. robusta* morphotypes, in addition to all intermediate forms.

*Subdivisions*. By merging *H. robusta* into *H. aspera* the resulting taxon exhibits a wide range of morphological variability. The morphotypes recognizable as these former species are easily recognized, but connected by a variety of intermediate morphologies. Recognizing *H. robusta*

as a subspecies of *H. aspera* would instigate confusion regarding the identity of these intermediate forms, which is why no subspecies are formally described here.

*Distribution in literature*. China; Gansu Province, Guangxi Province, Guizhou Province, Hubei Province, Hunan Province, Jiangsu Province, Shaanxi Province, Sichuan Province, Yunnan Province. India. Nepal. Sikkim. Nepal.

*Wild populations sampled in this study*. China; Sichuan Province (Niba Shan, Hailuogou, Lingguan, Tongla Shan)

10. ***Hydrangea platyarguta*** Y.De Smet & Granados. Taxon 64 (4), pp. 741-753. 2015

*Type information.* c.s. 14776 collected in China, Zhejiang.

*Synonyms. Platycrater arguta* Siebold & Zuccarini; *Platycrater arguta* var. *typica* C.K. Schneid.

*Cytological data.* 2n = 34 (Funamoto & Nakamura, 1988).

*Description*. Shrubs 0.5-3 m tall. Branchlets brown, subglabrous. Petiole 1-7 cm long; leaf blade lanceolate to elliptic, 9-15 × 3-6 cm, membranous to papery, both surfaces pubescent or adaxially subglabrous, secondary veins 7-9 on both sides of midvein, slender, abaxially slightly prominent, base narrowly cuneate, slightly decurrent, margin roughly serrate to serrulate. Inflorescence subglabrous; bracts linear. Marginal flowers with sepals 3 or 4, broadly ovate, connate from base to middle and forming a triangle or square 2.5-2.8 cm in diameter in fruit, translucent and thinly net veined. Central flowers with calyx tube turbinate, 4-5 mm; teeth 4 or 5, triangular-ovate to narrowly triangular, 4-5.5 mm, to 7 mm in fruit. Petals ovate, ca. 7 mm. Filaments filiform; anthers subglobose, ca. 1 mm in diam. Styles slender, ca. 1 cm in fruit; stigmas small. Capsule 8-9 mm, apically 6-8 mm in diam., striate. Seeds dark brown, compressed ellipsoid, 0.6-0.8 mm, thinly striate, shortly winged.

*Relationships*. The phylogenetic hypothesis proposed by De Smet et al. (2015a) shows this species to be part of sect. *Asperae*, in an unsupported (PP: 0.8) sister relationship with the Japanese *H. sikokiana.*

*Operational criteria*. The unique morphology detailed above provides evidence for the recognition of this taxon as independent evolutionary lineage.

*Discussion.* In order to create an infrageneric classification reflecting evolutionary relationships, sect. *Asperae* should include this morphologically unique taxon. Further research is required to confirm the exact relationships between *H. platyarguta* and the other taxa contained in the section, which might be complicated by the high level of molecular divergence. Evidence of the latter is found in the long branches recovered in the chloroplast based phylogenetic hypothesis by De Smet et al. (2015a, chapter 2), and the inability to amplify certain nuclear regions with sect. *Asperae* specific primers.

*Distribution*. China; Anhui Province, Fujian Province, Jiangxi Province, Zhejian Province. Japan.

*Wild populations sampled in this study.* No wild populations of this taxon were sampled within the framework of this study..

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Chapter VI

# General discussion

The overarching goal of this doctoral thesis can be summarized as increasing the evolutionary understanding of the genus *Hydrangea* at two levels. At the higher taxonomic level, the evolutionary history of the genus itself needed to be unraveled, with possible consequences for genus and higher- level classification. At a lower taxonomic level, species boundaries and phylogenetic relationships within *Hydrangea* sect. *Asperae* were in need of stabilization, after consecutive shifts in interpretation. For both these levels, the ever-increasing body of molecular tools available to biologists offered several interesting pathways, some of which had not been previously explored in the genus. This allowed for the evaluation of the usefulness of these methods in *Hydrangea* evolutionary research, and Spermatophytes as a whole. Advances made in these fields are presented in the following paragraphs, highlighting the contribution of this PhD to the evolutionary insight in the genus *Hydrangea* at the two abovementioned levels. Inevitably, integrating these results with the existing taxonomic and systematic situation in *Hydrangea* encountered several ongoing philosophical discussions regarding reconciliation of modern, molecular data-driven and traditional morphology-based taxonomy. Insights gained from navigating these often-opposing views have been summarized for each of the main research lines of this thesis, being the conundrum of unraveling polyphyletic or paraphyletic genera, and the issue of species delimitation.

**Advances in creating a stable classification for tribe Hydrangeeae**

Ever since the conception of tribe Hydrangeeae by Hufford et al. (2001) based on a combination of morphological (Hufford, 1997) and molecular (Soltis et al., 1995) data, the genus *Hydrangea* has been suggested to be polyphyletic. These earlier studies, however, were unable to garner sufficient support to draw strong conclusions regarding phylogenetic relations within the tribe. Subsequent studies sought to clarify the evolutionary history of the tribe, expanding on taxon sampling (Samain et al., 2010) or testing new markers for use in phylogenetic reconstruction (Granados Mendoza et al., 2013). Combining the findings of these recent studies, the current work was able to present the most comprehensive and supported phylogenetic hypothesis for tribe Hydrangeeae to date. Including representatives for all genera contained within the tribe, the current study achieved sufficient support to serve as a basis for proposing a new classification of tribe Hydrangeeae, reflecting evolutionary relationships. Moreover, as all sections making up the genus *Hydrangea* (McClintock, 1957) were represented by several taxa, a new infrageneric classification could be proposed (De Smet et al., 2015a; chapter 2). As earlier studies suggested, tribe Hydrangeeae was found to consist of eight monophyletic genera (*Broussaisia*, *Cardiandra*, *Decumaria*, *Deinanthe*, *Dichroa*, *Pileostegia*, *Platycrater* and *Schizophragma*), nested within the largely polyphyletic genus *Hydrangea*. Since each of these genera represent morphologically very distinct taxa, the then current classification of the tribe was in line with traditional conceptions of biological classification. Indeed, in this type of classifications, the hierarchy of ranks was used to represent relative levels of morphological divergence, not evolutionary relatedness (Kolanowska et al., 2016). However, since the emergence of evolutionary thinking and the increasing availability of molecular data, proposals were made to bring biological classifications in line with the evolutionary history of taxa (Hennig, 1965, 1966). This idea, as originally envisioned by Hennig, would require a complete rebuild of the current taxonomic and nomenclatural system. Indeed, in order for a taxonomic system to truly reflect phylogeny, its various rules and principles must be formulated in terms of the central tenet of evolution. One of these proposed systems is the PhyloCode (de Queiroz & Gauthier, 1990, 1992), which has not gained general acceptance, being the subject of some philosophical debate (de Queiroz & Donoghue, 2011, 2013; Platinck, 2012). Nevertheless, one of the central ideas in Hennig's proposal, the adherence to monophyletic taxa as a first step towards a phylogenetically

informed taxonomy seems commonly accepted by contemporary biologists (Xiang et al., 2012). Some discussion does remain regarding the acceptance of paraphyletic taxa, as some authors claim this type of assemblage reflects similarity and practicality (evolutionary systematics, e.g. Hörandl & Stuessy, 2010). Polyphyletic assemblages - groups of taxa not encompassing the most recent common ancestors of all constituting taxa - are however rejected by all sides in this argument. Undeniably, reconciliation of such assemblages with the evolutionary idea of common decent is not possible, rendering it an unwanted property of any taxon in a phylogenetically informed classification. Therefore, the current study proposed a new classification for tribe Hydrangeeae, addressing the polyphyletic nature of *Hydrangea*. The goals for this new classification were to reflect evolutionary relationships, and to be stable in the face of possible small changes in Hydrangeeae phylogeny. Indeed, as a limited number of nodes in the phylogeny of the tribe remain unresolved, future studies might affect evolutionary relationships, albeit to minor effect in most of the group. For these reasons, the most stable approach was deemed to merge the eight satellite genera into *Hydrangea*. The resulting larger genus is strictly monophyletic and is stable with regards to small changes in tribe Hydrangeeae phylogeny, such as resolution of the unsupported position of the type *H. arborescens* within the genus. Section names can be used to conserve the link to the well-known names of the satellite genera where possible, facilitating acceptance in horticulture. As could be seen in chapter 2 (De Smet et al., 2015a), the genus *Hydrangea* is subdivided into sections which largely coincide with those proposed by McClintock (1957), taking care to only recognize monophyletic taxa.

**Difficulties encountered in proposing a novel classification for tribe Hydrangeeae**

Reception of the proposed changes in classification followed the discrepancy discussed by de Queiroz & Gauthier (1992) concerning the acceptance of the evolutionary framework in phylogenetics versus nomenclature. Several authors accepted our proposed *Hydrangea* classification (Lin & Chung, 2017; Sodusta, 2019; Samain et al., 2019), while others accepted the presented phylogenetic hypothesis without explicitly following the nomenclatural changes (Wiedemann et al., 2015; Fu et al., 2019). In contrast, Ohba & Akiyama (2016) proposed to segregate several genera from this wider interpretation of *Hydrangea* (hereinafter referred to as *Hydrangea s.l.*), in order to rescue the morphologically recognizable genera

published by Engler (1890). No argument based on an evolutionary framework was provided, except for the statement that the phylogenetic hypothesis published in De Smet et al. (2015a) was followed. Remarkably, the authors propose separating five genera out of *Hydrangea* s.l., not discussing their views on the merger of the other former satellite genera. Indeed, these changes would render the genus *Hydrangea* polyphyletic, still containing the formerly recognized genera *Dichroa*, *Deinanthe*, *Pileostegia*, *Decumaria*, *Schizophragma*, *Broussaisia* and the former *Hydrangea* s.s. sections *Stylosae* and *Hydrangea* (Figure 6.1). Therefore, the classification system resulting from Ohba & Akiyama (2016) is unable to inform evolutionary relationships among its constituents. One of the counterintuitive features of this system would be that certain species retained in the genus *Hydrangea* would exhibit a smaller genetic distance to species in other genera than to other members of *Hydrangea*. For example, *Hydrangea stylosa* (part of *H.* sect. *Stylosae*) would be more closely related to *Hortensia chinensis* (formerly *Hydrangea chinensis*) than to *Hydrangea arborescens*. This proposal, however, did find limited support (Huang et al., 2018).

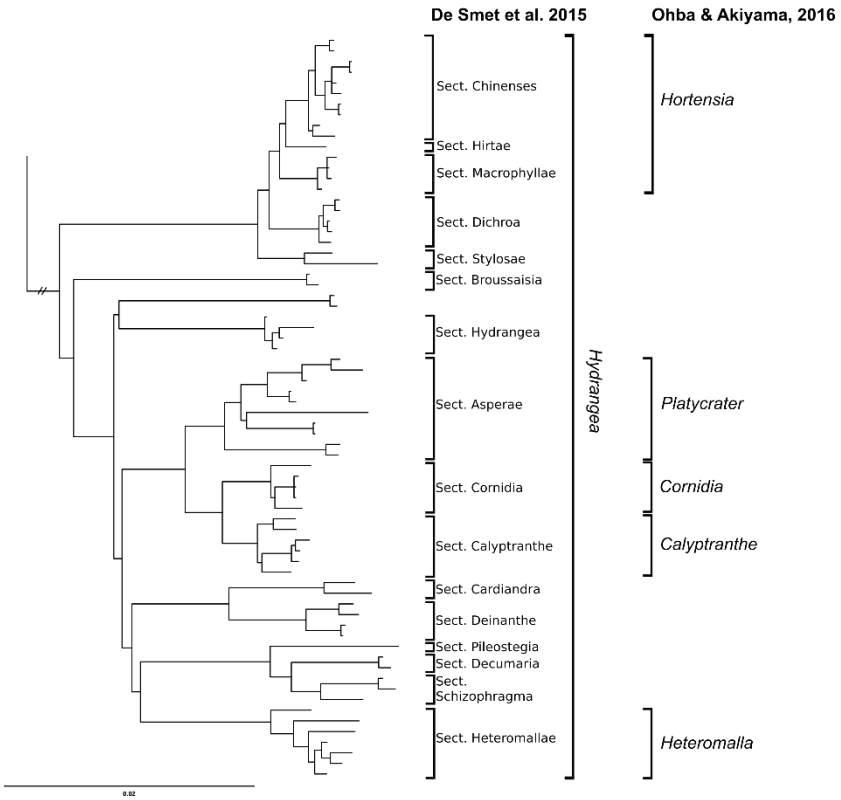

**Figure 6.1: classifications for tribe Hydrangeeae as proposed by De Smet et al. (2015a) and Ohba & Akiyama (2016).** Two classification schemes compared to the phylogenetic hypothesis inferred by De Smet et al. (2015a). The full topology, including support values and tip labels is presented as Figure 2.3.

### Advances in creating stable species boundaries in *Hydrangea* sect. *Asperae*

Owing to different interpretations of the taxonomic relevance for several diagnostic characters, a wide variation existed in the number of species recognized in *Hydrangea* sect. *Asperae* (Table 6.1) among and even within revisions (Rehder, 1911; McClintock, 1957; Wei & Bartholomew, 2001). At least part of this confusion can be ascribed to the lack of alternative lines of evidence, as well as the absence of explicit reference to the species concept adhered to. Without explicit adherence to a species concept, and whose characters or divergence therein are considered necessary to recognize separate species, the placing and moving of species boundaries by different authors is rendered highly subjective. In order to generate species boundaries as explicit hypotheses, based on several lines of evidence, this thesis adheres to the general lineage concept of species (de Queiroz, 1998). Therefore, species are equated to segments of independently evolving metapopulation lineages. During their divergence they may or may not evolve the various contingent properties employed by other species definitions (e.g. intrinsic reproductive isolation, distinct ecological niches, or fixed morphological character state differences), and they do not need to possess any of these properties to be considered species (de Queiroz, 2011). Nevertheless, these properties, which form the basis for the disagreements among rival species definitions, remain important in three ways. Firstly, they remain the empirically observable, albeit non-essential properties of species, termed "operational criteria". Since the central tenant of the general lineage concept is impossible to observe directly (lineages are evolutionary independent), these criteria form lines of evidence towards interpreting whether sampled individuals belong to diverging species or not. For example, fixed character state differences and reciprocal monophyly are unlikely to be maintained unless the lineages in which they occur are evolving independently of one another (de Queiroz, 2011). Secondly, these operational criteria might provide insight into the mechanisms driving or maintaining differentiation between species. Indeed, occupying different ecological niches, or displaying reproductive incompatibility, might provide strong hypotheses regarding the causal factors behind species diverging. Lastly, documenting which operational criteria form the basis for certain species hypothesis might

provide extra information regarding the biology of the taxon. In some cases, it could be relevant to only compare species who have achieved reproductive isolation, or show enhanced molecular divergence, in order to set up conservation schemes.

Adhering to this species concept, several potential lines of evidence were gathered in order to address species boundaries in *Hydrangea* sect. *Asperae*. With the aim of identifying fixed character states differentiating potential species, type specimens, protologues and previous revisions of the section were examined. Through these comparisons, pubescence of stems, petioles and abaxial leaf surface was identified as an important diagnostic feature, as described in chapter 3 (De Smet et al., 2017). In order to evaluate the operational criteria of reciprocal monophyly, and the newly developed coalescent based species delimitation (Yang & Rannala, 2010), molecular markers exhibiting sufficient variability within *H.* sect. *Asperae* were needed. Primers for the amplification of three chloroplast regions (trnV-ndhC IGS, rpl32-ndhF IGS, trnL-rpl32 IGS and ndhA intron) developed by Granados Mendoza et al. (2013, 2015) could be utilized. However, primers and amplification protocols for three nuclear regions (TIF3H1, SMC1-44, SMC1-22) were designed specifically for the section studied here. Additionally, the nuclear ITS region was shown to exhibit sufficient variation for species delimitation purposes. In order to compare these markers generated by traditional Sanger sequencing with the High-throughput based RADseq approach, the same dataset was employed in a second species delimitation study employing this novel technique (chapter 4). Moreover, SNPs generated in the latter study could be employed in the operational criteria of population structuring, as implemented in the STRUCTURE (Pritchard et al., 2000) algorithm. Considering these various lines of evidence, several well-supported species could be hypothesized within *H.* sect. *Asperae*. Receiving support as an independently evolving lineage from all tested operational criteria are the Japanese taxa *H. involucrata* and *H. sikokiana*, as well as the Taiwanese *H. longifolia*. For these three taxa, the overwhelming support gathered across the studies in chapters 3 and 4 signifies a high support in their status as species. For the nominal taxa *H. longipes* and *H. sargentiana*, none of the molecular-based operational criteria were able to provide support for their status as independent from one another. However, invoking their divergent morphology, and the fact that the single known population of *H. sargentiana* maintains its distinct morphotype in sympatry with the widespread *H. longipes*, provides evidence for their status as independent lineages. The hypothesis of these two

nominal taxa representing separate species could be further tested by examining their possibility to interbreed. Nevertheless, the current recognition of *H. sargentiana* as separate (morpho)species is pivotal in the conservation of this unique pool of variation within the genus, as it is currently known from a single, relatively small population (De Smet et al., 2015b; box 5.1). Nominal taxa *H. villosa* and *H. kawakamii* are supported as separate evolutionary lineages by most of the operational criteria examined here (reciprocal monophyly in the SNP data generated from RADseq, coalescent species delimitation based on both RADseq and traditional marker data). Both taxa were merged into *H. aspera* by previous revisions (e.g. McClintock, 1957; Bartholomew in Wei & Bartholomew, 2001), owing to differences in interpretation for subtle morphological differences. The current study, however, provides ample evidence for the recognition of both nominal taxa as segregate species, exemplifying the need for multiple lines of evidence in order to create stable species boundaries. For the nominal taxa *H. aspera*, *H. robusta* and *H. strigosa*, all available data pointed towards the recognition of a morphologically variable species consisting of the morphotypes relatable to *H. aspera* and *H. robusta*, and a species exhibiting the morphology ascribed to *H. strigosa*. Interestingly, when both occur in sympatry, gene flow has been observed, resulting in populations with intermediate morphologies, and shared molecular variation (see STRUCTURE analysis in chapter 4). Although hypothesizing *H. strigosa* as a separate species receives sufficient support from molecular-based operational criteria, the other hypothesized species remains in need of additional study. Part of the issues remaining with the interpretation of the *H. aspera* – *H. robusta* – *H. strigosa* species complex stem from the challenges of reconciling modern taxonomic insights with traditional taxonomy, as discussed further in the next paragraph.

**Table 6.1: species recognized within *Hydrangea* section *asperae* by different revisions.** Both authors of the Flora of China (FOC) (Wei & Bartholomew, 2011), explicitly mention different opinions regarding species status for several taxa. Furthermore *H. sikokiana* and *H. involucrata* are not mentioned in FOC, since this revision only pertains to Chinese taxa.

| McClintock (1957) | FOC (Wei) (2011) | FOC (Bartholomew) (2011) | This thesis |
|---|---|---|---|
| *H. sikokiana* | | | *H. sikokiana* |
| *H. involucrata* | | | *H. involucrata* |
| | *H. longifolia* | *H. longifolia* | *H. longifolia* |
| *H. aspera* subsp. *sargentiana* | *H. sargentiana* | *H. sargentiana* | *H. sargentiana* |

| | | | |
|---|---|---|---|
| | *H. longipes* | | *H. longipes* |
| | | | *H. villosa* |
| | *H. kawakamii* | | *H. kawakamii* |
| *H. aspera* subsp. *strigosa* | *H. strigosa* | *H. strigosa* | *H. strigosa* |
| *H. aspera* subsp. *aspera* | *H. aspera* | *H. aspera* | *H. aspera* |
| *H. aspera* subsp. *robusta* | *H. robusta* | *H. robusta* | |
| | *H. coacta* | | |
| | | | *H. platyarguta* |

**Challenges in reconciliating traditional taxonomic practices and molecular species delimitation**

The last few decades have seen an increase in the number of molecular methods aimed at species delimitation (summarized in: Sites & Marshall, 2003; Camargo & Sites, 2013), complementing a primarily morphological approach to taxonomy. In some cases, however, conflicts might arise between traditional taxonomic practices and genetic methods for species discovery and validation.

A first conflict is situated in the discovery of new, possibly cryptic diversity. Indeed, the discovery of new species based solely on molecular data is insufficient for formal taxonomic descriptions (e.g. Leache & Fujita, 2010; Bauer et al., 2010; Fujita & Leache, 2011), since morphology-based diagnoses are still required by nomenclatural codes. Despite being easily rectified by providing morphological diagnoses for discovered taxa, this conundrum endures for cryptic species, where morphological characters are not an adequate proxy for species boundaries. Applied to the current study of *Hydrangea*, no new cryptic species were discovered, although one nominal taxon (*H. strigosa*) seems to split into two independent lineages, irrespective of morphological characters. As described in more detail in chapters 3 and 4, one of these lineages is suggested to result from heavy gene flow between specimens identifiable as *H. strigosa*, *H. aspera* and *H. robusta*.

A second conflict relevant to the present study is related to assigning sampled individuals to nominal taxa in the Linnaean nomenclatural framework. Since published names are only inextricably linked to a type specimen and a verbose diagnostic description, newly collected specimens can exclusively be linked to published names by way of morphological identification. Although the case has been made by several authors (e.g. Cao et al., 2016; Gemeinholzer et al., 2020) to include diverse sources of information into species descriptions,

including sequence data, this is not a requirement under any of the nomenclatural codes. Therefore, when testing the validity of existing species boundaries through genetic methods, a level of uncertainty persists in deciding whether the studied lineage contains the type specimen. Per extension, the formally published binomial applied to the lineage remains uncertain. Availability of type specimen sequence data for systematic research, as is often the case in fungi (e.g. De Crop et al., 2017), could alleviate this uncertainty. Nevertheless, in plant systematics, this is rarely available. As an alternative strategy, genetic material can be obtained from populations morphologically identical to the type, residing at the type location. This strategy is nonetheless predicated on the persistence of these populations since description of the taxon, as well as their accessibility. Species in *H.* sect. *Asperae* were described before the widespread availability of sequence data. Linking lineages identified through molecular-based species delimitation algorithms to published names was therefore impeded in several cases.

Several morphospecies in *H.* sect. *Asperae* are distinguished from closely related taxa based on limited morphological divergences. Assignment of sampled individuals to these taxa is therefore highly subjective. This situation is exacerbated by the often-limited details in which protologues describe the distinguishing diagnostic character states. Unsurprisingly, many of these morphospecies have been merged into related taxa by subsequent authors and revisions. For example, the nominal taxon *H. coacta* is described as closely related to *H. aspera*, with slightly differing pubescence of the abaxial leaf surface. Based on this description, even after studying the type specimen, none of the collected specimens could be appointed to this nominal taxon. Furthermore, herbarium specimens identified as *H. coacta* did not differ sufficiently or consistently with specimens referable to *H. villosa*. Therefore, no material for molecular studies which could be assigned to *H. coacta* with high levels of certainty could be collected, and the merging of this taxon with *H. villosa* is based primarily on morphological similarity. A possible alleviation for this issue would be the collection of fresh material for DNA extraction from the type location of *H. coacta*, if it is still accessible. This would add another line of evidence (for example Bayesian species delimitation) for assessing the species status of the taxon.

In some cases, type locations can become inaccessible due to geopolitical reasons. This is the case for the type location of *H. aspera* var. *velutina*, which is located in a region with restricted access for foreigners. Since no material for DNA extraction could be acquired for this nominal taxon, taxonomic decisions are based solely on morphological comparison of the type, descriptions and new collections.

The *H. aspera* – *H. robusta* – *H. strigosa* species complex exemplifies the difficulties associated with acquiring sequence data reliably linked to nominal taxa. During fieldwork, it was possible to collect specimens identifiable as *H. strigosa* from various locations, including the type location. For *H. aspera* and *H. robusta*, however, specimens complying morphologically with the descriptions and type specimens were gathered, but collecting at the type location was beyond the scope of the current PhD (type locations are situated in Nepal and India, respectively). Interestingly, molecular data recovered two lineages in *H. strigosa* (see chapters 3 and 4), one of each larger region of collection. One lineage was linked to the type location, and therefore clearly associated with the formally published binomial. The other lineage, however, was not molecularly distinct from the specimens identified as *H. robusta* and *H. aspera* which occur in sympatry. Since an independent lineage associated with the name *H. strigosa* was identified based on molecular data, and showed a distinct morphology, this was interpreted as sufficient evidence to recognize *H. strigosa* at the species level. For the other lineage no molecular divergence between the morphospecies *H. strigosa*, *H. robusta* and *H. aspera* led to assigning this lineage to *H. aspera*. However, since each of these nominal taxa is only linked to the collected specimens based on morphology, it is possible that both *H. aspera* and *H. robusta* form independent lineages when occurring in allopatry from their close relatives, as is inferred to be the case for *H. strigosa*. Further confidence in species boundaries within the complex can be achieved by sampling at the type location for both taxa, which are described to occur in allopatry.

**Conservation perspectives**

Fieldwork in the Chinese Provinces Sichuan and Hubei allowed to assess the conservation status of *H.* sect. *Asperae* in its center of diversity. Since these species generally occur along steep mountain or valley slopes, the majority of the sampled populations only experienced minor anthropogenic influences. Nevertheless, several populations documented in literature

(e.g. *H. strigosa* on mt. Emei) could not be sampled due to conversion of natural forest. In other areas, development of tourist or industrial infrastructure might encroach on natural habitats of *H.* sect. *Asperae*. The taxa most vulnerable to these threats are those exhibiting a limited geographic distribution. In this regard, fieldwork in conjunction with taxonomic and phylogenetic study identified two taxa vulnerable to the increasing deforestation linked to timber trade, agriculture or industry in China (Volis, 2018). Both of these taxa (*H. villosa* and *H. sargentiana*) were previously merged into the widespread taxon *H. aspera* (Table 6.1). However, as shown in chapters 3, 4 and 5, both taxa merit recognition at the species level as they represent independent evolutionary lineages. In order to conserve these unique pools of variation within the genus *Hydrangea*, steps should be undertaken to protect their limited distribution areas. Fortunately, part of the area where the only known *H. sargentiana* population occurs already resides within a protected forestry area.

**Future perspectives**

The current study proposed a new classification for the genus *Hydrangea* (De Smet et al., 2015a; chapter 2) based on a largely resolved phylogenetic hypothesis. Nevertheless, several nodes within the topology remain unsupported. Future studies attempting to increase support and resolution at this level will require additional molecular markers. With the increasing availability of High-throughput sequencing tools for phylogenetic studies, several approaches are available to further the evolutionary understanding of tribe Hydrangeeae. For example, RAD sequencing data have been successfully adopted to resolve phylogenetic relationships in taxa where traditional molecular markers provided insufficient variation (e.g. Eaton et al., 2016; Vargas et al., 2017; Wagner et al., 2018; Clugston et al., 2019). Alternatively, the low copy nuclear markers utilized in chapter 3 (De Smet et al., 2017) could be used on the scale of the genus if the *H.* sect. *Asperae*-specific primers are adapted to different clades. Integrating these results into the classification proposed here (De Smet et al., 2015a; chapter 2), would increase the evolutionary content of tribe Hydrangeeae classification.

Species hypotheses proposed in chapters 3 and 4 (De Smet et al., 2017) are based on an expansive set of operational criteria (de Queiroz, 1998), and could therefore be considered as highly supported. However, the *H. aspera* – *H. robusta* – *H. strigosa* species complex might benefit from a population-level approach in elucidating their evolutionary history. A study

aiming to address this complex could benefit from the insights provided in chapter 4, where the RADseq technique was tested for applicability in *H.* sect. *Asperae*. Resolving this recalcitrant species complex would, however, require samples to be taken from the type location of each of its constituent nominal taxa, to gage whether these taxa represent independent evolutionary lineages when in allopatry. Next, the intermediate morphological forms occurring when these taxa grow in sympatry need to be identified. A rich sampling of individuals covering a wide altitudinal gradient on several Chinese mountain slopes was collected during fieldwork within the frame of the current study. These specimens were used here to assess whether the reported morphological intermediates existed, but could be used in a more population level gene-flow study.

Application of the general lineage concept of species to *Hydrangea* sect. *Asperae* has brought clarity to several contested nominal taxa (e.g. *H. longifolia* which was considered as a form of *H. involucrata*). Therefore, other sections in the genus could benefit from the same treatment. Using newly published molecular markers (De Smet et al., 2017) or novel sequencing techniques (e.g. RADseq, chapter 4), well-supported species hypotheses could be generated for other sections in need of a revision. For example, during the testing of chloroplast and nuclear molecular markers (chapter 2), one nominal taxon classified in *H.* section *Heteromallae* was found to contain two distinct, well-supported clades. It is therefore proposed that this section could be a candidate for the same treatment as *H.* sect. *Asperae*, providing well-supported species boundaries.

**General conclusions**

The study presented in this doctoral thesis aimed at increasing the understanding of evolutionary relationships and boundaries within and around the genus *Hydrangea*. In order to gain these insights, a representative sample of taxa classified in the Hydrangeaceae tribe Hydrangeeae were collected, and a well-resolved phylogenetic hypothesis was generated. At the lower taxonomic level, species boundaries within *Hydrangea* section *Asperae* were tested, by sampling wild populations and, where possible, type locations.
At the genus level, the inferred phylogenetic hypothesis corroborated the polyphyletic nature of the genus *Hydrangea*. This in turn highlighted a conflict between the traditional, morphology-based concept of the genus and the evolutionary relationships with its closely

related sister genera of tribe Hydrangeeae. To re-align the classification with these new evolutionary insights, a novel classification was proposed, merging the eight satellite genera *Broussaisia*, *Cardiandra*, *Decumaria*, *Deinanthe*, *Dichroa*, *Pileostegia*, *Platycrater* and *Schizophragma* into *Hydrangea*. The proposed classification was however not unanimously accepted by authors working in the group, exemplifying the difficulty in uniting traditional classifications with molecular insights.

At the level of species boundaries, a targeted sampling of representatives of *H.* section *Asperae* allowed for the testing of species boundaries in this group according to different operational criteria. Integrating these lines of evidence within the framework of the general lineage concept of species ensures that these recognized species (independent evolutionary lineages) can be treated as falsifiable hypotheses. With the available samples and data, it can be concluded that *H.* sect. *Asperae* contains ten recognizable species, coinciding with the nominal taxa *H. sikokiana*, *H. involucrata*, *H. longifolia*, *H. sargentiana*, *H. longipes*, *H. villosa*, *H. kawakamii*, *H. strigosa*, *H. aspera* and *H. platyarguta*. Testing these hypotheses using the massive parallel sequencing technique RADseq, further corroborated the findings of the sanger-sequencing based study. Furthermore, the utility of this novel technique for species delimitation in the genus *Hydrangea* was confirmed.

## Summary

The Cornales family Hydrangeaceae contains two tribes, Philadelpheae and Hydrangeeae. The latter consists of the genus *Hydrangea*, containing several well-known garden ornamentals, and eight smaller satellite genera: *Broussaisia*, *Cardiandra*, *Decumaria*, *Deinanthe*, *Dichroa*, *Pileostegia*, *Platycrater* and *Schizophragma.* Through consecutive morphology- and molecular-based studies into tribe Hydrangeeae phylogenetic relations, a consistent pattern of a para- or polyphyletic genus *Hydrangea* emerged. These observations were further corroborated by studies utilizing a more expansive sampling of taxa and genetic markers, focused specifically representing each genus and subgeneric taxon classification unit in the tribe. These studies highlighted the incongruence between tribe Hydrangeeae classification and evolutionary relationships among its constituent taxa. Additionally, the infrageneric classification scheme for *Hydrangea*, as proposed in the most recent revision of the genus is at least in part incompatible with the most recent phylogenetic hypotheses. Most notably, this classification splits the genus into two sections, *Hydrangea* and *Cornidia*, while this bifurcation is not supported by any of the available phylogenetic evidence. Furthermore, evolutionary cohesion for several subsections (subsect. *Asperae*, subsect. *Americanae* and subsect. *Macrophyllae*) could not be supported by any of the available studies.

Within the genus *Hydrangea*, species boundaries are obscured by widely differing interpretations of morphological variability among and within subsequent revisions. One of the more striking cases can be found in *H.* subsect. *Asperae* (*H.* sect. *Asperae* in the classification proposed here) in which the last worldwide revision of the genus recognizes three species. One of these species, *H. aspera* is recognized as being a widespread, morphologically variable taxon, containing four subspecies to organize this variation. Other authors, however, recognize these subspecies at the species level, along with several other morphotypes previously described in the *H. aspera* species complex.

The current thesis aims to alleviate the abovementioned challenges faced by the genus *Hydrangea*, exploring the applicability of novel molecular techniques and algorithms to address following aims: 1) inferring a robust phylogenetic hypothesis for tribe Hydrangeeae, 2) proposing a new classification scheme for tribe Hydrangeeae and the genus *Hydrangea*, 3) identifying molecular markers containing sufficient variability for species level studies within

the genus *Hydrangea*, using both traditional Sanger, and High-throughput sequencing, 4) amassing several independent lines of evidence to generate stable species boundaries for *Hydrangea* subsection *Asperae* within the framework of the general lineage concept of species. To address these issues, a representative sample of herbarium and fresh specimens was assembled, partly by making collections of wild populations during this study.

Chapter 1 provides the general framework within which this thesis is situated. Furthermore, several concepts pivotal to later chapters are described in short. Finally, the general aims of this study are outlined.

Chapter 2 proposes a new classification for tribe Hydrangeeae based on an extensive phylogenetic hypothesis. To achieve this a representative sampling of taxa contained in the tribe was assembled, comprising of at least one accession for all nine genera, and multiple samples for each infrageneric unit of classification in *Hydrangea*. Sequencing four noncoding plastid regions previously shown to be phylogenetically informative for the tribe, in addition to the ribosomal ITS, a highly resolved phylogeny could be inferred. Since the sampling contained the type species for each of the genera and infrageneric taxa, the resulting phylogenetic hypothesis could be used to propose a new classification. This classification merged all satellite genera into genus *Hydrangea*, creating a monophyletic genus. This type of taxa are considered preferable, since they reflect the evolutionary history of the contained species. An infrageneric classification was proposed, in which monophyletic sections coincide with previously recognized genera, retaining these recognizable names where possible.

Chapter 3 presents a multilocus coalescent-based species delimitation for *Hydrangea* sect. *Asperae*, comparing these results with morphologically defined species boundaries. Several of the molecular markers necessary for the coalescent based species delimitation algorithm were specifically designed for this study, and comprised three low copy nuclear markers. In addition, four plastid regions and ribosomal ITS were sequenced. The acquired sequences were used to generate species trees, to be used as a base for a multilocus, coalescent-based species delimitation algorithm. The results from this analysis were combined with morphological characters within the framework of the general lineage concept of species to identify independent evolutionary lineages in the studied group. One of these morphological characters, adaxial leaf pubescence, was studied in detail and documented using scanning

electron microscopy. The identified lineages corresponded with the nominal taxa *H. sikokiana*, *H. involucrata*, *H. longifolia*, *H. longipes*, *H. sargentiana*, *H. villosa*, *H. kawakamii*, *H. aspera* and *H. strigosa*. The latter of these presented a challenge in that two lineages identifiable as *H. strigosa* were found, only one of which could be linked to the type specimen. The other was hypothesized to be the result of hybridization between the closely related taxa *H. aspera* and *H. robusta*.

Chapter 4 explores the applicability of RADseq to phylogenetic reconstruction and species delimitation in *H.* sect. *Asperae*. Despite the issue of low and uneven coverage across the sampled individuals, the acquired SNPs and RAD loci could be used in several species delimitation algorithms. Since the sampling of specimens for this chapter was almost identical to that of chapter 3, efficiency of both methodologies could be compared. Additionally, data generated in both studies could be analyzed conjointly to propose highly supported species hypotheses for *H.* sect. *Asperae*. These supported species are identical as those recovered in the previous chapter, albeit more insight is gained into their evolutionary background.

Chapter 5 provides formal species descriptions based on the integration of results from the previous two chapters. Species garnering sufficient lines of evidence are provided with a morphological description, as well as a formal taxonomic treatment allocating previously published names to recognized taxa. In addition, a morphological key and discussion of collected and studied specimens are provided.

Chapter 6 discusses the general advances this thesis provides to tribe Hydrangeeae taxonomy and species boundaries in *H.* sect. *Asperae*. Challenges and conflicts arising from reconciling molecular-based evolutionary insights with more traditional views on *Hydrangea* taxonomy are outlined. At the level of the tribe classification these conflicts were situated around the recognition of para- or polyphyletic taxa. Since the current work focusses on the evolutionary relationships in the group, a brief rationalization for the adherence to monophyletic taxa is presented. At the species level, the general lineage concept of species provides a robust theoretical framework for the nature of the entity of "species". By following this species concept, the species delimited here represent well-supported hypotheses, backed-up by several objective lines of evidence.

In conclusion, the work summarized in this thesis provides additional insight into tribe Hydrangeeae evolutionary history. A new classification is proposed for the tribe, merging the eight satellite genera into the larger genus *Hydrangea*, rendering the latter monophyletic. Through amassing molecular and morphological evidence, stabile species boundaries are proposed in *Hydrangea* sect. *Asperae*. In doing this, the applicability of several low copy nuclear markers, and RADseq for species delimitation in *Hydrangea* is validated. Future studies could apply these techniques in other sections, further fleshing out the species boundaries throughout the genus.

## Samenvatting

De familie Hydrangeaceae omvat twee tribus: Philadelpheae en Hydrangeeae. Deze laatste bestaat uit het genus *Hydrangea*, dat verschillende bekende sierplanten omvat, en acht kleinere genera: *Broussaisia*, *Decumaria*, *Dichroa*, *Pileostegia*, *Platycrater* en *Schizophragma.* Opeenvolgende moleculaire en morfologische studies in de tribus vormden een consistent beeld van de para- of polyfyletische aard van het genus *Hydrangea*. Deze observaties werden verder bevestigd in enkele recentere studies specifiek toegespitst op het genus *Hydrangea*. Hierbij kon een duidelijke incongruentie worden aangetoond tussen de classificatie van tribus Hydrangeeae en de evolutionaire relaties tussen de taxa die tot deze tribus behoren. Daarenboven werd duidelijk dat de verdere indeling van het genus *Hydrangea* in secties en subsecties zoals voorgesteld in de meest recente revisie niet overeenstemde met de evolutionaire relaties binnen de groep. Het meest opvallend hierbij is dat deze classificatie het genus opsplitst in twee secties: *Hydrangea* en *Cornidia*, terwijl deze opsplitsing niet ondersteund wordt door de meest recente fylogenetische hypotheses. Verder leverde geen van de beschikbare studies ondersteuning voor de evolutionaire cohesie van verscheidene subsecties (subsect. *Asperae*, subsect. *Americanae* en subsect. *Macrophyllae*).

Soortsgrenzen binnen het genus *Hydrangea* zijn moeilijk te interpreteren door de verschillende meningen aangaande morfologische variatie in en tussen de opeenvolgende revisies van de groep. Een sprekend voorbeeld van deze verwarring is te vinden in *H*. subsect. *Asperae* (*H.* sect. *Asperae* in de hier voorgestelde classificatie). De laatste wereldwijde revisie van het genus herkent drie soorten in deze groep. Een van deze soorten, *H. aspera*, wordt erkend als een wijdverspreid taxon, met grote morfologische variatie, dat opgedeeld kan worden in vier ondersoorten. Andere auteurs erkennen deze ondersoorten echter als soorten, samen met een aantal additionele morfotypes toegeschreven aan het *H. aspera* soortscomplex.

Deze thesis heeft als doel de bovenvermelde onduidelijkheden in het genus *Hydrangea* aan te pakken. Hierbij wordt de toepasbaarheid van nieuwe moleculaire technieken en analyse algoritmen uitgetest om volgende doelen te bereiken: 1) een ondersteunde fylogenetische hypothese voor tribus Hydrangeeae opstellen, 2) het voorstellen van een nieuwe classificatie voor tribus Hydrangeeae, 3) het identificeren van moleculaire merkers bruikbaar voor het bestuderen van soortsgrenzen in het genus *Hydrangea*, 4) het verzamelen van verschillende

onafhankelijke bewijsvoeringen gelieerd aan het opstellen van stabiele soortsgrenzen in *Hydrangea* subsect. *Asperae*, binnen een expliciet soortsconcept. Om deze verschillende vraagstellingen aan te pakken werd een representatieve staalname van herbarium en verse specimens ingezameld. Een deel van deze specimens kon zelf ingezameld worden uit wilde populaties.

Hoofdstuk 1 situeert het huidige werk in een algemene achtergrond. Verder worden enkele sleutelconcepten gehanteerd in latere hoofdstukken kort beschreven. Tenslotte worden de algemene doelen van deze studie gepresenteerd.

Hoofdstuk 2 omvat een nieuw classificatiesysteem voor de tribus Hydrangeeae, vertrekkende van een uitgebreide fylogenetische hypothese voor de groep. Om dit te verwezenlijken werd een representatieve staalname van de taxa in de groep genomen. Hierbij werden meerdere stalen voor elk van de negen genera opgenomen, evenals meerdere stalen voor elke infragenerische eenheid van classificatie in *Hydrangea*. Een sterk opgeloste fylogenetische hypothese werd opgesteld aan de hand van vier chloroplast regio's in combinatie met ribosomaal ITS. Aangezien de typesoorten voor alle aanwezige infragenerische taxa werden opgenomen in de analyse kon de resulterende fylogenie gebruikt worden om een nieuwe classificatie voor de tribus op te stellen. In deze classificatie worden de acht satellietgenera opgenomen in het grotere genus *Hydrangea*, waardoor een monofyletische groep ontstaat. Een dergelijke classificatie geniet de voorkeur boven de voorgaande, aangezien deze de evolutionaire relaties in de groep beter weerspiegelt. Een verdere opdeling van het resulterende genus in secties werd voorgesteld, waarbij zoveel mogelijk de voorgaande genusnamen werden gebruikt om de overgang naar dit nieuwe systeem te vergemakkelijken.

Hoofdstuk 3 stelt de resultaten voor van een coalescentie-gebaseerde benadering voor het afbakenen van soorten in *Hydrangea* sect. *Asperae*, gebaseerd om meerdere moleculaire merkers. De resultaten van deze analyse worden vergeleken met soortsgrenzen gedefinieerd op basis van morfologische kenmerken. Drie van de moleculaire merkers gebruikt voor dit algoritme werden specifiek voor deze studie en groep ontworpen. Daarenboven werden sequenties verkregen voor vier chloroplast regio's en ribosomaal ITS. De verkregen sequentiedata werden gebruikt voor het opstellen van fylogenetische bomen die de relaties tussen soorten weergeven, gebaseerd op een combinatie van meerdere genetische regio's

(zogenaamde "species trees"). De resulterende fylogenetische hypotheses konden daaropvolgend gebruikt worden in een coalescentie-gebaseerd algoritme voor soortsafbakening. Door de resultaten van dit algoritme te combineren met morfologische gegevens konden ondersteunde onafhankelijke evolutionaire lijnen binnen de sectie herkend worden. Een van deze morfologische kenmerken, de beharing aan de onderzijde van de bladeren, werd in beeld gebracht en gedocumenteerd voor verder gebruik aan de hand van scanning elektronen microscopie. De geïdentificeerde evolutionaire lijnen kwamen overeen met de nominale taxa *H. sikokiana*, *H. involucrata*, *H. longifolia*, *H. longipes*, *H. sargentiana*, *H. villosa*, *H. kawakamii*, *H. aspera* en *H. strigosa*. Deze laatste vertegenwoordigde echter een uitdaging door de aanwezigheid van twee evolutionaire lijnen die morfologisch geleken op *H. strigosa*. Slechts een van deze lijnen vertoonde relaties met het type specimen, en kon dus worden gelinkt aan de gepubliceerde naam. Voor de andere lijn werd een hypothese opgesteld die hybridisatie omvat met de nauw verwante taxa *H. aspera* en *H. robusta*.

Hoofdstuk 4 verkent de toepasbaarheid van RADseq voor fylogenetische reconstructie en soortsafbakening in *H.* sect. *Asperae*. Ondanks een lage en ongelijke verspreiding van de sequentie data over de gebruikte stalen, konden de resulterende SNPs en RAD loci gebruikt worden in verschillende algoritmen voor soortsafbakening. Aangezien de specimens gebruikt in deze studie nagenoeg identiek zijn aan deze in hoofdstuk 3 kon een vergelijking gemaakt worden tussen beide methodes voor het bekomen van sequentie data (Sanger vs. RADseq). Daarenboven konden de data gegenereerd in beide studie gezamenlijk geanalyseerd worden, om zo sterkere ondersteuning te bekomen van de in *H.* sect. *Asperae* voorgestelde soortsgrenzen. Deze grenzen zijn identiek aan deze voorgesteld in hoofdstuk 3, er werd echter wel meer inzicht vergaard in diens evolutionaire achtergrond.

Hoofdstuk 5 omvat de formele omschrijvingen voor de soorten die onderscheiden worden op basis van de resultaten uit de twee voorgaande hoofdstukken. Evolutionaire lijnen die erkend kunnen worden op het niveau van soort worden voorzien van een morfologische omschrijving, evenals een formele taxonomische behandeling. Deze brengt reeds gepubliceerde namen in verband brengt met de hier erkende taxa. Verder omvat dit hoofdstuk een morfologische sleutel en een korte bespreking van het materiaal dat werd ingezameld en bestudeerd voor dit werk.

Hoofdstuk 6 bespreekt de algemene bijdrage die dit werk levert aan de kennis van tribus Hydrangeeae en *H.* sect. *Asperae*. Gedurende deze studie werden enkele conflicten blootgelegd tussen moleculaire data en de traditionele visie op de taxonomie van *Hydrangea*. Deze conflicten en uitdagingen worden in detail besproken. Op het niveau van classificatie van de tribus zijn deze gecentreerd rond de discussie aangaande para- en polyfyletische taxa. Aangezien het huidige werk zich verdiept in de evolutionaire relaties in de groep, wordt kort aangehaald waarom een voorkeur wordt gegeven aan monofyletische eenheden van classificatie. Op soortsniveau wordt het "general lineage concept" voor soorten gevolgd, hetgeen een robuuste theoretische achtergrond voorziet voor de entiteit "soort". Door het volgen van dit concept kunnen de hier gedefinieerde soorten gezien worden als hypothesen, ondersteund door verschillende objectieve bewijzen.

In conclusie draagt het werk samengevat in deze thesis bij aan het inzicht in de evolutionaire geschiedenis van tribus Hydrangeeae. Een nieuwe classificatie voor deze tribus werd voorgesteld, waarin de acht satelliet genera samengevoegd worden met het grotere genus *Hydrangea*. Hierdoor wordt deze laatste monofyletisch. Door het verzamelen van verschillende morfologische en moleculaire bewijsvoeringen konden stabiele soortsgrenzen worden voorgesteld voor *Hydrangea* sect. *Asperae*. Hierbij kan eveneens de toepasbaarheid van bepaalde nucleaire merkers en RADseq voor soortsafbakening binnen *Hydrangea* gevalideerd worden. Toekomstige studies kunnen deze technieken toepassen in de andere secties, om zo soortsgrenzen binnen het genus verder te verkennen.

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# Appendix 1: supplementary data chapter 2

**Table S2.1: Regions excluded from phylogenetic reconstruction and Bayesian hypothesis testing.** Original positions in the master alignments are provided for each chloroplast region.

| Chloroplast marker | Position in master alignment | Length (bp) |
|---|---|---|
| *rpl32-ndhF* IGS | 90-97 | 7 |
| *rpl32-ndhF* IGS | 344-347 | 3 |
| *rpl32-ndhF* IGS | 671-687 | 16 |
| *rpl32-ndhF* IGS | 799-827 | 28 |
| *rpl32-ndhF* IGS | 833-836 | 3 |
| *rpl32-ndhF* IGS | 1151-1154 | 3 |
| *rpl32-ndhF* IGS | 1157-1161 | 4 |
| *rpl32-ndhF* IGS | 1424-1449 | 25 |
| *ndhA* intron | 618-628 | 10 |
| *ndhA* intron | 643-648 | 5 |
| *ndhA* intron | 671-684 | 13 |
| *ndhA* intron | 743-757 | 14 |
| *trnL-rpl32* IGS | 71 | 1 |
| *trnL-rpl32* IGS | 311 | 1 |
| *trnL-rpl32* IGS | 324-333 | 9 |
| *trnL-rpl32* IGS | 378-403 | 25 |
| *trnL-rpl32* IGS | 961-971 | 10 |
| *trnV-ndhC* IGS | 501-517 | 16 |

**Table S2.2: Datasheet with all recognized Hydrangeeae species names assigned to sections.**

| Name | Epithet | Author | Section |
|---|---|---|---|
| *Broussaisia* | | Gaudichaud | *Broussaisia* |
| *Broussaisia* | *arguta* | Gaudichaud | *Broussaisia* |
| *Broussaisia* | *pellucida* | Gaudichaud | *Broussaisia* |
| *Cardiandra* | | Siebold & Zuccarini | *Cardiandra* |
| *Cardiandra* | *alternifolia* | (Siebold) Siebold & Zuccarini | *Cardiandra* |
| *Cardiandra* | *amamiohsimensis* | Koidzumi | *Cardiandra* |
| *Cardiandra* | *densifolia* | C.F.Wei | *Cardiandra* |
| *Cardiandra* | *formosana* | Hayata | *Cardiandra* |
| *Cardiandra* | *laxiflora* | H.L.Li | *Cardiandra* |
| *Cardiandra* | *moellendorffii* | (Hance) Migo | *Cardiandra* |
| *Cardiandra* | *oppositifolia* | Honda | *Cardiandra* |
| *Cardiandra* | *sinensis* | Hemsley | *Cardiandra* |
| *Cardiandra* | *x agricola* | J.M.H.Shaw | *Cardiandra* |
| *Decumaria* | | L. | *Decumaria* |
| *Decumaria* | *barbara* | L. | *Decumaria* |
| *Decumaria* | *forsythia* | Michaux | *Decumaria* |
| *Decumaria* | *prostrata* | Loddiges ex Loudon | *Decumaria* |
| *Decumaria* | *radicans* | Moench | *Decumaria* |
| *Decumaria* | *sarmentosa* | Bosc | *Decumaria* |
| *Decumaria* | *scandens* | (Walter) Salisbury | *Decumaria* |
| *Decumaria* | *sinensis* | Oliver | *Decumaria* |
| *Deinanthe* | | Maximowicz | *Deinanthe* |
| *Deinanthe* | *bifida* | Maximowicz | *Deinanthe* |
| *Deinanthe* | *caerulea* | Stapf | *Deinanthe* |
| *Dichroa* | | Loureiro | *Dichroa* |
| *Dichroa* | *celebica* | Warburg | *Dichroa* |
| *Dichroa* | *cyanea* | (Wallich) Schlechter | *Dichroa* |
| *Dichroa* | *cyanitis* | Miquel | *Dichroa* |
| *Dichroa* | *daimingshanensis* | Y.C.Wu | *Dichroa* |
| *Dichroa* | *febrifuga* | Loureiro | *Dichroa* |
| *Dichroa* | *henryi* | H.Léveillé | *Dichroa* |
| *Dichroa* | *hirsuta* | Gagnepain | *Dichroa* |
| *Dichroa* | *latifolia* | Miquel | *Dichroa* |
| *Dichroa* | *mollissima* | Merrill | *Dichroa* |
| *Dichroa* | *parviflora* | Schlechter | *Dichroa* |
| *Dichroa* | *pentandra* | Schlechter | *Dichroa* |
| *Dichroa* | *philippinensis* | Schlechter | *Dichroa* |
| *Dichroa* | *platyphylla* | Merrill | *Dichroa* |
| *Dichroa* | *pubescens* | Miquel | *Dichroa* |
| *Dichroa* | *sarasinorum* | Warburg | *Dichroa* |

| | | | |
|---|---|---|---|
| *Dichroa* | *schumanniana* | Schlechter | *Dichroa* |
| *Dichroa* | *sylvatica* | (Reinwardt ex Blume) Merrill | *Dichroa* |
| *Dichroa* | *thyrsoidea* | Elmer | *Dichroa* |
| *Dichroa* | *tomentosa* | Warburg | *Dichroa* |
| *Dichroa* | *tristyla* | W.T.Wang & M.X.Nie | *Hirtae* ? |
| *Dichroa* | *versicolor* | (Fortune) D.R.Hunt | *Dichroa* |
| *Dichroa* | *yaoshanensis* | Y.C.Wu | *Dichroa* |
| *Dichroa* | *yunnanensis* | S.M.Hwang | *Dichroa* |
| | | | |
| *Hydrangea* | | L. | *Hydrangea* |
| *Hydrangea* | *acuminata* | Siebold & Zuccarini | *Macrophyllae* |
| *Hydrangea* | *acuta* | Rafinesque | *Hydrangea* |
| *Hydrangea* | *alba* | Reinward ex Miquel | *Asperae* |
| *Hydrangea* | *albostellata* | Samain, Najarro & E.Martínez | *Cornidia* |
| *Hydrangea* | *alternifolia* | Siebold | *Cardiandra* |
| *Hydrangea* | *altissima* | Wallich | *Calyptranthe* |
| *Hydrangea* | *amamiohsimensis* | (Koidzumi) Y. De Smet & Granados | *Cardiandra* |
| *Hydrangea* | *ampla* | (Chun) Y. De Smet & Granados | *Schizophragma* |
| *Hydrangea* | *amplifolia* | Rafinesque | *Hydrangea* |
| *Hydrangea* | *angulata* | Tausch | unplaced |
| *Hydrangea* | *angustifolia* | Hayata | *Chinenses* |
| *Hydrangea* | *angustipetala* | Hayata | *Chinenses* |
| *Hydrangea* | *"angustisepala"* | Hayata | *Chinenses* |
| *Hydrangea* | *anomala* | D.Don | *Calyptranthe* |
| *Hydrangea* | *antioquiensis* | Engler | *Cornidia* |
| *Hydrangea* | *arborescens* | L. | *Hydrangea* |
| *Hydrangea* | *arbostiana* | H.Léveillé | *Chinenses* |
| *Hydrangea* | *arguta* | (Gaudichaud) Y.De Smet & Granados | *Broussaisia* |
| *Hydrangea* | *ashei* | Harbison | *Hydrangea* |
| *Hydrangea* | *aspera* | Buch.-Ham. ex D.Don | *Asperae* |
| *Hydrangea* | *asterolasia* | Diels | *Cornidia* |
| *Hydrangea* | *azisai* | Siebold | *Macrophyllae* |
| *Hydrangea* | *bangii* | Engler | *Cornidia* |
| *Hydrangea* | *barbara* | (L.) Bernd Schulz | *Decumaria* |
| *Hydrangea* | *belzonii* | Siebold & Zuccarini | *Macrophyllae* |
| *Hydrangea* | *bifida* | (Maximowicz) Y. De Smet & Granados | *Deinanthe* |
| *Hydrangea* | *borealis* | (Nakai) Nakai | *Chinenses* |
| *Hydrangea* | *bracteata* | Siebold & Zuccarini | *Calyptranthe* |
| *Hydrangea* | *bretschneideri* | Dippel | *Heteromallae* |
| *Hydrangea* | *brevipes* | Chun | *Stylosae* ? |
| *Hydrangea* | *briquetii* | Engler | *Cornidia* |
| *Hydrangea* | *buergeri* | Siebold & Zuccarini | *Macrophyllae* |
| *Hydrangea* | *caerulea* | (Stapf) Y. De Smet & Granados | *Deinanthe* |
| *Hydrangea* | *candida* | Chun | *Hirtae* ? |
| *Hydrangea* | *caucana* | Engler | *Cornidia* |
| *Hydrangea* | *caudatifolia* | W.T. Wang & M.X. Nie | *Chinenses* ? *Stylosae* ? |
| *Hydrangea* | *chinensis* | Maximowicz | *Chinenses* |
| *Hydrangea* | *chloroleuca* | Diels | *Chinenses* |

| | | | |
|---|---|---|---|
| *Hydrangea* | *chungii* | Rehder | *Chinenses* ? *Stylosae* ? |
| *Hydrangea* | *cinerea* | Small | *Hydrangea* |
| *Hydrangea* | *coacta* | C.F. Wei | *Asperae* |
| *Hydrangea* | *coenobialis* | Chun | *Hirtae* ? |
| *Hydrangea* | *cordata* | Pursh | *Hydrangea* |
| *Hydrangea* | *cordifolia* | Siebold & Zuccarini | *Calyptranthe* |
| *Hydrangea* | *corylifolia* | (Chun) Y. De Smet & Granados | *Schizophragma* |
| *Hydrangea* | *crassa* | (Handel-Mazzetti) Y. De Smet & Granados | *Schizophragma* |
| *Hydrangea* | *cuneatifolia* | Elmer | *Cornidia* |
| *Hydrangea* | *cuspidata* | (Thunberg) Makino | *Macrophyllae* |
| *Hydrangea* | *cuspidata* | (Thunberg) Miquel | *Macrophyllae* |
| *Hydrangea* | *cyanema* | Nuttall | *Asperae* ? |
| *Hydrangea* | *daimingshanensis* | (Y.C.Wu) Y. De Smet & Granados | *Dichroa* |
| *Hydrangea* | *davidii* | Franchet | *Chinenses* |
| *Hydrangea* | *densifolia* | (C.F.Wei) Y. De Smet & Granados | *Cardiandra* |
| *Hydrangea* | *diplostemona* | (J. Donnell Smith) Standley | *Cornidia* |
| *Hydrangea* | *discocarpa* | C.F. Wei | *Asperae* |
| *Hydrangea* | *discolor* | Rafinesque | *Hydrangea* |
| *Hydrangea* | *dumicola* | W.W.Smith | *Heteromallae* |
| *Hydrangea* | *durifolia* | Briquet | *Cornidia* |
| *Hydrangea* | *ecuadorensis* | Briquet | *Cornidia* |
| *Hydrangea* | *epiphytica* | Morton ex Haworth-Booth | *Cornidia* |
| *Hydrangea* | *fauriei* | (Hayata) Y. De Smet & Granados | *Schizophragma* |
| *Hydrangea* | *febrifuga* | (Loureiro) Y. De Smet & Granados | *Dichroa* |
| *Hydrangea* | *florida* | Salisbury | *Macrophyllae* |
| *Hydrangea* | *formosana* | Koidzumi | *Chinenses* |
| *Hydrangea* | *frutescens* | Moench | *Hydrangea* |
| *Hydrangea* | *fulvescens* | Rehder | *Asperae* |
| *Hydrangea* | *giraldii* | Diels | *Heteromallae* |
| *Hydrangea* | *glabra* | Hayata | *Calyptranthe* |
| *Hydrangea* | *glabrifolia* | Hayata | *Chinenses* |
| *Hydrangea* | *glabripes* | Rehder | *Asperae* |
| *Hydrangea* | *glandulosa* | Elmer | *Cornidia* |
| *Hydrangea* | *glauca* | Rafinesque | *Hydrangea* |
| *Hydrangea* | *glaucescens* | (Rehder) Y. De Smet & Granados | *Schizophragma* |
| *Hydrangea* | *glaucophylla* | C.C. Yang | *Calyptranthe* |
| *Hydrangea* | *goudotii* | Briquet | *Cornidia* |
| *Hydrangea* | *gracilis* | W.T. Wang & M.X. Nie | *Hirtae* ? |
| *Hydrangea* | *grosseserrata* | Engler | *Chinenses* |
| *Hydrangea* | *hattoriana* | Nakai | *Macrophyllae* |
| *Hydrangea* | *hedyotidea* | Chun | *Stylosae* ? |
| *Hydrangea* | *hemsleyana* | Diels | *Asperae* |
| *Hydrangea* | *heteromalla* | D.Don | *Heteromallae* |
| *Hydrangea* | *heterophylla* | Rafinesque | *Hydrangea* |
| *Hydrangea* | *hirsuta* | (Gagnepain) Y. De Smet & Granados | *Dichroa* |
| *Hydrangea* | *hirta* | (Thunberg) Siebold | *Hirtae* |
| *Hydrangea* | *hortensia* | Seringe | *Macrophyllae* |
| *Hydrangea* | *hortensia* | Siebold | *Macrophyllae* |

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|---|---|---|---|
| *Hydrangea* | *hortensis* | Smith | *Macrophyllae* |
| *Hydrangea* | *hydrangeoides* | (Siebold & Zuccarini) Bernd Schulz | *Schizophragma* |
| *Hydrangea* | *hypoglauca* | Rehder | *Heteromallae* |
| *Hydrangea* | *indochinensis* | Merrill | *Stylosae* |
| *Hydrangea* | *inornata* | Standley | *Cornidia* |
| *Hydrangea* | *integerrima* | (Hooker & Arnott) Engler | *Cornidia* |
| *Hydrangea* | *integra* | Hayata | *Cornidia* |
| *Hydrangea* | *integrifolia* | Hayata | *Cornidia* |
| *Hydrangea* | *involucrata* | Siebold | *Asperae* |
| *Hydrangea* | *japonica* | Siebold | *Macrophyllae* |
| *Hydrangea* | *jelskii* | Szyszylowicz | *Cornidia* |
| *Hydrangea* | *jelskii* | Zahlbruckner | *Cornidia* |
| *Hydrangea* | *jiangxiensis* | W.T. Wang & M.X. Nie | *Chinenses* |
| *Hydrangea* | *kamienskii* | Léveillé | *Heteromallae* |
| *Hydrangea* | *kawagoeana* | Koidzumi | *Chinenses* |
| *Hydrangea* | *kawakamii* | Hayata | *Asperae* |
| *Hydrangea* | *khasiana* | Hooker f. & Thomson | *Heteromallae* |
| *Hydrangea* | *kwangsiensis* | Hu | *Stylosae* ? |
| *Hydrangea* | *kwangtungensis* | Merrill | *Stylosae* ? |
| *Hydrangea* | *lehmannii* | Engler | *Cornidia* |
| *Hydrangea* | *lindleyana* | G.Nicholson | *Macrophyllae* |
| *Hydrangea* | *lingii* | G.Hoo | *Hirtae* ? |
| *Hydrangea* | *linkweiensis* | Chun | *Chinenses* |
| *Hydrangea* | *liukiuensis* | Nakai | *Chinenses* |
| *Hydrangea* | *lobbii* | Maximowicz | *Chinenses* |
| *Hydrangea* | *longialata* | C.F. Wei | *Asperae* |
| *Hydrangea* | *longifolia* | Hayata | *Asperae* |
| *Hydrangea* | *longipes* | Franchet | *Asperae* |
| *Hydrangea* | *longipes* | Hemsley ex Forbes & Hemsley | *Asperae* |
| *Hydrangea* | *luteovenosa* | Koidzumi | *Chinenses* |
| *Hydrangea* | *macrocarpa* | Handel-Mazzetti | *Heteromallae* |
| *Hydrangea* | *macrophylla* | (Thunberg) Seringe | *Macrophyllae* |
| *Hydrangea* | *macrosepala* | Hayata | *Chinenses* |
| *Hydrangea* | *mandarinorum* | Diels | *Heteromallae* |
| *Hydrangea* | *mangshanensis* | C.F. Wei | *Chinenses* ? *Stylosae* ? |
| *Hydrangea* | *maritima* | Haworth-Booth | *Macrophyllae* |
| *Hydrangea* | *mathewsii* | Briquet | *Cornidia* |
| *Hydrangea* | *maximowiczii* | Léveillé | *Asperae* |
| *Hydrangea* | *minnanica* | W.D. Han | *Hirtae* ? |
| *Hydrangea* | *moellendorffii* | Hance | *Cardiandra* |
| *Hydrangea* | *mollis* | (Rehder) W.T. Wang | *Heteromallae* |
| *Hydrangea* | *mollissima* | (Merrill) Y. De Smet & Granados | *Dichroa* |
| *Hydrangea* | *mutabilis* | Steudel | *Macrophyllae* |
| *Hydrangea* | *nebulicola* | Nevling & Gomez-Pompa | *Cornidia* |
| *Hydrangea* | *neesiana* | Steudel | *Hydrangea* |
| *Hydrangea* | *nivea* | Michaux | *Hydrangea* |
| *Hydrangea* | *oblongifolia* | Blume | *Dichroa* ? |
| *Hydrangea* | *obovatifolia* | Hayata | *Chinenses* |

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|---|---|---|---|
| *Hydrangea* | *obtusifolia* | (Hu) Y. De Smet & Granados | *Decumaria* |
| *Hydrangea* | *oerstedii* | Briquet | *Cornidia* |
| *Hydrangea* | *opuloides* | (Lamarck) K. Koch | *Macrophyllae* |
| *Hydrangea* | *opuloides* | Steudel | *Macrophyllae* |
| *Hydrangea* | *otaksa* | Siebold & Zuccarini | *Macrophyllae* |
| *Hydrangea* | *panamensis* | Standley | *Cornidia* |
| *Hydrangea* | *paniculata* | Siebold | *Heteromallae* |
| *Hydrangea* | *peruviana* | Moricand ex Seringe | *Cornidia* |
| *Hydrangea* | *petiolaris* | Siebold & Zuccarini | *Calyptranthe* |
| *Hydrangea* | *platyarguta* | Y. De Smet & Granados | *Asperae* |
| *Hydrangea* | *platyphylla* | Briquet | *Cornidia* |
| *Hydrangea* | *pottingeri* | Prain | *Chinenses* |
| *Hydrangea* | *preslii* | Briquet | *Cornidia* |
| *Hydrangea* | *pubescens* | Decaisne | *Heteromallae* |
| *Hydrangea* | *pubescens* | Koehne | *Heteromallae* |
| *Hydrangea* | *pubescens* | Nees ex Steudel | *Hydrangea* |
| *Hydrangea* | *pubinervis* | Rehder | *Heteromallae* |
| *Hydrangea* | *pubiramea* | Merrill | *Chinenses* |
| *Hydrangea* | *quercifolia* | Bartram | unplaced |
| *Hydrangea* | *radiata* | J.E. Smith | unplaced |
| *Hydrangea* | *radiata* | Walter | *Hydrangea* |
| *Hydrangea* | *rehderiana* | C.K. Schneider | *Asperae* |
| *Hydrangea* | *robusta* | J.D.Hooker & Thomson | *Asperae* |
| *Hydrangea* | *rosthornii* | Diels | *Asperae* |
| *Hydrangea* | *rotundifolia* | C.F. Wei | *Asperae* |
| *Hydrangea* | *rotundifolia* | Rafinesque | *Hydrangea* |
| *Hydrangea* | *sachalinensis* | Léveillé | *Heteromallae* |
| *Hydrangea* | *sargentiana* | Rehder | *Asperae* |
| *Hydrangea* | *scandens* | (L.f.) Seringe | *Chinenses* |
| *Hydrangea* | *scandens* | Maximowicz | *Calyptranthe* |
| *Hydrangea* | *scandens* | Poeppig ex DC. | *Cornidia* |
| *Hydrangea* | *schindleri* | Engler | *Heteromallae* |
| *Hydrangea* | *schizomollis* | Y. De Smet & Granados | *Schizophragma* |
| *Hydrangea* | *schlimii* | Briquet | *Cornidia* |
| *Hydrangea* | *seemannii* | L.Riley | *Cornidia* |
| *Hydrangea* | *serrata* | (Thunberg) Seringe | *Macrophyllae* |
| *Hydrangea* | *serratifolia* | (Hooker & Arnott) F. Philippi | *Cornidia* |
| *Hydrangea* | *shaochingii* | Chun | *Hirtae* ? |
| *Hydrangea* | *sikokiana* | Maximowicz | *Asperae* |
| *Hydrangea* | *sitsitan* | Siebold | *Macrophyllae* |
| *Hydrangea* | *sprucei* | Briquet | *Cornidia* |
| *Hydrangea* | *stellata* | Siebold & Zuccarini | *Macrophyllae* |
| *Hydrangea* | *stenophylla* | Merrill & Chun | *Stylosae* ? |
| *Hydrangea* | *steyermarkii* | Standley | *Cornidia* |
| *Hydrangea* | *strigosa* | Rehder | *Asperae* |
| *Hydrangea* | *stylosa* | J.D.Hooker & Thomson | *Stylosae* |
| *Hydrangea* | *subferruginea* | W.W. Smith | *Chinenses* |
| *Hydrangea* | *subintegra* | Merrill | *Chinenses* |

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|---|---|---|---|
| *Hydrangea* | *sungpanensis* | Handel-Mazzetti | *Heteromallae* |
| *Hydrangea* | *taiwaniana* | Y.C. Liu & F.Y. Lu | *Cornidia* |
| *Hydrangea* | *taquetii* | H.Léveillé | *Schizophragma* |
| *Hydrangea* | *tarapotensis* | Briquet | *Cornidia* |
| *Hydrangea* | *taronensis* | Handel-Mazzetti | *Stylosae* |
| *Hydrangea* | *thunbergii* | Siebold | *Macrophyllae* |
| *Hydrangea* | *tiliaefolia* | Léveillé | *Calyptranthe* |
| *Hydrangea* | *tomentella* | (Handel-Mazzetti) Y. De Smet & Granados | *Pileostegia* |
| *Hydrangea* | *trianae* | Briquet | *Cornidia* |
| *Hydrangea* | *umbellata* | Rehder | *Chinenses* |
| *Hydrangea* | *umbellata* | (Ruiz & Pavon) Briquet | *Cornidia* |
| *Hydrangea* | *verticillata* | W.H. Gao | *Heteromallae* |
| *Hydrangea* | *vestita* | Wallich | *Heteromallae* |
| *Hydrangea* | *viburnifolia* | Salisbury | *Hydrangea* |
| *Hydrangea* | *viburnoides* | (J.D.Hooker & Thomson) Y. De Smet & Granados | *Pileostegia* |
| *Hydrangea* | *villosa* | Rehder | *Asperae* |
| *Hydrangea* | *vinicolor* | Chun | *Hirtae* ? |
| *Hydrangea* | *virens* | (Thunberg) Siebold | *Chinenses* |
| *Hydrangea* | *vulgaris* | Michaux | *Hydrangea* |
| *Hydrangea* | *weberbaueri* | Engler | *Cornidia* |
| *Hydrangea* | *xanthoneura* | Diels | *Heteromallae* |
| *Hydrangea* | *yaoshanensis* | (Y.C.Wu) Y. De Smet & Granados | *Dichroa* |
| *Hydrangea* | *yayeyamensis* | Koidzumi | *Chinenses* |
| *Hydrangea* | *yesoensis* | Koidzumi | Macrophyllae |
| *Hydrangea* | *yunnanensis* | Rehder | Chinenses |
| *Hydrangea* | *zhewanensis* | P.S. Hsu & X.P. Zhang | Stylosae ? |
| | | | |
| *Pileostegia* | | J.D.Hooker & T.Thomson | Pileostegia |
| *Pileostegia* | *mexicana* | Turczaninow | = *Ilex cassine* subsp. *mexicana* |
| *Pileostegia* | *obtusifolia* | (Hu) Hu | Decumaria |
| *Pileostegia* | *subansiriana* | H.B.Naithani & Bennet | Pileostegia |
| *Pileostegia* | *tomentella* | Handel-Mazzetti | Pileostegia |
| *Pileostegia* | *urceolata* | Hayata | Pileostegia |
| *Pileostegia* | *viburnoides* | J.D.Hooker & Thomson | Pileostegia |
| | | | |
| *Platycrater* | | Siebold & Zuccarini | Asperae |
| *Platycrater* | *arguta* | Siebold & Zuccarini | Asperae |
| *Platycrater* | *serrata* | (Thunberg) Makino | Macrophyllae |
| | | | |
| *Schizophragma* | | Siebold & Zuccarini | Schizophragma |
| *Schizophragma* | *amplum* | Chun | Schizophragma |
| *Schizophragma* | *choufenianum* | Chun | Schizophragma |
| *Schizophragma* | *corylifolium* | Chun | Schizophragma |
| *Schizophragma* | *crassum* | Handel-Mazzetti | Schizophragma |
| *Schizophragma* | *elliptifolium* | C.F.Wei | Schizophragma |
| *Schizophragma* | *fauriei* | Hayata | Schizophragma |
| *Schizophragma* | *glaucescens* | (Rehder) Chun | Schizophragma |

| | | | |
|---|---|---|---|
| *Schizophragma* | *hsitaoanum* | Chun | Schizophragma |
| *Schizophragma* | *hydrangeoides* | Siebold & Zuccarini | Schizophragma |
| *Schizophragma* | *hypoglaucum* | Rehder | Schizophragma |
| *Schizophragma* | *integrifolium* | Oliver | Schizophragma |
| *Schizophragma* | *macrosepalum* | Hu | Schizophragma |
| *Schizophragma* | *megalocarpum* | Chun | Schizophragma |
| *Schizophragma* | *molle* | (Rehder) Chun | Schizophragma |
| *Schizophragma* | *obtusifolium* | Hu | Decumaria |
| *Schizophragma* | *tomentellum* | (Handel-Mazzetti) Stapf | Pileostegia |
| *Schizophragma* | *viburnoides* | (J.D.Hooker & Thomson) Stapf | Pileostegia |

**Figure S2.1: The 50% majority rule consensus tree for chloroplast regions.** This phylogenetic hypothesis was inferred based on the combined dataset of chloroplast regions without indel data. Posterior probabilities obtained from Bayesian Inference are indicated on the respective branches when below 1.

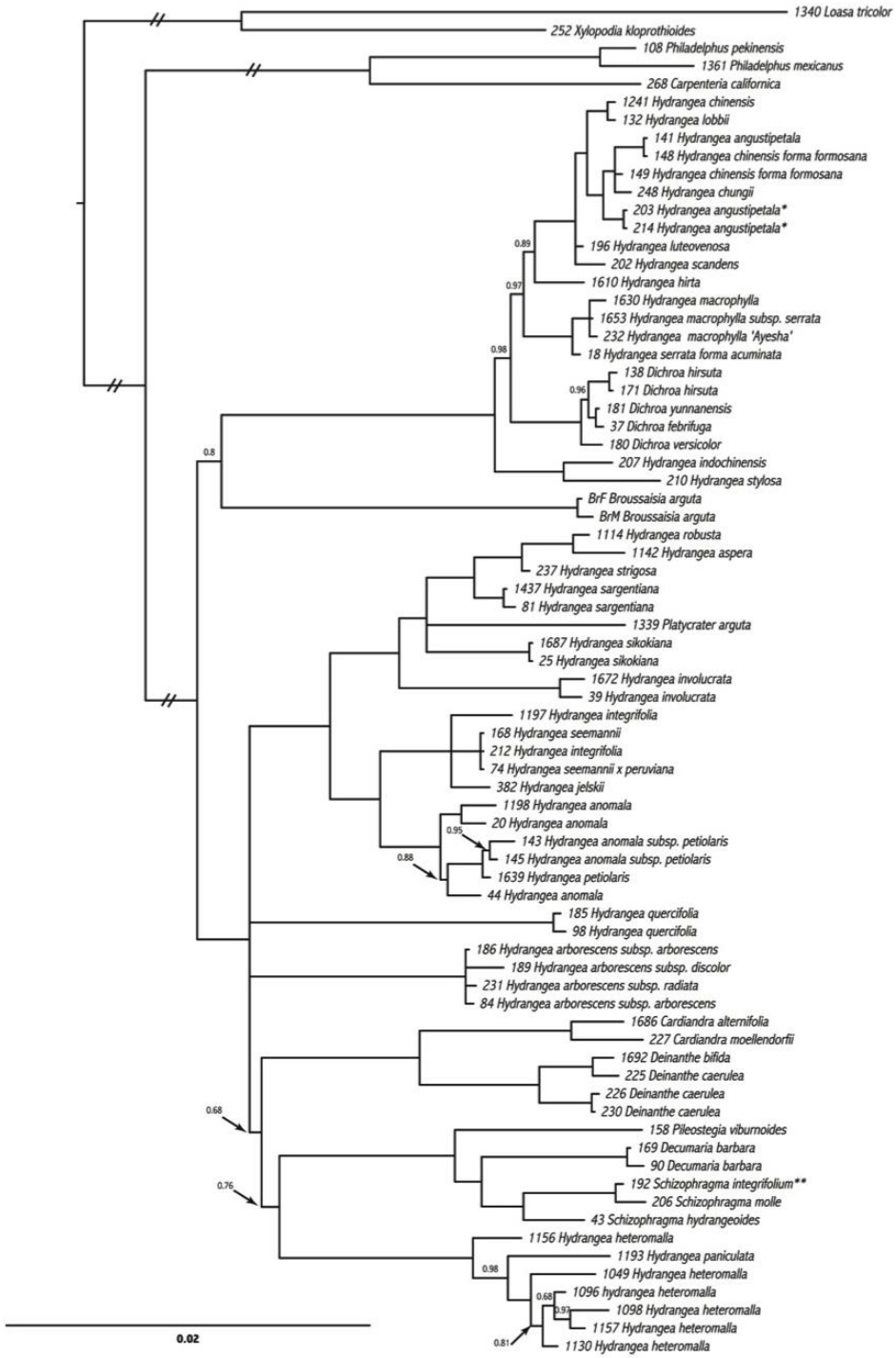


**Figure S2.2: Single gene trees based on the analysis containing coded indels.**

**Figure S2.2A:** The 50% majority rule consensus tree based on the *rpl32-ndhF* IGS with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. Supported topological differences with the phylogenetic tree based on only nucleotide data (not shown): *Broussaisia arguta* sister to Hydrangea II (PP: 0.82) in analyses without indel data.

**Figure S2.2B:** The 50% majority rule consensus tree based on the *trnV-ndhC* IGS with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1.No supported topological differences with the phylogenetic tree based on only nucleotide data (not shown).

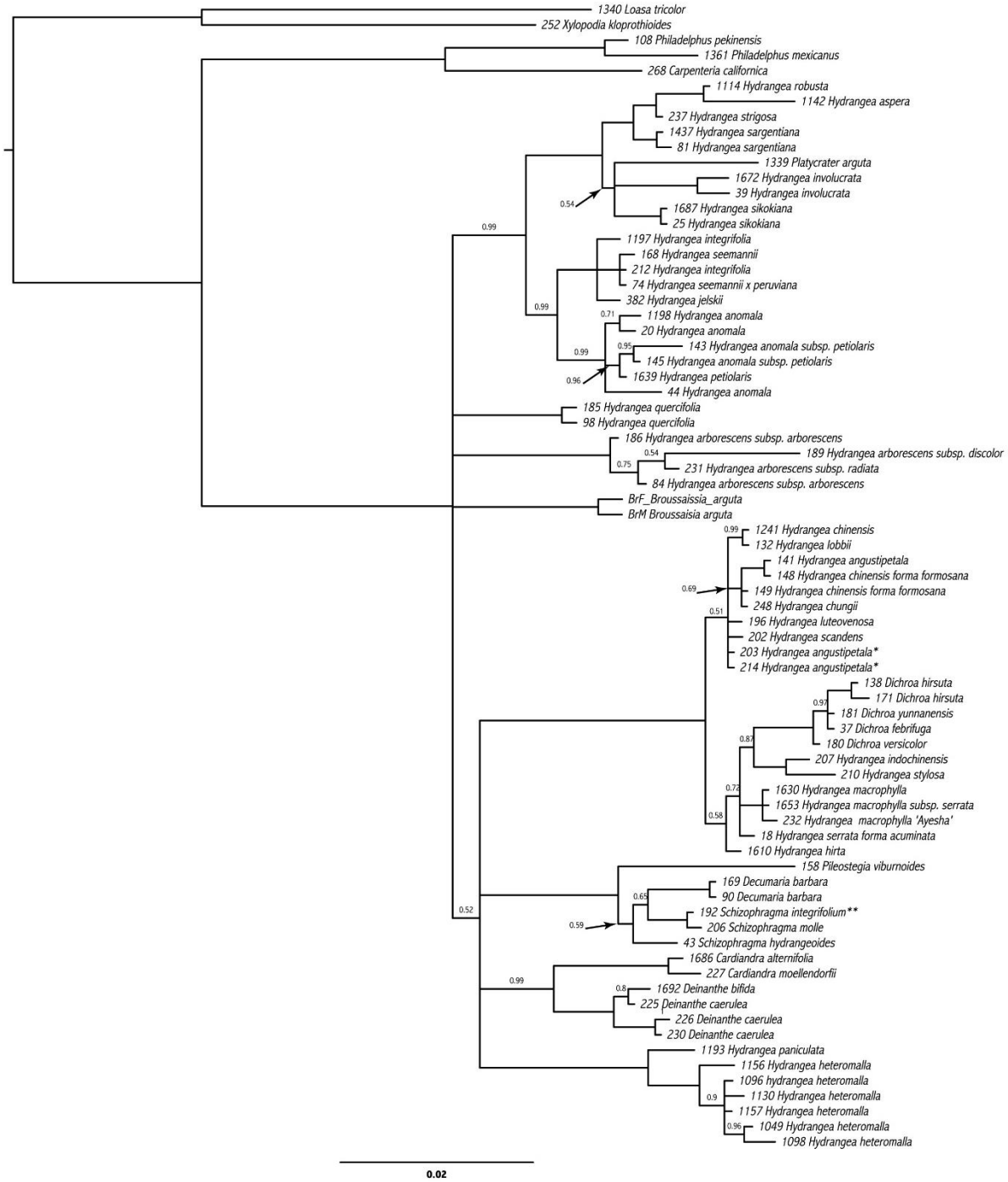


**Figure S2.2C:** The 50% majority rule consensus tree based on the *trnL-rpl32* IGS with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1.No supported topological differences with the phylogenetic tree based on only nucleotide data (not shown).

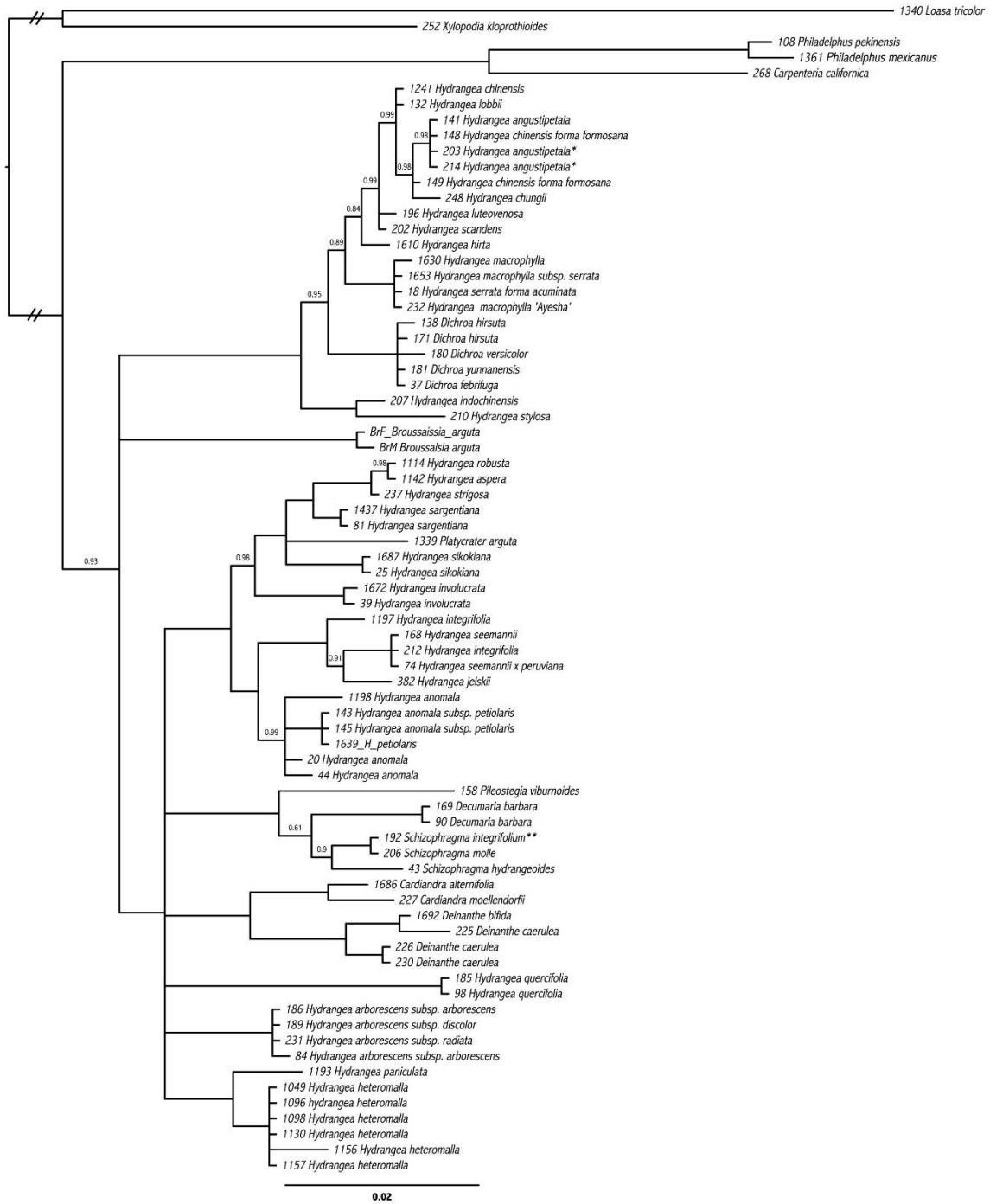


**Figure S2.2D:** The 50% majority rule consensus tree based on the *ndhA* intron without indel information, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. No supported topological differences with the phylogenetic tree based on only nucleotide data (not shown).

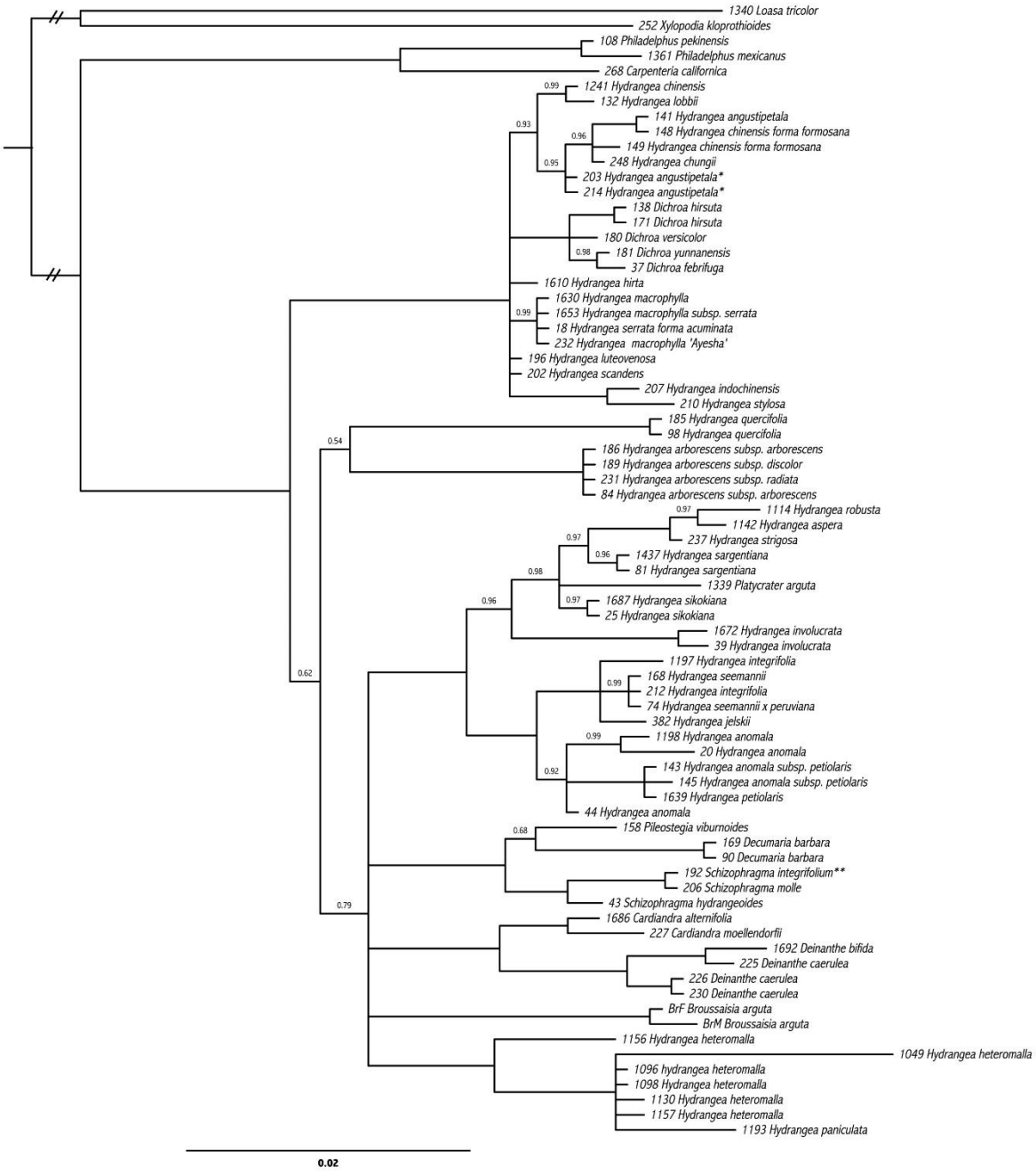
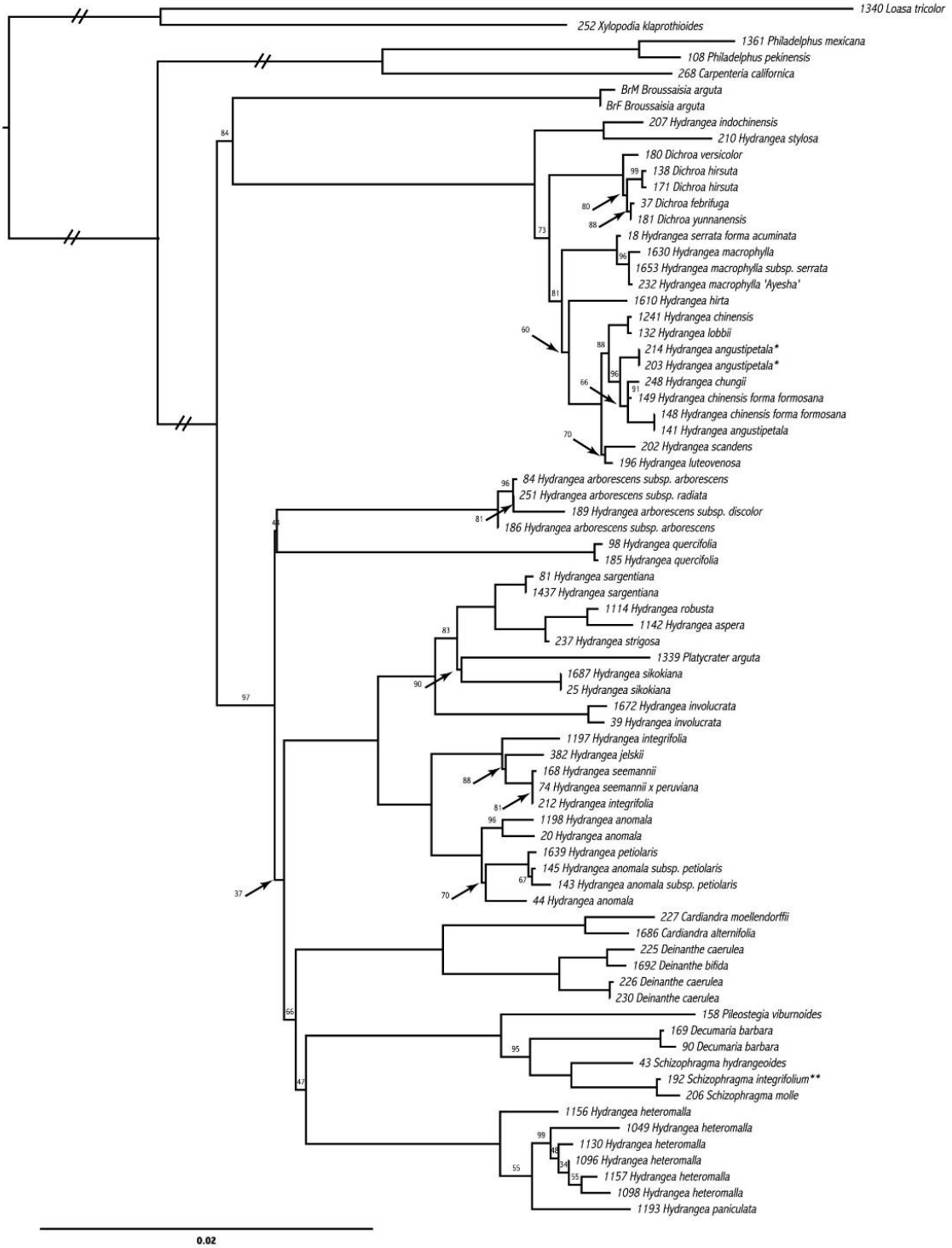


**Figure S2.2E:** The 50% majority rule consensus tree based on ITS with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. No supported topological differences with the phylogenetic tree based on only nucleotide data (not shown).

**Figure S2.3: Best scoring ML tree for the plastid dataset.** Bootstrap values lower than 100 are displayed on the branches. *Hydrangea angustipetala** = *Hydrangea angustipetala* forma *macrosepala*. *Schizophragma integrifolium*** = *Schizophragmaintegrifolium* var. *fauriei*.



# Appendix 2: supplementary data chapter 3

**Table S3.1: Voucher specimens for the sequences analyzed in the present study**. For each specimen the full name, voucher ID, locality (if available), type of material for DNA extraction, altitude of collection, name of collectors (where 1: Y. De Smet and E. Rodriguez, 2: Y. De Smet and K. Bauters, 3: Y. De Smet, L. Reyserhove and T. Uemachi, 4: Banerjee & P.R. Shakya, 5: F. Kingdon Ward.), and EMBL nucleotide sequence database accession numbers are given. Collectors: The type of the material is given as W: Wild collected or H: Herbarium specimen.

| Taxon | Voucher | Locality | Material | Altitude | Collector | ITS | rpl32-ndhF | trnL-rpl32 | trnV-ndhC | ndhA | SMC22 | SMC44 | TIF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *H. integrifolia* | YDS1197 | Taiwan, Yilan (24.49, 122) | W | 1921m | 1 | LT838907 | LT838936 | LT838965 | LT838995 | LT854706 | LT854735 | LT854762 | LT854792 |
| *H. strigosa* | YDS1032 | China, Sichuan (29.57, 103.44) | W | 541m | 1 | LT838911 | LT838937 | LT838966 | LT838996 | | | LT854763 | LT854793 |
| *H. strigosa* | YDS1035 | China, Sichuan (29.57, 103.44) | W | 548m | 1 | LT838912 | LT838938 | LT838967 | LT838997 | LT854707 | LT854736 | LT854764 | LT854794 |
| *H. robusta* | YDS1086 | China, Sichuan (29.67, 102.94) | W | 1906m | 1 | LT838913 | LT838939 | LT838968 | LT838998 | LT854708 | LT854737 | LT854765 | LT854795 |
| *H. aspera* | YDS1101 | China, Sichuan (29.83, 102.7) | W | 919m | 1 | LT838914 | LT838940 | LT838969 | LT838999 | LT854709 | LT854738 | LT854766 | LT854796 |
| *H. robusta* | YDS1114 | China, Sichuan (29.69, 102.61) | W | 1862m | 1 | | LT838941 | LT838970 | LT839000 | LT854710 | LT854739 | LT854767 | LT854797 |
| *H. aspera* | YDS1137 | China, Sichuan (29.6, 102.06) | W | 2194m | 1 | LT838915 | LT838942 | LT838971 | LT839001 | LT854711 | | LT854768 | LT854798 |
| *H. kawakamii* | YDS1176 | Taiwan, Yilan (24.39, 121.36) | W | 1974m | 1 | LT838929 | LT838943 | LT838972 | LT839004 | LT854712 | LT854756 | LT854769 | LT854799 |
| *H. longifolia* | YDS1183 | Taiwan, Yilan (24.54, 121.51) | W | 803m | 1 | LT838922 | LT838944 | LT838973 | LT839002 | LT854713 | LT854740 | LT854770 | LT854800 |
| *H. longifolia* | YDS1203 | Taiwan, Taichung (24.2, 121.48) | W | 952m | 1 | LT838923 | LT838945 | LT838974 | LT839003 | LT854714 | LT854741 | LT854771 | LT854801 |
| *H. kawakamii* | YDS1227 | Taiwan, Taichung (24.22, 121.27) | W | 2030m | 1 | LT838930 | LT838946 | LT838975 | LT839005 | LT854715 | LT854757 | LT854772 | LT854802 |
| *H. aspera* | YDS1349 | Nepal, Khinti Khola | H | 1898m | 4 | LT838916 | | LT838976 | LT839008 | LT854716 | LT854742 | LT854773 | LT854803 |
| *H. robusta* | YDS1351 | India, Delei valley (28.33, 96.58) | H | | 5 | LT838917 | LT838947 | LT838977 | LT839009 | LT854717 | LT854743 | LT854774 | LT854804 |
| *H. longipes* | YDS1400 | China, Hubei (31.05, 110.95) | W | | 2 | LT838921 | LT838948 | LT838978 | LT839010 | LT854718 | LT854746 | LT854775 | LT854805 |
| *H. strigosa* | YDS1434 | China, Hubei (31.33, 110.48) | W | 1331m | 2 | LT838931 | LT838949 | LT838979 | LT839012 | LT854719 | LT854758 | LT854788 | LT854806 |
| *H. sargentiana* | YDS1437 | China, Hubei (31.33, 110.48) | W | 1315m | 2 | LT838918 | LT838950 | LT838980 | LT839011 | LT854720 | LT854744 | LT854776 | LT854807 |
| *H. strigosa* | YDS1459 | China, Hubei (31.31, 110.48) | W | 1357m | 2 | LT838932 | LT838951 | LT838981 | LT839014 | LT854721 | LT854759 | LT854789 | LT854808 |
| *H. strigosa* | YDS1462 | China, Hubei (31.31, 110.48) | W | 1403m | 2 | LT838933 | LT838952 | LT838982 | LT839007 | LT854722 | LT854760 | LT854790 | LT854809 |
| *H. sargentiana* | YDS1468 | China, Hubei (31.31, 110.48) | W | 1443m | 2 | LT838919 | LT838953 | LT838983 | LT839015 | LT854723 | LT854745 | LT854777 | LT854810 |
| *H. strigosa* | YDS1485 | China, Hubei (31.34, 110.51) | W | 740m | 2 | LT838934 | LT838954 | LT838984 | LT839013 | LT854724 | LT854761 | LT854787 | LT854811 |
| *H. longipes* | YDS1489 | China, Hubei (31.53, 110.34) | W | 1725m | 2 | LT838920 | LT838955 | LT838985 | LT839016 | LT854725 | LT854747 | LT854778 | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *H. villosa* | YDS1524 | China, Hubei (30.17, 110.97) | W | 882m | 2 | LT838908 | LT838956 | LT838986 | LT839017 | LT854726 | LT854748 | LT854779 | LT854812 |
| *H. villosa* | YDS1538 | China, Hubei (30.16, 110.78) | W | 1055m | 2 | LT838909 | LT838957 | LT838987 | LT839018 | LT854727 | LT854749 | LT854780 | LT854813 |
| *H. villosa* | YDS1545 | China, Hubei (30.18, 110.72) | W | 1136m | 2 | LT838910 | LT838958 | LT838988 | LT839019 | LT854728 | LT854750 | LT854781 | LT854814 |
| *H. strigosa* | YDS1554 | China, Hubei (30.69, 110.56) | W | 1114m | 2 | LT838935 | LT838959 | LT838989 | LT839006 | LT854729 | | LT854791 | |
| *H. involucrata* | YDS1600 | Japan, Hinohara (35.72, 139.11) | W | 263m | 3 | LT838924 | LT838960 | LT838990 | LT839020 | LT854730 | LT854751 | LT854782 | LT854815 |
| *H. involucrata* | YDS1638 | Japan, Oshima (34.71, 139.43) | W | 367m | 3 | LT838925 | LT838961 | LT838991 | LT839021 | LT854731 | LT854752 | LT854783 | LT854816 |
| *H. involucrata* | YDS1645 | Japan, Shiga (35.93, 137.13) | W | 626m | 3 | LT838926 | LT838962 | LT838992 | LT839022 | LT854732 | LT854753 | LT854784 | LT854817 |
| *H. sikokiana* | YDS1674 | Japan, Tokushima (33.95, 134.4) | W | 925m | 3 | LT838927 | LT838963 | LT838993 | LT839023 | LT854733 | LT854754 | LT854785 | LT854818 |
| *H. sikokiana* | YDS1689 | Japan, Tokushima (33.91, 134.29) | W | 1143m | 3 | LT838928 | LT838964 | LT838994 | LT839024 | LT854734 | LT854755 | LT854786 | LT854819 |

**Table S3.2: Primer sequences specifically designed for *Hydrangea* sect. *Asperae*.**

| Fragment | Primers | Sequence (5'-3') | size |
|---|---|---|---|
| TIF3H1 | 840_asp_1F | ATGGAACTTCACCGTAGTA | ~ 693bp |
| | 840_asp_6R | GTTGTAGCCGGTCATAGTCA | |
| SMC1-22 | SMC1as_2R | TAYTGACGCATGATGTACC | ~ 1070bp |
| | SMC1as_2F | GGTGGACATTCTATTGGTG | |
| SMC1-44 | SMC1as_4F | GAGGCTCTCAAACGCCTATT | ~ 529bp |
| | SMC1as_4R | ATTGGATCACATCAAAAATCAGC | |

**Figure S3.1: The 50% majority-rule consensus tree based on the *trnV-ndhC* IGS**. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

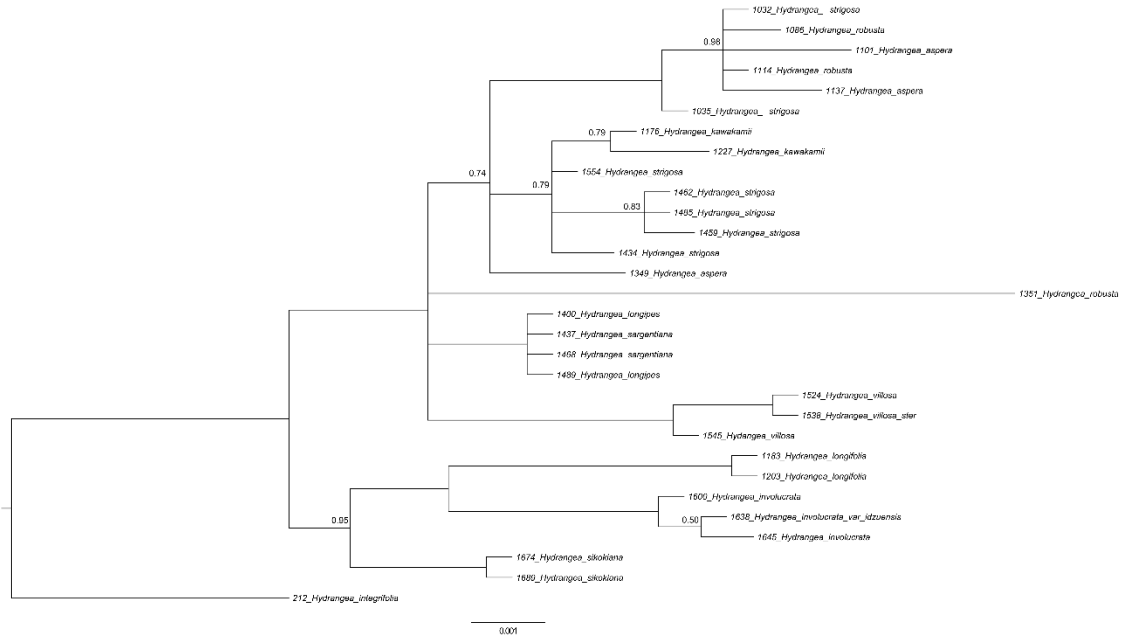


**Figure S3.2: The 50% majority-rule consensus tree based on the *rpl32-ndhF* IGS.** Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

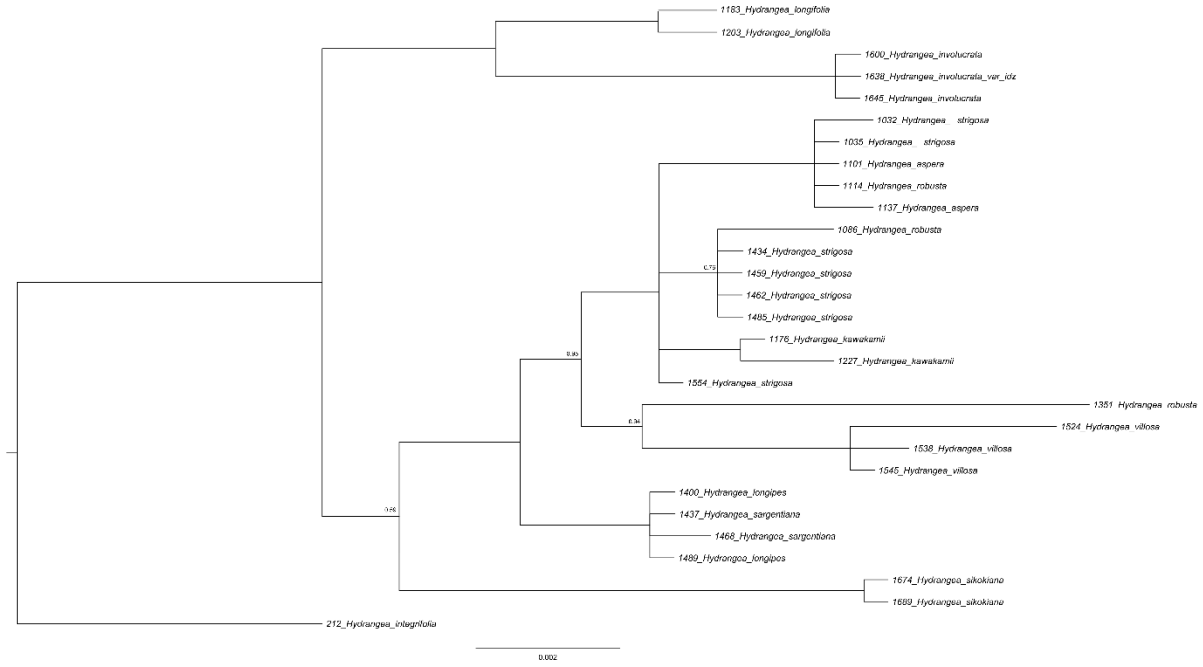



**Figure S3.3: The 50% majority-rule consensus tree based on the *ndhA* intron.** Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

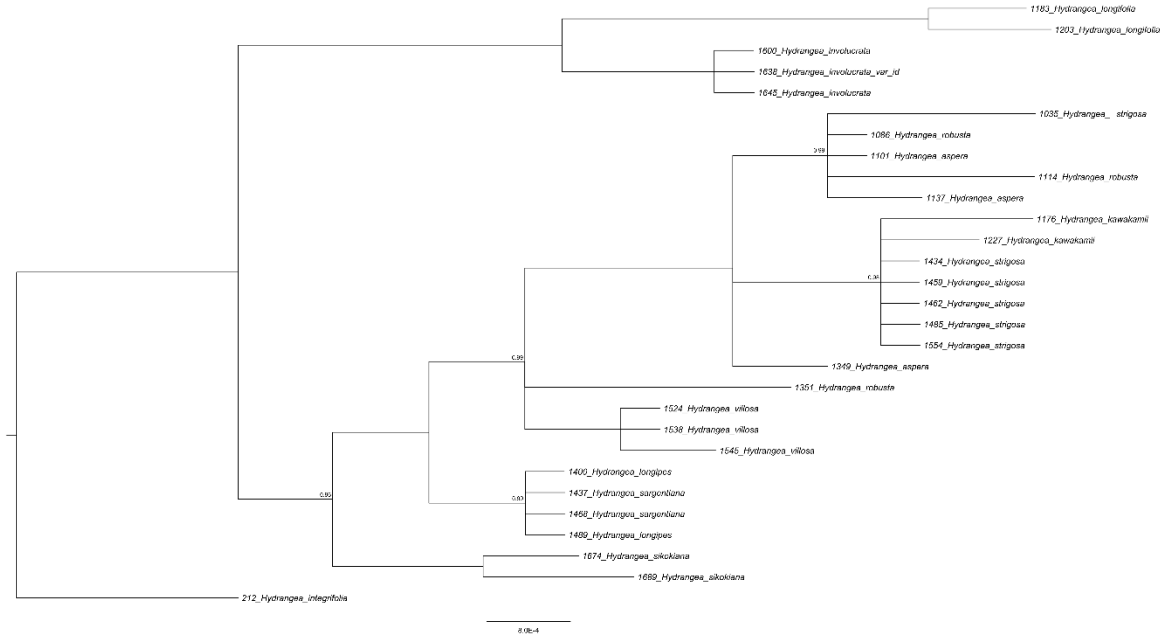


**Figure S3.4: The 50% majority-rule consensus tree based on the *trnL-rpl32* IGS.** Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

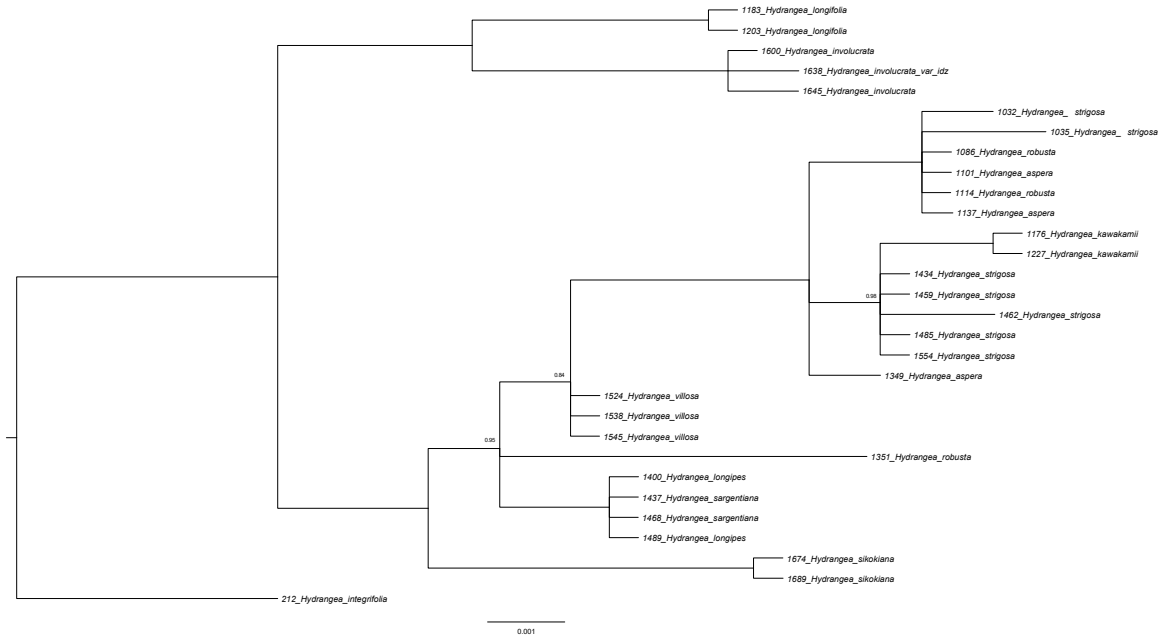



**Figure S3.5: The 50% majority-rule consensus tree based on the ITS region.** Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

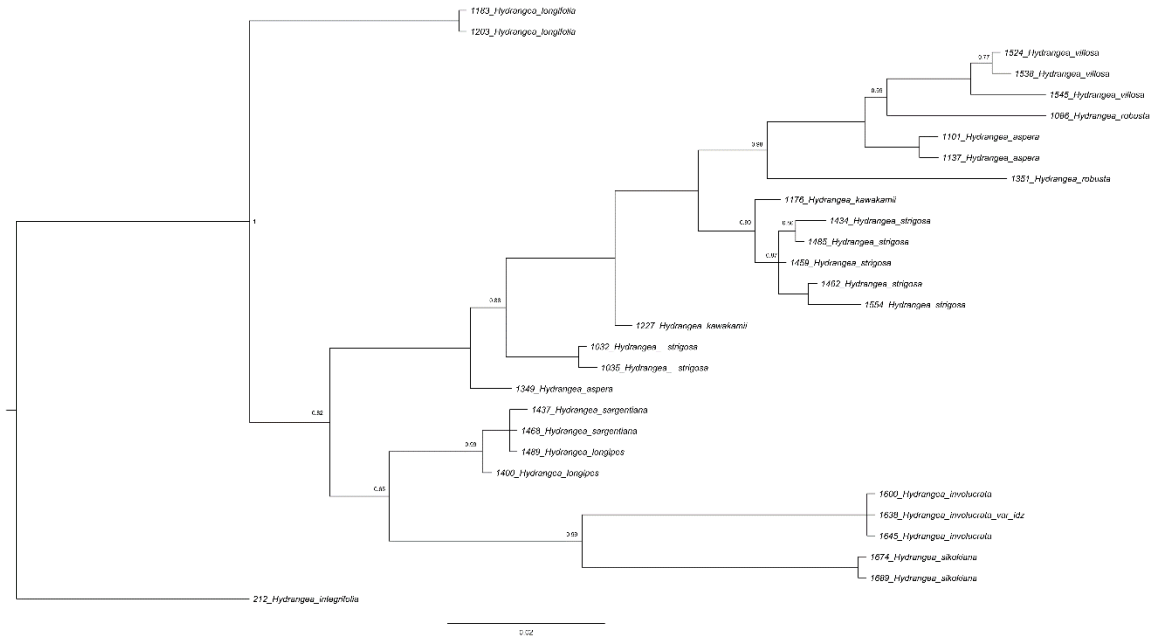


**Figure S3.6: The 50% majority-rule consensus tree based on the *SMC1*-22 region.** Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

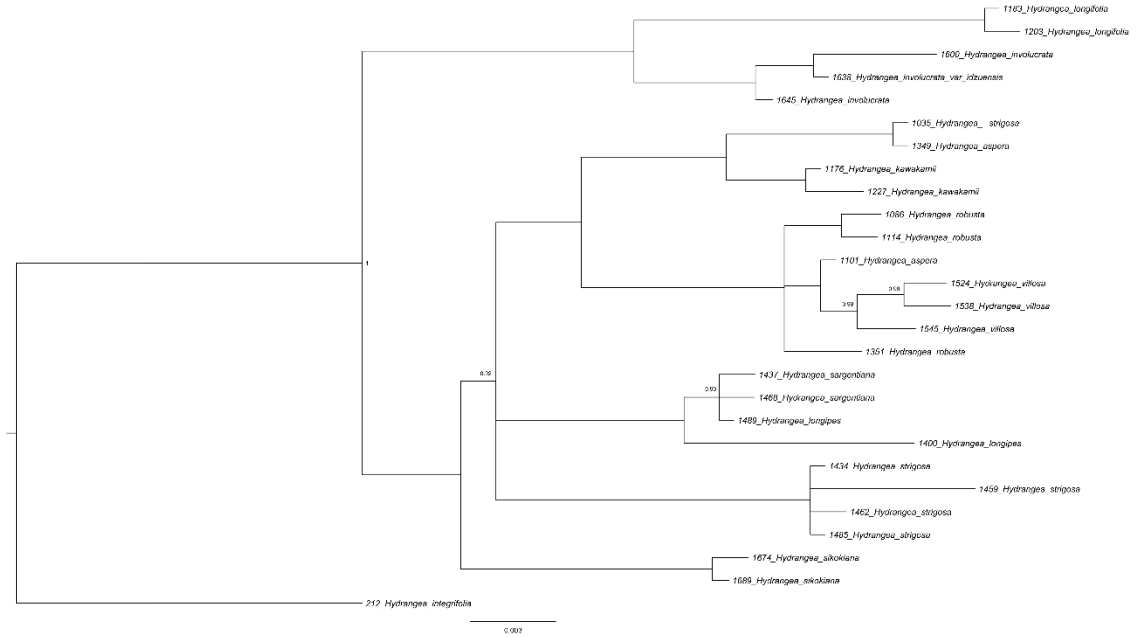



**Figure S3.7: The 50% majority-rule consensus tree based on the *SMC1-44*.** Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

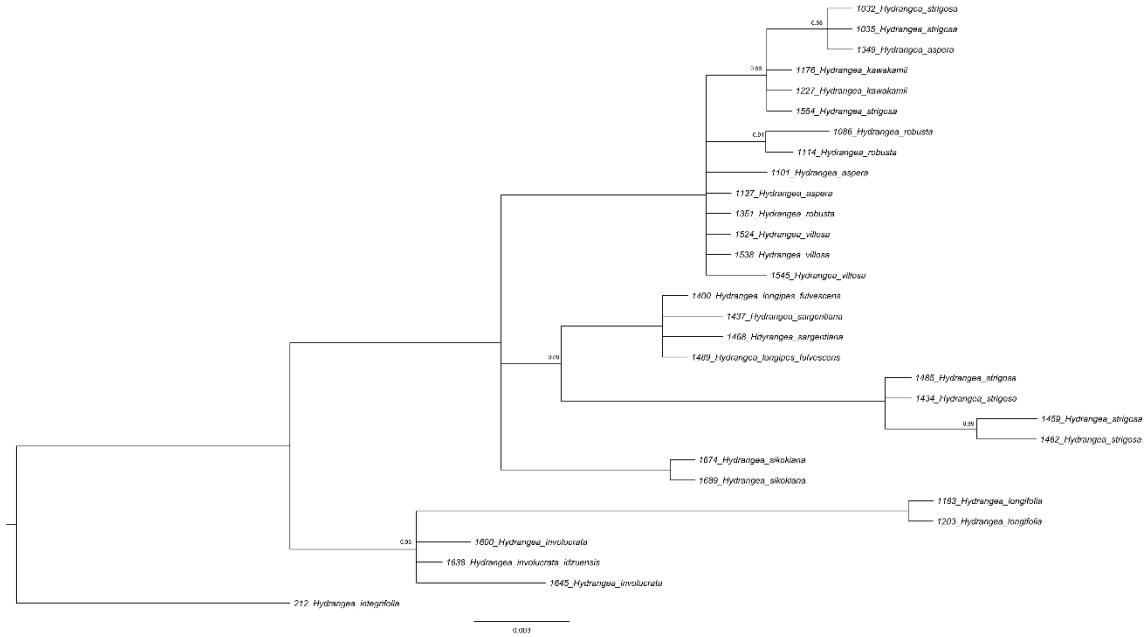
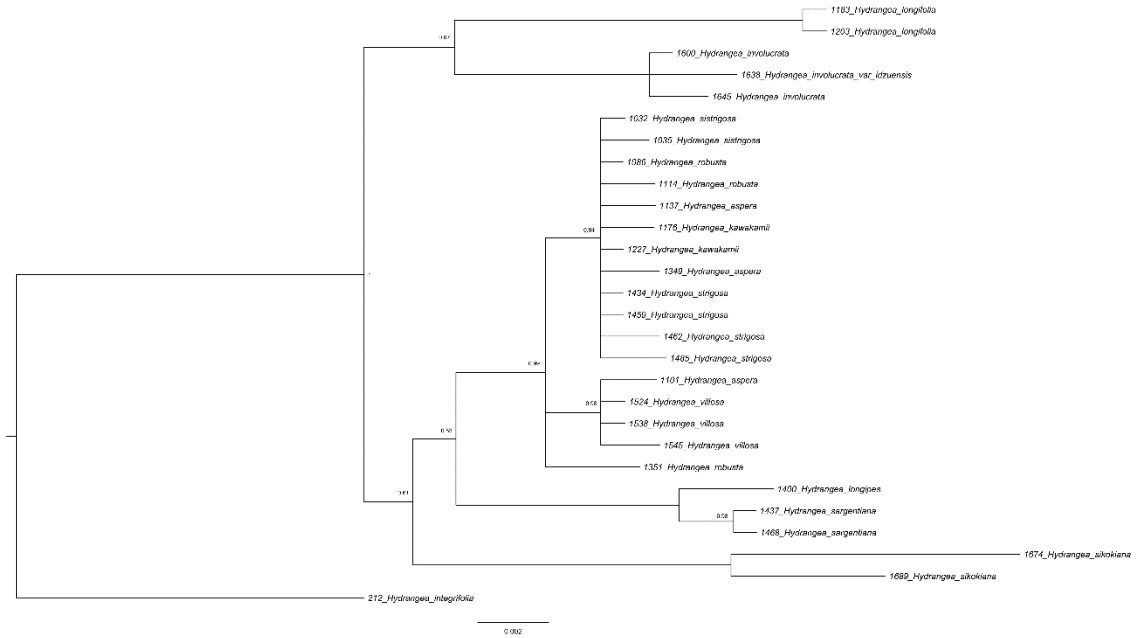


**Figure S3.8: The 50% majority-rule consensus tree based on the *TIF3H1* region**. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.



# Appendix 3: supplementary data chapter 4

**Table S4.1: Voucher specimens for the DNA material utilized in the present study**. For each specimen the full name, voucher ID, locality (if available), altitude of collection and name of collectors are given (where 1: Y. De Smet and E. Rodriguez, 2: Y. De Smet and K. Bauters, 3: Y. De Smet, L. Reyserhove and T. Uemachi) . All specimens were collected from natural populations, with the exception of *Philadelphus* sp., which was grown from seed.

| Taxon | ID | Voucher | Locality | Altitude | Collectors |
|---|---|---|---|---|---|
| *Hydrangea strigosa* | 1035 | YDS1035 | China, Sichuan (29.57, 103.44) | 548m | 1 |
| *Hydrangea robusta* | 1086 | YDS1086 | China, Sichuan (29.67, 102.94) | 1906m | 1 |
| *Hydrangea aspera* | 1101 | YDS1101 | China, Sichuan (29.83, 102.7) | 919m | 1 |
| *Hydrangea robusta* | 1114 | YDS1114 | China, Sichuan (29.69, 102.61) | 1862m | 1 |
| *Hydrangea aspera* | 1137 | YDS1137 | China, Sichuan (29.6, 102.06) | 2194m | 1 |
| *Hydrangea kawakamii* | 1176 | YDS1176 | Taiwan, Yilan (24.39, 121.36) | 1974m | 1 |
| *Hydrangea longifolia* | 1183 | YDS1183 | Taiwan, Yilan (24.54, 121.51) | 803m | 1 |
| *Hydrangea longifolia* | 1203 | YDS1203 | Taiwan, Taichung (24.2, 121.48) | 952m | 1 |
| *Hydrangea kawakamii* | 1227 | YDS1227 | Taiwan, Taichung (24.22, 121.27) | 2030m | 1 |
| *Hydrangea longipes* | 1400 | YDS1400 | China, Hubei (31.05, 110.95) | ~1300m | 2 |
| *Hydrangea strigosa* | 1434 | YDS1434 | China, Hubei (31.33, 110.48) | 1331m | 2 |
| *Hydrangea sargentiana* | 1437 | YDS1437 | China, Hubei (31.33, 110.48) | 1315m | 2 |
| *Hydrangea strigosa* | 1459 | YDS1459 | China, Hubei (31.31, 110.48) | 1357m | 2 |
| *Hydrangea sargentiana* | 1468 | YDS1468 | China, Hubei (31.31, 110.48) | 1443m | 2 |
| *Hydrangea strigosa* | 1485 | YDS1485 | China, Hubei (31.34, 110.51) | 740m | 2 |
| *Hydrangea longipes* | 1489 | YDS1489 | China, Hubei (31.53, 110.34) | 1725m | 2 |
| *Hydrangea villosa* | 1524 | YDS1524 | China, Hubei (30.17, 110.97) | 882m | 2 |
| *Hydrangea villosa* | 1538 | YDS1538 | China, Hubei (30.16, 110.78) | 1055m | 2 |
| *Hydrangea involucrata* | 1600 | YDS1600 | Japan, Hinohara (35.72, 139.11) | 263m | 3 |
| *Hydrangea involucrata* | 1638 | YDS1638 | Japan, Oshima island (34.71, 139.43) | 367m | 3 |
| *Hydrangea involucrata* | 1645 | YDS1645 | Japan, Shiga (35.93, 137.13) | 626m | 3 |
| *Hydrangea sikokiana* | 1674 | YDS1674 | Japan, Tokushima (33.95, 134.4) | 925m | 3 |
| *Hydrangea sikokiana* | 1689 | YDS1689 | Japan, Tokushima (33.91, 134.29) | 1143m | 3 |
| *Hydrangea strigosa* | 1074 | YDS1074 | China, Sichuan (29.63, 103.04) | 1108m | 1 |
| *Hydrangea aspera* | 1164 | YDS1164 | China, Sichuan (30.41, 102.65) | 1532m | 1 |
| *Philadelphus sp.* | Ph01 | YDSPh | | | |

**Table S4.2 Number of loci recovered after preprocessing.** For each of the specimens used in the study, the number of RAD fragments retained after two subsequent processing steps is given: the five step preprocessing and the process-radtags script distributed with the Stacks pipeline.

| Collection nr. | Species | Nominal taxon in study | Loci recovered after: 5 step preprocessing | process-radtags |
|---|---|---|---|---|
| 1137 | *Hydrangea aspera* | *H. aspera* | 389810 | 148253 |
| 1101 | *Hydrangea aspera* | | 233040 | 149502 |
| 1164 | *Hydrangea aspera* | | 163646 | 123587 |
| 1035 | *Hydrangea strigosa* | *H. strigosa* (Sichuan) | 269034 | 86699 |
| 1074 | *Hydrangea strigosa* | | 140790 | 66232 |
| 1434 | *Hydrangea strigosa* | *H. strigosa* (Hubei) | 254674 | 93134 |
| 1485 | *Hydrangea strigosa* | | 203732 | 182717 |
| 1459 | *Hydrangea strigosa* | | 12445 | 2717 |
| 1114 | *Hydrangea robusta* | *H. robusta* | 222527 | 94025 |
| 1086 | *Hydrangea robusta* | | 36254 | 16957 |
| 1400 | *Hydrangea longipes* | *H. longipes* | 475037 | 426147 |
| 1489 | *Hydrangea longipes* | | 330479 | 285614 |
| 1674 | *Hydrangea sikokiana* | *H. sikokiana* | 23915 | 7634 |
| 1689 | *Hydrangea sikokiana* | | 8220 | 640 |
| 1203 | *Hydrangea longifolia* | *H. longifolia* | 30002 | 10062 |
| 1183 | *Hydrangea longifolia* | | 133584 | 106622 |
| 1227 | *Hydrangea kawakamii* | *H. kawakamii* | 89496 | 67155 |
| 1176 | *Hydrangea kawakamii* | | 171414 | 96335 |
| 1437 | *Hydrangea sargentiana* | *H. sargentiana* | 539876 | 405803 |
| 1468 | *Hydrangea sargentiana* | | 635799 | 486655 |
| 1538 | *Hydrangea villosa* | *H. villosa* | 77441 | 55717 |
| 1524 | *Hydrangea villosa* | | 266962 | 218499 |
| 1600 | *Hydrangea involucrata* | *H. involucrata* | 205205 | 126591 |
| 1645 | *Hydrangea involucrata* | | 286282 | 256380 |
| 1638 | *Hydrangea involucrata* | | 214194 | 192466 |
| Ph | *Philadelphus sp.* | *Philadelphus* sp. | 209385 | 97098 |

# Appendix 4: supplementary data chapter 5

**Table S5.1 Herbarium specimens for representatives of *Hydrangea* sect. *Asperae* studied.** Species names in this list are those on the labels, and do not represent confirmation of these identifications by the author.

| Taxon on label | Herb. | Herb. number | Country | Collector | Coll. Number |
|---|---|---|---|---|---|
| *Hydrangea sargentiana* | K | 111 | China | Kirkham, Flanagan, Howick & McNamara | SICH 1801 |
| *Hydrangea aspera subsp. robusta* | E | E00360046 | China | Sino Amer. Exped. | 1353 |
| *Hydrangea aspera* | E | E00360063 | China | Wen-Pen Leu | 1224 |
| *Hydrangea aspera* | AAU | N.A. | China | D.E. Boufford & B. Bartholomew | 24340 |
| *Hydrangea aspera* | CAS | 775626 | China | Sino-american Guizhou Botanical Expedition | 957 |
| *Hydrangea aspera* | MICH | N.A. | China | Sino-american Guizhou Botanical Expedition | 957 |
| *Hydrangea aspera* | AAU | N.A. | China | Sino-american Guizhou Botanical Expedition | 957 |
| *Hydrangea aspera* | US | 1575066 | China | Y. Tsiang | 8412 |
| *Hydrangea aspera* | S | 09-45965 | China | Y. Tsiang | 5589 |
| *Hydrangea aspera* | WU | op-212/32 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 209099 |
| *Hydrangea aspera* | CAS | 826337 | China | D.E. Boufford & B. Bartholomew | 24340 |
| *Hydrangea aspera* | CAS | 1078707 | China | D.E. Boufford | 27176 |
| *Hydrangea aspera* | CAS | 826167 | China | D.E. Boufford & B. Bartholomew | 23946 |
| *Hydrangea aspera* | CAS | 1005427 | China | Kirkham, Cole, Flanagan and McNamara | SICH no.2002 |
| *Hydrangea aspera* | CAS | 1004957 | China | Kirkham, Cole, Flanagan and McNamara | Sich no. 2069 |
| *Hydrangea aspera* | CAS | 1055746 | China | Zhu Da-Hai | 1704 |
| *Hydrangea aspera* | MICH | N.A. | China | D.E. Boufford & B. Bartholomew | 24340 |
| *Hydrangea aspera* | US | 1757819 | China | W.P. Fang | 12619 |
| *Hydrangea aspera* | US | 1968439 | China | C.L. Sun | 1295 |
| *Hydrangea aspera* | AAU | E.H. Wilson C207 | China | E.H. Wilson | 757 |
| *Hydrangea aspera* | E | E00103256 | China | Gaoligong Shan Expedition 1997 | 8687 |
| *Hydrangea aspera* | E | E00246426 | China | Li Heng | 11122 |

| | | | | | |
|---|---|---|---|---|---|
| *Hydrangea aspera* | E | E00248090 | China | Li Heng | 10303 |
| *Hydrangea aspera* | E | E00156371 | China | Gaoligong Shan Expedition | 7392 |
| *Hydrangea aspera* | E | E00073242 | China | Cox, P. & Hutchinson, P. | 7127 |
| *Hydrangea aspera* | E | E00103265 | China | Gaoligong Shan Expedition | 8545 |
| *Hydrangea aspera* | E | E00360056 | China | N.A. | N.A. |
| *Hydrangea aspera* | WU | op-212/30 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 1789 |
| *Hydrangea aspera* | WU | op-212/31 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 711 |
| *Hydrangea aspera* | WU | op-212/35 | China | N.A. | N.A. |
| *Hydrangea aspera* | GB | N.A. | China | Alpine Garden Society expedition to China | 386 |
| *Hydrangea aspera* | E | E00360061 | China | Sino-amer. Bot. Exped. | 1648 |
| *Hydrangea aspera* | E | E00360064 | China | B. Alden | 1243 |
| *Hydrangea aspera* | E | E00360019 | China | George forrest | 30030 |
| *Hydrangea aspera* | CAS | 724743 | China | Sino-amer. Bot. Exped. | 1648 |
| *Hydrangea aspera* | CAS | 796362 | China | B. Alden et al. | 1499 |
| *Hydrangea aspera* | US | 1271045 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 711 |
| *Hydrangea aspera* | G | G00163792 | China | C. Schneider | 2616 |
| *Hydrangea aspera* | AAU | Forrest 30030 | China | Forrest | 30030 |
| *Hydrangea aspera* | E | E00360060 | China | Coll. J. Cavalerie | N.A. |
| *Hydrangea aspera* | E | E00360057 | China | George Forrest | 29867 |
| *Hydrangea aspera* | E | E00198443 | China | N.A. | ACE 386 |
| *Hydrangea aspera* | E | E00102648 | China | Gaoligong shan expedition | 9039 |
| *Hydrangea aspera* | E | E00102649 | China | Gaoligong shan expedition | 9167 |
| *Hydrangea aspera* | E | E00360059 | China | N.A. | N.A. |
| *Hydrangea aspera* | WU | op-212/29 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 2094 |
| *Hydrangea aspera* | WU | op-212/33 | China | leg. E. Faber | N.A. |
| *hydrangea aspera* | WU | op-212/34 | China | leg. E. Faber | N.A. |
| *Hydrangea aspera* | S | S 06-200 | India | Erik Emanuelsson | 3071 |
| *Hydrangea aspera* | US | 2581377 | Nepal | D. Banesjee | 5580 |
| *Hydrangea aspera* | US | 3293208 | Taiwan | Wen-Pen Leu | 1224 |
| *Hydrangea aspera* | CAS | 1104975 | Taiwan | Chien-Hua Liu | 523 |

| | | | | | |
|---|---|---|---|---|---|
| *Hydrangea aspera* | CAS | 865235 | Taiwan | Chi-Cheng Liao | 475 |
| *Hydrangea aspera* | CAS | 927566 | Taiwan | C.M. Wang | 1763 |
| *Hydrangea aspera* | E | E00003162 | Taiwan | Yih-Ren Lin | 132 |
| *Hydrangea aspera* | S | 09-45984 | Taiwan | T. Shimizu | 20435 |
| *Hydrangea aspera* | E | E00210591 | Taiwan | B. Bartholomew | 7636 |
| *Hydrangea aspera* | CAS | 944983 | Taiwan | B. Bartholomew | 7636 |
| *Hydrangea aspera* | CAS | 1002807 | Taiwan | S.L. Kelley | 98-98 |
| *Hydrangea aspera* | E | E00037397 | Taiwan | Edinburgh Taiwan Expedition | 139 |
| *Hydrangea aspera* | CAS | 1051522 | Tibet | Gary h. Bolton | 93-27 |
| *Hydrangea aspera* | AAU | 3058 | Vietnam | D.D. Tirvengadum | 3058 |
| *Hydrangea aspera* | WU | 4533 | Nepal | Sajan Subedi | 315 |
| *Hydrangea aspera* | S | 09-46075 | Nepal | herbarium of the late East Indian Company | 2493 |
| *Hydrangea aspera* | US | 1990383 | China | F.C. Tai | 4077 |
| *Hydrangea aspera* | E | E00360058 | N.A. | N.A. | 960 |
| *Hydrangea aspera* | US | 281963 | N.A. | H.O. Forbes | 9479 |
| *Hydrangea aspera* | K | 130 | China | Y.W. Law | 1344 |
| *Hydrangea aspera* | K | 227 | China | Fliegner, Howick, McNamara & Staniforth | SICH 1052 |
| *Hydrangea aspera* | K | 108 | China | Kirkham, Flanagan, Howick & McNamara | SICH 1757 |
| *Hydrangea aspera* | K | 107 | China | Kirkham, Flanagan, Howick & McNamara | SICH 1708 |
| *Hydrangea aspera* | K | 109 | China | Kirkham, Cole, Flanagan and McNamara | SICH 2069 |
| *Hydrangea aspera* | K | 110 | China | Kirkham, Cole, Flanagan and McNamara | SICH 2002 |
| *Hydrangea aspera* | K | 112 | China | Simmons, Erksine, Howick & McNamara | SICH 772 |
| *Hydrangea aspera* | K | 228 | China | Alpine Garden Society expedition to China | ACE 386 |
| *Hydrangea aspera* | K | 307 | Nepal | A.D. Schilling | 1025 |
| *Hydrangea aspera* | K | 287 | Sumatra | W.J.J.O. De Wilde and B.E.E. de Wilde-Duyfjes | N.A. |
| *Hydrangea aspera* | K | 290 | Sumatra | N. Walter & C.M. Bangham | 959 |
| *Hydrangea aspera* | K | 113 | Taiwan | Kirkham & Flanagan | ETOT 55 |
| *Hydrangea aspera subsp. strigosa* | K | 121 | China | C.R. Lancaster | L. 1054 |
| *Hydrangea aspera ssp. strigosa* | K | 97 | China | C.R. Lancaster | L. 1054 |
| *Hydrangea aspera subsp robusta* | CAS | 775825 | China | Sino-American Guizhou Botanical expedition no. 677 | N.A. |

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|---|---|---|---|---|---|
| *Hydrangea aspera subsp robusta* | CAS | 826349 | China | D.E. Boufford & B. Bartholomew | 24740 |
| *Hydrangea aspera subsp. aspera* | E | E00360062 | China | D. Chamberlain | CEE 244 |
| *Hydrangea aspera subsp. strigosa* | MICH | N.A. | China | Sino-American Guizhou Botanical Expedition | 1278 |
| *Hydrangea aspera subsp. strigosa* | AAU | N.A. | China | Sino-American Guizhou Botanical Expedition | 1278 |
| *Hydrangea aspera subsp. strigosa* | CAS | 773169 | China | Sino-American Guizhou Botanical Expedition | 1997 |
| *Hydrangea aspera subsp. strigosa* | CAS | 773253 | China | Sino-American Guizhou Botanical Expedition | 1978 |
| *Hydrangea aspera subsp. strigosa* | CAS | 801296 | China | Sino-American Guizhou Botanical Expedition | 1278 |
| *Hydrangea aspera subsp. strigosa* | CAS | 776947 | China | Sino-American Guizhou Botanical Expedition | 812 |
| *Hydrangea aspera subsp. strigosa* | CAS | 801236 | China | Sino-American Guizhou Botanical Expedition | 1249 |
| *Hydrangea aspera subsp. strigosa* | G | G00163787 | China | Sino-American Guizhou Botanical expedition | 116 |
| *Hydrangea aspera subsp. strigosa* | E | E00360054 | China | Sino-Amer. Exped. | 1106 |
| *Hydrangea aspera subsp. strigosa* | E | E00360053 | China | Sino-Amer. Exped. | 467 |
| *Hydrangea aspera subsp. strigosa* | MICH | N.A. | China | D.E. Boufford & B. Bartholomew | 24018 |
| *Hydrangea aspera subsp. strigosa* | AAU | N.A. | China | D.E. Boufford & B. Bartholomew | 24018 |
| *Hydrangea aspera subsp. strigosa* | CAS | 826168 | China | D.E. Boufford & B. Bartholomew | 24018 |
| *Hydrangea aspera subsp. strigosa* | US | 1990473 | China | C.L. Chow | 4721 |
| *Hydrangea aspera subsp. strigosa* | US | 1991014 | China | Wen-Kuang Hu | 8836 |
| *Hydrangea aspera subsp. strigosa* | US | 1991039 | China | Wen-Kuang Hu | 8984 |
| *Hydrangea aspera subsp. strigosa* | US | 1525757 | China | N.A. | N.A. |
| *Hydrangea aspera var. velutina* | E | E00296416 | China | E.H. Wilson | 2405 |
| *Hydrangea cf. robusta* | K | 2814 | Bhutan | A.J.C. Grierson & D.G. Long | 2021 |
| *Hydrangea cf. sargentiana* | G | G00163843 | China | E.E. Maire | N.A. |
| *Hydrangea coacta* | CAS | 1056231 | China | Zhu Da-Hai etc. | 2325 |
| *Hydrangea coacta* | CAS | 1056227 | China | Zhu Da-Hai etc. | 2258 |
| *Hydrangea fulvescens* | E | E00296421 | China | E.H. Wilson | 1373 |
| *Hydrangea glabripes* | E | E00296417 | China | E.H. Wilson | 2391 |
| *Hydrangea glabripes* | US | bc00096996 | China | E.H. Wilson | 2391 |
| *Hydrangea kawakamii* | S | 09-46066 | Taiwan | J. L. Gressitt | 458 |
| *Hydrangea longifolia* | CAS | 943166 | Taiwan | Shu-Mei Liu | 351 |
| *Hydrangea longifolia* | CAS | 1104996 | Taiwan | Ya-Yi Huang | 560 |

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|---|---|---|---|---|---|
| *Hydrangea longifolia* | CAS | 798941 | Taiwan | Tsui-Ya Lui | 863 |
| *Hydrangea longifolia* | US | N.A. | N.A. | N.A. | N.A. |
| *Hydrangea longipes* | AAU | N.A. | China | Yuan Yong-ming | N.A. |
| *Hydrangea longipes* | CAS | 846671 | China | Yuan Yong-ming | 1102 |
| *Hydrangea longipes* | WU | op-212/28 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 12386 |
| *Hydrangea longipes* | US | 597016 | China | E.H. Wilson | 2514 |
| *Hydrangea longipes* | CAS | 1056224 | China | Zhu Da-Hai etc | 2393 |
| *Hydrangea longipes* | K | 237 | China | J.F. Rock | 14782 |
| *Hydrangea longipes* | K | 238 | China | W. Purdom | 977 |
| *Hydrangea longipes* | K | 241 | China | Fliegner, Howick, McNamara & Staniforth | SICH 1240 |
| *Hydrangea longipes* | K | 239 | China | E.H. Wilson | 2406 |
| *Hydrangea maximowiczii* | E | E00296415 | China | J. Cavalerie | 22 |
| *Hydrangea maximowiczii* | E | E00296414 | China | E. Bodinier | 1654 |
| *Hydrangea robusta* | K | 292 | N.A. | F. Kingdon | 8525 |
| *Hydrangea rosthornii* | S | 09-45959 | China | A.N. Steward | 952 |
| *Hydrangea rosthornii* | US | 1757304 | China | Tsang W.T. | 27885 |
| *Hydrangea rosthornii* | US | 1757175 | China | Tsang, W.T. | 27728 |
| *Hydrangea rosthornii* | US | 1757667 | China | Tsang W.T. | 28361 |
| *Hydrangea rosthornii* | S | 09-45961 | China | Y. Tsiang | 8915 |
| *Hydrangea rosthornii* | S | 09-46084 | China | Y. Tsiang | 5869 |
| *Hydrangea rosthornii* | G | G00163791 | China | Y. Tsiang | 5869 |
| *Hydrangea rosthornii* | E | E00360044 | China | E.H. Wilson | 2414 |
| *Hydrangea rosthornii* | E | E00360043 | China | Mc Laren | AD167 |
| *Hydrangea rosthornii* | E | E00360045 | China | N.A. | N.A. |
| *Hydrangea rosthornii* | US | 1757135 | China | W.T. Tsang | 27675 |
| *Hydrangea rosthornii* | K | 105 | China | Fliegner, Howick, McNamara & Staniforth | SICH 916 |
| *Hydrangea rosthornii* | K | 106 | China | W.F. Fang | 6697 |
| *Hydrangea sargentiana* | CAS | 1012878 | China | Wilson E.H. | 772 |
| *Hydrangea sargentiana* | US | 1279991 | China | W.Y. Chun | 3891 |
| *Hydrangea sargentiana* | US | N.A. | China | N.A. | N.A. |

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|---|---|---|---|---|---|
| *Hydrangea sargentiana* | K | 247 | China | Kirkham, Ruddy, Flanagan, McNamara | SICH 2107 |
| *Hydrangea strigosa* | AAU | N.A. | China | K.S. Chow | 35 |
| *Hydrangea strigosa* | WU | 14800 | China | C.Y. Chiao | N.A. |
| *Hydrangea strigosa* | US | 1247171 | China | R.C. Ching | 2340 |
| *Hydrangea strigosa* | US | 1427111 | China | C.Y. Chiao | 1389 |
| *Hydrangea strigosa* | CAS | 763555 | China | K. Yao | 9148 |
| *Hydrangea strigosa* | WU | op-212/38 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 2686 |
| *Hydrangea strigosa* | AAU | N.A. | China | K.S.S Chow | 133 |
| *Hydrangea strigosa* | US | 3179320 | China | K.S. Chow et al. | 133 |
| *Hydrangea strigosa* | US | 3179064 | China | Li Zhen-yu et al. | 1557 |
| *Hydrangea strigosa* | US | 3467679 | China | Hu Zhong-hui | 507 |
| *Hydrangea strigosa* | E | E00360049 | China | E.H. Wilson | 2390 |
| *Hydrangea strigosa* | E | E00360048 | China | E.H. Wilson | 2396 |
| *Hydrangea strigosa* | US | 598478 | China | E.H. Wilson | 2390 |
| *Hydrangea strigosa* | US | 598480 | China | E.H. Wilson | 2392 |
| *Hydrangea strigosa* | US | 1279992 | China | W.Y. Chun | 3961 |
| *Hydrangea strigosa* | US | 1969506 | China | P.C. Silvestri | 4350 |
| *Hydrangea strigosa* | CAS | 845044 | China | S.L. Liu | 890002 |
| *Hydrangea strigosa* | AAU | N.A. | China | K. Yao | 9148 |
| *Hydrangea strigosa* | US | 3532687 | China | Tan Ce-ming | 95535 |
| *Hydrangea strigosa* | AAU | N.A. | China | Tan Ce -ming | 95535 |
| *Hydrangea strigosa* | AAU | N.A. | China | C.M. Tan | 9611108 |
| *Hydrangea strigosa* | S | 09-45966 | China | Liang Feng Yah | 4 |
| *Hydrangea strigosa* | S | 09-45963 | China | Ta Ho Yen | 741 |
| *Hydrangea strigosa* | S | 09-46854 | China | Y. Tsiang | 5161 |
| *Hydrangea strigosa* | S | 09-46848 | China | Y. Tsiang | 4849 |
| *Hydrangea strigosa* | US | 1575103 | China | Y. Tsiang | 4849 |
| *Hydrangea strigosa* | US | 1598783 | China | A.N. Steward et al. | 4 |
| *Hydrangea strigosa* | WU | op-212/37 | China | Dr. Heinr. Frh. V. Handel-Mazzetti | 2089 |
| *Hydrangea strigosa* | CAS | 778968 | China | J.L. Reveal | 5934 |

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|---|---|---|---|---|---|
| *Hydrangea strigosa* | CAS | 1078710 | China | D.E. Boufford et al. | 32842 |
| *Hydrangea strigosa* | S | 09-46837 | China | C.Y. Chiao | 2075 |
| *Hydrangea strigosa* | CAS | 706327 | China | C.Y. Chiao | 87 |
| *Hydrangea strigosa* | CAS | N.A. | China | W.P. Fang | 7382 |
| *Hydrangea strigosa* | E | E00360055 | China | W.P. Fang | 2313 |
| *Hydrangea strigosa* | US | 597019 | China | E.H. Wilson | 2527 |
| *Hydrangea strigosa* | US | 598482 | China | E.H. Wilson | 2395 |
| *Hydrangea strigosa* | E | E00360052 | China | George Forrest | 9426 |
| *Hydrangea strigosa* | E | E00360051 | China | George Forrest | 18847 |
| *Hydrangea strigosa* | US | 1332734 | China | J.F. Rock | 7134 |
| *Hydrangea strigosa* | US | 1332733 | China | J.F. Rock | 7086 |
| *Hydrangea strigosa* | E | E00360050 | China | George Forrest | 27704 |
| *Hydrangea strigosa* | US | 1674257 | China | W.C. Cheng | 3924 |
| *Hydrangea strigosa* | K | 94 | China | B.-Z. Xiao | 4405 |
| *Hydrangea strigosa* | K | 137 | China | K. Yao | N.A. |
| *Hydrangea strigosa* | K | 104 | China | W.T. Tsang | 20671 |
| *Hydrangea strigosa* | K | 138 | China | Z.-T. Wang etc. | 870419 |
| *Hydrangea strigosa* | K | 98 | China | W.P. Fang | 7435 |
| *Hydrangea strigosa* | K | 101 | China | W. Hancock | 358 |
| *Hydrangea strigosa* | K | 125 | China | A. Henry | N.A. |
| *Hydrangea strigosa* | K | 127 | Taiwan | A. Henry | 2167 |
| *Hydrangea strigosa* | K | 121 | China | K. Yao | 9148 |
| *Hydrangea cfr. strigosa* | S | S09-46851 | China | E. Dahlström | 140 |
| *Hydrangea strigosa var. angustifolia* | K | 103 | China | Y. Tsiang | 4849 |
| *Hydrangea strigosa var. macrophylla* | K | 131 | China | R.-C. Ching | 3130 |
| *Hydrangea vestita* | CAS | 486343 | Nepal | N.A. | N.A. |
| *Hydrangea vestita* | E | E00360023 | China | G. Forrest | N.A. |
| *Hydrangea vestita* | S | 09-10791 | China | N.A. | N.A. |
| *Hydrangea vestita* | S | 09-46344 | China | N.A. | N.A. |
| *Hydrangea vestita* | S | 09-46349 | China | N.A. | N.A. |

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|---|---|---|---|---|---|
| *Hydrangea vestita* | G | G00219583 | Nepal | M. Wallich | N.A. |
| *Hydrangea vestita* | G | G00219584 | Nepal | M. Wallich | N.A. |
| *Hydrangea vestita* | K | 212 | China | G. Forrest | 2830 |
| *Hydrangea vestita var. pubescens* | K | 221 | China | Moellendorff | 45 |
| *Hydrangea villosa* | CAS | 1067206 | China | N.A. | N.A. |
| *Hydrangea villosa* | CAS | 842207 | China | Wang Zhong-tao etc | 870300 |
| *Hydrangea villosa* | CAS | 841333 | China | Zhao Qing-sheng | N.A. |
| *Hydrangea villosa* | CAS | 843298 | China | Wang Zhong-tao etc | 870198 |
| *Hydrangea villosa* | E | E00296418 | China | E.H. Wilson | 1227 |
| *Hydrangea villosa* | WU | op-212/36 | China | N.A. | N.A. |
| *Hydrangea villosa* | K | 122 | China | Z.-Y. Li | 896421 |
| *Hydrangea villosa* | K | 141 | China | Cheng et Hwa | 1197 |
| *Hydrangea villosa* | K | 118 | China | Fliegner, Howick, McNamara & Staniforth | SICH 925 |
| *Hydrangea villosa* | K | 119 | China | Fliegner, Howick, McNamara & Staniforth | SICH 9000 |
| *Hydrangea villosa* | K | 120 | China | Z.-T. Wang etc. | 870300 |
| *Hydrangea villosa* | K | 121 | China | Z.-T. Wang etc. | 870198 |
| *Hydrangea villosa form. sterilis* | E | E00296393 | China | E.H. Wilson | 1473 |
| *Hydrangea villosa var. sterilis* | K | 124 | China | W.H. Qun | W102 |
| *Hydrangea villosa var. sterilis* | K | 123 | China | Z.-T. Wang etc. | 870333 |

**Table S5.2 Collections carried out in the framework of this project.** These specimens were utilized to study diversity of *Hydrangea* sect. *Asperae***.** For each specimen, the voucher number is given, as well as the location of collection, date of collection and the collectors are mentioned (where 1: Y. De Smet and E. Rodriguez, 2: Y. De Smet and K. Bauters, 3: Y. De Smet, L. Reyserhove and T. Uemachi).

| Voucher | Species | Country | Region | Locality | Altitude (m) | lat. ° | lat. ' | lat. " | lat dir. | long. ° | long. ' | long. " | long. dir. | Collector | Collection Date |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1556 | *H. cf. aspera* | China | Hubei | Changyang city | 1282 | 30 | 42 | 19,7 | N | 110 | 34 | 11,1 | E | 2 | 22/07/2012 |
| YDS1557 | *H. cf. aspera* | China | Hubei | Changyang city | 1285 | 30 | 42 | 19,6 | N | 110 | 34 | 11 | E | 2 | 22/07/2012 |
| YDS1558 | *H. cf. aspera* | China | Hubei | Changyang city | 1308 | 30 | 42 | 21,1 | N | 110 | 34 | 23,9 | E | 2 | 22/07/2012 |
| YDS1559 | *H. cf. aspera* | China | Hubei | Changyang city | 1311 | 30 | 42 | 21,1 | N | 110 | 34 | 24,7 | E | 2 | 22/07/2012 |
| YDS1560 | *H. cf. aspera* | China | Hubei | Changyang city | 1311 | 30 | 42 | 21,1 | N | 110 | 34 | 25,3 | E | 2 | 22/07/2012 |
| YDS1084 | *H. aspera* | China | Sichuan | Wawu Shan | 1908 | 29 | 39 | 54,7 | N | 102 | 56 | 27,1 | E | 1 | 25/07/2011 |
| YDS1086 | *H. aspera* | China | Sichuan | Wawu Shan | 1906 | 29 | 39 | 54,6 | N | 102 | 56 | 27,2 | E | 1 | 25/07/2011 |
| YDS1087 | *H. aspera* | China | Sichuan | Wawu Shan | 1912 | 29 | 39 | 54 | N | 102 | 56 | 27,2 | E | 1 | 25/07/2011 |
| YDS1089 | *H. aspera* | China | Sichuan | Wawu Shan | 1934 | 29 | 40 | 5,1 | N | 102 | 56 | 48 | E | 1 | 25/07/2011 |
| YDS1108 | *H. aspera* | China | Sichuan | Niba Shan | 1802 | 29 | 41 | 49,7 | N | 102 | 36 | 36,9 | E | 1 | 27/07/2011 |
| YDS1114 | *H. aspera* | China | Sichuan | Niba Shan | 1862 | 29 | 41 | 39,1 | N | 102 | 36 | 43,4 | E | 1 | 27/07/2011 |
| YDS1115 | *H. aspera* | China | Sichuan | Niba Shan | 2142 | 29 | 39 | 55,5 | N | 102 | 36 | 35 | E | 1 | 27/07/2011 |
| YDS1116 | *H. aspera* | China | Sichuan | Niba Shan | 2283 | 29 | 39 | 17,6 | N | 102 | 37 | 10,5 | E | 1 | 27/07/2011 |
| YDS1117 | *H. aspera* | China | Sichuan | Niba Shan | 2289 | 29 | 39 | 14,8 | N | 102 | 37 | 11 | E | 1 | 27/07/2011 |
| YDS1118 | *H. aspera* | China | Sichuan | Niba Shan | 2291 | 29 | 39 | 14,2 | N | 102 | 37 | 11,2 | E | 1 | 31/07/2011 |
| YDS1129 | *H. aspera* | China | Sichuan | Erlang Shan | 2090 | 29 | 51 | 44,2 | N | 102 | 18 | 49,4 | E | 1 | 01/08/2011 |
| YDS1526 | *H. villosa* | China | Hubei | Wufeng county | 957 | 30 | 10 | 19,7 | N | 110 | 57 | 27 | E | 2 | 20/07/2012 |
| YDS1542 | *H. villosa* | China | Hubei | Wufeng county | 1205 | 30 | 9 | 27,2 | N | 110 | 43 | 19 | E | 2 | 20/07/2012 |
| YDS1543 | *H. villosa* | China | Hubei | Wufeng county | 1205 | 30 | 9 | 27,3 | N | 110 | 43 | 19,1 | E | 2 | 20/07/2012 |
| YDS1038 | *H. aspera* | China | Sichuan | Xiu Shui | 1374 | 29 | 32 | 11 | N | 103 | 20 | 3,7 | E | 1 | 23/07/2011 |
| YDS1058 | *H. aspera* | China | Sichuan | Jinkouhe | 1009 | 29 | 27 | 59,2 | N | 103 | 21 | 51,2 | E | 1 | 24/07/2011 |
| YDS1059 | *H. aspera* | China | Sichuan | Jinkouhe | 917 | 29 | 27 | 35,6 | N | 103 | 21 | 46,5 | E | 1 | 24/07/2011 |
| YDS1060 | *H. aspera* | China | Sichuan | Jinkouhe | 934 | 29 | 18 | 19 | N | 103 | 16 | 30,3 | E | 1 | 24/07/2011 |
| YDS1063 | *H. aspera* | China | Sichuan | Wa Shan | 923 | 29 | 19 | 8,2 | N | 103 | 5 | 48,5 | E | 1 | 24/07/2011 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1072 | *H. aspera* | China | Sichuan | Wawu Shan | 1118 | 29 | 38 | 7,3 | N | 103 | 4 | 14,3 | E | 1 | 25/07/2011 |
| YDS1092 | *H. aspera* | China | Sichuan | Wawu Shan | 1635 | 29 | 42 | 24,1 | N | 102 | 57 | 13 | E | 1 | 25/07/2011 |
| YDS1094 | *H. aspera* | China | Sichuan | Wawu Shan | 1313 | 29 | 41 | 56,9 | N | 102 | 58 | 7,5 | E | 1 | 26/07/2011 |
| YDS1101 | *H. aspera* | China | Sichuan | Niba Shan | 919 | 29 | 49 | 47,6 | N | 102 | 41 | 46,2 | E | 1 | 27/07/2011 |
| YDS1106 | *H. aspera* | China | Sichuan | Niba Shan | 1289 | 29 | 44 | 18,7 | N | 102 | 37 | 33,9 | E | 1 | 27/07/2011 |
| YDS1109 | *H. aspera* | China | Sichuan | Niba Shan | 1806 | 29 | 41 | 49,5 | N | 102 | 36 | 37 | E | 1 | 27/07/2011 |
| YDS1110 | *H. aspera* | China | Sichuan | Niba Shan | 1824 | 29 | 41 | 45,2 | N | 102 | 36 | 38,8 | E | 1 | 27/07/2011 |
| YDS1112 | *H. aspera* | China | Sichuan | Niba Shan | 1835 | 29 | 41 | 45,3 | N | 102 | 36 | 41,1 | E | 1 | 27/07/2011 |
| YDS1137 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2194 | 29 | 35 | 58,4 | N | 102 | 3 | 27,3 | E | 1 | 01/08/2011 |
| YDS1138 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2180 | 29 | 35 | 59,5 | N | 102 | 3 | 30,3 | E | 1 | 01/08/2011 |
| YDS1139 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2181 | 29 | 35 | 59,6 | N | 102 | 3 | 31,2 | E | 1 | 01/08/2011 |
| YDS1140 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2177 | 29 | 36 | 1,4 | N | 102 | 3 | 32,4 | E | 1 | 01/08/2011 |
| YDS1141 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2173 | 29 | 36 | 2,4 | N | 102 | 3 | 33,3 | E | 1 | 01/08/2011 |
| YDS1142 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2153 | 29 | 36 | 8,7 | N | 102 | 3 | 49,2 | E | 1 | 01/08/2011 |
| YDS1143 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2104 | 29 | 36 | 11 | N | 102 | 4 | 0,6 | E | 1 | 01/08/2011 |
| YDS1144 | *H. aspera* | China | Sichuan | Hailuogou Glacier Park | 2031 | 29 | 36 | 13,3 | N | 102 | 4 | 16,6 | E | 1 | 01/08/2011 |
| YDS1146 | *H. aspera* | China | Sichuan | Lingguan | 825 | 30 | 16 | 55,4 | N | 102 | 50 | 50,68 | E | 1 | 03/08/2011 |
| YDS1148 | *H. aspera* | China | Sichuan | Lingguan | 825 | 30 | 16 | 55,4 | N | 102 | 50 | 50,68 | E | 1 | 03/08/2011 |
| YDS1149 | *H. aspera* | China | Sichuan | Lingguan | 825 | 30 | 16 | 55,4 | N | 102 | 50 | 50,68 | E | 1 | 03/08/2011 |
| YDS1163 | *H. aspera* | China | Sichuan | Tongla Shan | 1531 | 30 | 24 | 28,7 | N | 102 | 38 | 51,8 | E | 1 | 04/08/2011 |
| YDS1164 | *H. aspera* | China | Sichuan | Tongla Shan | 1532 | 30 | 24 | 28,7 | N | 102 | 38 | 52,3 | E | 1 | 04/08/2011 |
| YDS1165 | *H. aspera* | China | Sichuan | Tongla Shan | 1535 | 30 | 24 | 27,7 | N | 102 | 38 | 51,36 | E | 1 | 04/08/2011 |
| YDS1167 | *H. kawakamii* | Taiwan | Yilan | Kefa bridge | 1572 | 24 | 24 | 22 | N | 121 | 21 | 23,4 | E | 1 | 15/08/2011 |
| YDS1168 | *H. kawakamii* | Taiwan | Yilan | Kefa bridge | 1593 | 24 | 24 | 21,3 | N | 121 | 21 | 22,8 | E | 1 | 15/08/2011 |
| YDS1169 | *H. kawakamii* | Taiwan | Yilan | Kefa bridge | 1595 | 24 | 24 | 21,2 | N | 121 | 21 | 22,7 | E | 1 | 15/08/2011 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1170 | *H. kawakamii* | Taiwan | Yilan | Suyuan | 1556 | 24 | 24 | 17,3 | N | 121 | 21 | 37,6 | E | 1 | 15/08/2011 |
| YDS1171 | *H. kawakamii* | Taiwan | Yilan | Suyuan | 1571 | 24 | 24 | 12,6 | N | 121 | 21 | 40 | E | 1 | 15/08/2011 |
| YDS1172 | *H. kawakamii* | Taiwan | Yilan | Suyuan | 1615 | 24 | 24 | 10,7 | N | 121 | 21 | 59,2 | E | 1 | 15/08/2011 |
| YDS1173 | *H. kawakamii* | Taiwan | Yilan | Shun Guan | 1842 | 24 | 22 | 19,3 | N | 121 | 20 | 23,4 | E | 1 | 15/08/2011 |
| YDS1174 | *H. kawakamii* | Taiwan | Yilan | Shun Guan | 1853 | 24 | 22 | 17,2 | N | 121 | 20 | 22,9 | E | 1 | 15/08/2011 |
| YDS1175 | *H. kawakamii* | Taiwan | Yilan | Shun Guan | 1852 | 24 | 20 | 19,1 | N | 121 | 20 | 23,7 | E | 1 | 15/08/2011 |
| YDS1176 | *H. kawakamii* | Taiwan | Yilan | Szyuan Yakou | 1974 | 24 | 23 | 18,4 | N | 121 | 21 | 19,7 | E | 1 | 15/08/2011 |
| YDS1177 | *H. kawakamii* | Taiwan | Yilan | Szyuan Yakou | 1990 | 24 | 23 | 14,5 | N | 121 | 21 | 24,9 | E | 1 | 15/08/2011 |
| YDS1178 | *H. kawakamii* | Taiwan | Yilan | Szyuan Yakou | 1995 | 24 | 23 | 14,2 | N | 121 | 21 | 26,2 | E | 1 | 15/08/2011 |
| YDS1179 | *H. kawakamii* | Taiwan | Yilan | Szyuan Yakou | 2035 | 24 | 23 | 12,9 | N | 121 | 21 | 24,8 | E | 1 | 15/08/2011 |
| YDS1180 | *H. kawakamii* | Taiwan | Yilan | Szyuan Yakou | 2033 | 24 | 23 | 13 | N | 121 | 21 | 25,2 | E | 1 | 15/08/2011 |
| YDS1188 | *H. kawakamii* | Taiwan | Yilan | Szyuan Yakou | 1137 | 24 | 32 | 0,4 | N | 121 | 30 | 59,5 | E | 1 | 17/08/2011 |
| YDS1199 | *H. kawakamii* | Taiwan | Yilan | Cueifong lake | 1879 | 24 | 30 | 41 | N | 121 | 36 | 31,2 | E | 1 | 17/08/2011 |
| YDS1208 | *H. kawakamii* | Taiwan | Taichung | Shing Bai Lang | 1631 | 24 | 11 | 49,4 | N | 121 | 25 | 45 | E | 1 | 23/08/2011 |
| YDS1209 | *H. kawakamii* | Taiwan | Taichung | Shing Bai Lang | 1639 | 24 | 11 | 49 | N | 121 | 25 | 41,2 | E | 1 | 23/08/2011 |
| YDS1211 | *H. kawakamii* | Taiwan | Taichung | Shing Bai Lang | 1792 | 24 | 11 | 41,5 | N | 121 | 24 | 24,9 | E | 1 | 23/08/2011 |
| YDS1212 | *H. kawakamii* | Taiwan | Taichung | Shing Bai Lang | 1817 | 24 | 11 | 37 | N | 121 | 24 | 12 | E | 1 | 23/08/2011 |
| YDS1215 | *H. kawakamii* | Taiwan | Taichung | Shing Bai Lang | 1978 | 24 | 11 | 35,4 | N | 121 | 23 | 7,5 | E | 1 | 23/08/2011 |
| YDS1216 | *H. kawakamii* | Taiwan | Taichung | Shing Bai Lang | 2027 | 24 | 11 | 7,4 | N | 121 | 23 | 4,9 | E | 1 | 23/08/2011 |
| YDS1224 | *H. kawakamii* | Taiwan | Taichung | Bilu river | 2196 | 24 | 13 | 31,6 | N | 121 | 17 | 9,5 | E | 1 | 24/08/2011 |
| YDS1225 | *H. kawakamii* | Taiwan | Taichung | Bilu river | 2225 | 24 | 13 | 32,2 | N | 121 | 17 | 9,6 | E | 1 | 24/08/2011 |
| YDS1226 | *H. kawakamii* | Taiwan | Taichung | Bilu river | 2072 | 24 | 13 | 40,6 | N | 121 | 15 | 50,4 | E | 1 | 24/08/2011 |
| YDS1227 | *H. kawakamii* | Taiwan | Taichung | Bilu river | 2030 | 24 | 13 | 22,5 | N | 121 | 16 | 4,2 | E | 1 | 24/08/2011 |
| YDS1228 | *H. kawakamii* | Taiwan | Taichung | Hehuan river | 1974 | 24 | 12 | 45,1 | N | 121 | 16 | 1,2 | E | 1 | 24/08/2011 |
| YDS1229 | *H. kawakamii* | Taiwan | Taichung | Hehuan river | 1976 | 24 | 12 | 46,2 | N | 121 | 15 | 58,4 | E | 1 | 24/08/2011 |
| YDS1230 | *H. kawakamii* | Taiwan | Taichung | Hehuan river | 1978 | 24 | 12 | 48,1 | N | 121 | 15 | 59,3 | E | 1 | 24/08/2011 |
| YDS1231 | *H. kawakamii* | Taiwan | Taichung | Hehuan river | 1972 | 24 | 12 | 46,5 | N | 121 | 15 | 57,6 | E | 1 | 24/08/2011 |
| YDS1232 | *H. kawakamii* | Taiwan | Taichung | Hehuan river | 1973 | 24 | 12 | 47,7 | N | 121 | 15 | 56,9 | E | 1 | 24/08/2011 |
| YDS1238 | *H. kawakamii* | Taiwan | Taichung | Bilyu | 2190 | 24 | 10 | 38,4 | N | 121 | 24 | 17,4 | E | 1 | 24/08/2011 |
| YDS1239 | *H. kawakamii* | Taiwan | Taichung | Bilyu | 2194 | 24 | 10 | 47,5 | N | 121 | 24 | 12,7 | E | 1 | 24/08/2011 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1561 | *H. cf. aspera* | China | Hubei | Changyang city | 1315 | 30 | 42 | 21,2 | N | 110 | 34 | 25,3 | E | 2 | 27/07/2012 |
| YDS1562 | *H. cf. aspera* | China | Hubei | Changyang city | | 30 | 42 | 21,5 | N | 110 | 34 | 26,1 | E | 2 | 27/07/2012 |
| YDS1600 | *H. involucrata* | Japan | | Hinohara | 263m | 35 | 43 | 20,6 | N | 139 | 6 | 49,5 | E | 3 | 01/08/2012 |
| YDS1603 | *H. involucrata* | Japan | | Hinohara | 370m | 35 | 42 | 5 | N | 139 | 7 | 40 | E | 3 | 01/08/2012 |
| YDS1605 | *H. involucrata* | Japan | | Hinohara | 372m | 35 | 42 | 4,7 | N | 139 | 7 | 39,5 | E | 3 | 01/08/2012 |
| YDS1606 | *H. involucrata* | Japan | | Hinohara | 375m | 35 | 42 | 2,9 | N | 139 | 7 | 39,3 | E | 3 | 01/08/2012 |
| YDS1608 | *H. involucrata* | Japan | | Hinohara | 376m | 35 | 42 | 0,1 | N | 139 | 7 | 39,8 | E | 3 | 01/08/2012 |
| YDS1609 | *H. involucrata* | Japan | | Hinohara | 384m | 35 | 42 | 0,4 | N | 139 | 7 | 36,8 | E | 3 | 01/08/2012 |
| YDS1612 | *H. involucrata* | Japan | | Hakone Park | 799m | 35 | 11 | 11,3 | N | 139 | 0 | 56,8 | E | 3 | 01/08/2012 |
| YDS1613 | *H. involucrata* | Japan | | Hakone Park | 802m | 35 | 12 | 28,2 | N | 139 | 1 | 19 | E | 3 | 01/08/2012 |
| YDS1614 | *H. involucrata* | Japan | | Hakone Park | 804m | 35 | 12 | 28,1 | N | 139 | 1 | 19 | E | 3 | 01/08/2012 |
| YDS1616 | *H. involucrata* | Japan | | Hakone Park | 793m | 35 | 12 | 32,4 | N | 139 | 1 | 17 | E | 3 | 01/08/2012 |
| YDS1617 | *H. involucrata* | Japan | | Hakone Park | 792m | 35 | 12 | 33 | N | 139 | 1 | 17,2 | E | 3 | 01/08/2012 |
| YDS1618 | *H. involucrata* | Japan | | Hakone Park | 792m | 35 | 12 | 33,9 | N | 139 | 1 | 15,9 | E | 3 | 01/08/2012 |
| YDS1620 | *H. involucrata* | Japan | Oshima island | | 105m | 34 | 46 | 55,2 | N | 139 | 23 | 23,9 | E | 3 | 02/08/2012 |
| YDS1621 | *H. involucrata* | Japan | Oshima island | | 158m | 34 | 46 | 53,9 | N | 139 | 23 | 35,5 | E | 3 | 02/08/2012 |
| YDS1623 | *H. involucrata* | Japan | Oshima island | | | 34 | 46 | 53,7 | N | 139 | 23 | 36,5 | E | 3 | 02/08/2012 |
| YDS1624 | *H. involucrata* | Japan | Oshima island | | 192m | 34 | 46 | 44,2 | N | 139 | 23 | 55,4 | E | 3 | 02/08/2012 |
| YDS1625 | *H. involucrata* | Japan | Oshima island | | 195m | 34 | 46 | 45,5 | N | 139 | 24 | 9,4 | E | 3 | 02/08/2012 |
| YDS1626 | *H. involucrata* | Japan | Oshima island | | 121m | 34 | 46 | 46,2 | N | 139 | 24 | 34,7 | E | 3 | 02/08/2012 |
| YDS1628 | *H. involucrata* | Japan | Oshima island | | 107m | 34 | 46 | 47,8 | N | 139 | 24 | 38,5 | E | 3 | 02/08/2012 |
| YDS1629 | *H. involucrata* | Japan | Oshima island | | 52m | 34 | 46 | 56,4 | N | 139 | 24 | 34,4 | E | 3 | 02/08/2012 |
| YDS1631 | *H. involucrata* | Japan | Oshima island | | 52m | 34 | 46 | 55,7 | N | 139 | 24 | 44 | E | 3 | 02/08/2012 |
| YDS1632 | *H. involucrata* | Japan | Oshima island | | 433m | 34 | 45 | 28,9 | N | 139 | 23 | 42,1 | E | 3 | 02/08/2012 |
| YDS1634 | *H. involucrata* | Japan | Oshima island | | 434m | 34 | 45 | 28 | N | 139 | 23 | 36,5 | E | 3 | 02/08/2012 |
| YDS1635 | *H. involucrata* | Japan | Oshima island | | 79m | 34 | 41 | 32,2 | N | 139 | 26 | 18,4 | E | 3 | 02/08/2012 |
| YDS1636 | *H. involucrata* | Japan | Oshima island | | 77m | 34 | 41 | 33,5 | N | 139 | 26 | 19,3 | E | 3 | 02/08/2012 |
| YDS1637 | *H. involucrata* | Japan | Oshima island | | 160m | 34 | 41 | 41,5 | N | 139 | 26 | 4,1 | E | 3 | 02/08/2012 |
| YDS1638 | *H. involucrata* | Japan | Oshima island | | 367m | 34 | 42 | 46,3 | N | 139 | 26 | 5,9 | E | 3 | 02/08/2012 |
| YDS1644 | *H. involucrata* | Japan | Shiga prefecture | Gero | 672m | 35 | 57 | 11,2 | N | 137 | 8 | 2,5 | E | 3 | 04/08/2012 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1645 | *H. involucrata* | Japan | Shiga prefecture | Gero | 626m | 35 | 55 | 40,2 | N | 137 | 7 | 54,9 | E | 3 | 04/08/2012 |
| YDS1646 | *H. involucrata* | Japan | Shiga prefecture | Gero | 628m | 35 | 55 | 4,3 | N | 137 | 7 | 58,6 | E | 3 | 04/08/2012 |
| YDS1647 | *H. involucrata* | Japan | Shiga prefecture | Gero | 597m | 35 | 52 | 35,8 | N | 137 | 10 | 34,6 | E | 3 | 04/08/2012 |
| YDS1649 | *H. involucrata* | Japan | Shiga prefecture | Gero | 610m | 35 | 52 | 4,1 | N | 137 | 10 | 43,4 | E | 3 | 04/08/2012 |
| YDS1650 | *H. involucrata* | Japan | Shiga prefecture | Gero | 648m | 35 | 55 | 21,3 | N | 137 | 19 | 2,7 | E | 3 | 04/08/2012 |
| YDS1651 | *H. involucrata* | Japan | Shiga prefecture | Gero | 645m | 35 | 55 | 12 | N | 137 | 19 | 19,1 | E | 3 | 04/08/2012 |
| YDS1652 | *H. involucrata* | Japan | Shiga prefecture | Gero | 656m | 35 | 55 | 12,4 | N | 137 | 19 | 19,2 | E | 3 | 04/08/2012 |
| YDS1655 | *H. involucrata* | Japan | Shiga prefecture | Gero | 665m | 35 | 55 | 9,9 | N | 137 | 19 | 23,4 | E | 3 | 04/08/2012 |
| YDS1656 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 852m | 35 | 34 | 33,4 | N | 137 | 50 | 15 | E | 3 | 05/08/2012 |
| YDS1659 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 845m | 35 | 34 | 26,8 | N | 137 | 50 | 11,1 | E | 3 | 05/08/2012 |
| YDS1660 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 1038m | 35 | 35 | 13,7 | N | 137 | 49 | 53,5 | E | 3 | 05/08/2012 |
| YDS1661 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 998m | 35 | 35 | 5,8 | N | 137 | 49 | 58,4 | E | 3 | 05/08/2012 |
| YDS1668 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 982m | 35 | 34 | 58,8 | N | 137 | 49 | 59,9 | E | 3 | 05/08/2012 |
| YDS1669 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 971m | 35 | 34 | 56,2 | N | 137 | 49 | 58,9 | E | 3 | 05/08/2012 |
| YDS1671 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 953m | 35 | 34 | 52,6 | N | 137 | 50 | 1 | E | 3 | 05/08/2012 |
| YDS1672 | *H. involucrata* | Japan | Nagano prefecture | Takamori-cho | 941m | 35 | 34 | 49,4 | N | 137 | 49 | 59,3 | E | 3 | 05/08/2012 |
| YDS1166 | *H. longifolia* | Taiwan | Yilan | Kefa bridge | 1318 | 24 | 25 | 46,1 | N | 121 | 21 | 47,5 | E | 1 | 15/08/2011 |
| YDS1181 | *H. longifolia* | Taiwan | Yilan | Kefa bridge | 1380 | 24 | 25 | 47,6 | N | 121 | 21 | 49,1 | E | 1 | 15/08/2011 |
| YDS1182 | *H. longifolia* | Taiwan | Yilan | Kefa bridge | 1381 | 24 | 25 | 49,4 | N | 121 | 21 | 49,2 | E | 1 | 15/08/2011 |
| YDS1183 | *H. longifolia* | Taiwan | Yilan | Taiping Shan | 803 | 24 | 32 | 20,6 | N | 121 | 30 | 40,1 | E | 1 | 17/08/2011 |
| YDS1184 | *H. longifolia* | Taiwan | Yilan | Taiping Shan | 805 | 24 | 32 | 20,7 | N | 121 | 30 | 40,2 | E | 1 | 17/08/2011 |
| YDS1185 | *H. longifolia* | Taiwan | Yilan | Taiping Shan | 818 | 24 | 32 | 20,6 | N | 121 | 30 | 38,9 | E | 1 | 17/08/2011 |
| YDS1186 | *H. longifolia* | Taiwan | Yilan | Taiping Shan | 1096 | 24 | 32 | 8,8 | N | 121 | 31 | 8,2 | E | 1 | 17/08/2011 |
| YDS1187 | *H. longifolia* | Taiwan | Yilan | Taiping Shan | 1100 | 24 | 32 | 8,4 | N | 121 | 31 | 8 | E | 1 | 17/08/2011 |
| YDS1203 | *H. longifolia* | Taiwan | Taichung | Taroko National Park | 952 | 24 | 11 | 50,9 | N | 121 | 28 | 59,5 | E | 1 | 22/08/2011 |
| YDS1204 | *H. longifolia* | Taiwan | Taichung | Taroko National Park | 999 | 24 | 11 | 47,8 | N | 121 | 278 | 27,8 | E | 1 | 22/08/2011 |
| YDS1205 | *H. longifolia* | Taiwan | Taichung | Taroko National Park | 997 | 24 | 11 | 47,2 | N | 121 | 28 | 28,6 | E | 1 | 22/08/2011 |
| YDS1206 | *H. longifolia* | Taiwan | Taichung | Taroko National Park | 1200 | 24 | 12 | 22,8 | N | 121 | 26 | 11,8 | E | 1 | 22/08/2011 |
| YDS1207 | *H. longifolia* | Taiwan | Taichung | Taroko National Park | 1200 | 24 | 12 | 22,9 | N | 121 | 26 | 12,6 | E | 1 | 22/08/2011 |
| YDS1400 | *H. longipes* | China | Hubei | Dalaoling | | 31 | 2 | 56,8 | N | 110 | 56 | 49,5 | E | 2 | 08/07/2012 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1401 | *H. longipes* | China | Hubei | Dalaoling | | 31 | 2 | 57,8 | N | 110 | 56 | 48 | E | 2 | 08/07/2012 |
| YDS1402 | *H. longipes* | China | Hubei | Dalaoling | 1301 | 31 | 2 | 58 | N | 110 | 56 | 47,2 | E | 2 | 08/07/2012 |
| YDS1403 | *H. longipes* | China | Hubei | Dalaoling | | 31 | 2 | 55,8 | N | 110 | 56 | 51,7 | E | 2 | 08/07/2012 |
| YDS1404 | *H. longipes* | China | Hubei | Dalaoling | 1347 | 31 | 2 | 55,8 | N | 110 | 56 | 51,8 | E | 2 | 08/07/2012 |
| YDS1405 | *H. longipes* | China | Hubei | Dalaoling | 1300 | 31 | 2 | 59,1 | N | 110 | 56 | 45,8 | E | 2 | 08/07/2012 |
| YDS1406 | *H. longipes* | China | Hubei | Dalaoling | 1312 | 31 | 3 | 5,4 | N | 110 | 56 | 47,9 | E | 2 | 08/07/2012 |
| YDS1407 | *H. longipes* | China | Hubei | Dalaoling | 1313 | 31 | 3 | 6,2 | N | 110 | 56 | 46,8 | E | 2 | 08/07/2012 |
| YDS1410 | *H. longipes* | China | Hubei | Dalaoling | 1682 | 31 | 3 | 46,7 | N | 110 | 55 | 4,5 | E | 2 | 08/07/2012 |
| YDS1411 | *H. longipes* | China | Hubei | Dalaoling | 1677 | 31 | 3 | 48,3 | N | 110 | 55 | 5,2 | E | 2 | 08/07/2012 |
| YDS1412 | *H. longipes* | China | Hubei | Dalaoling | 1669 | 31 | 3 | 50,3 | N | 110 | 55 | 5,8 | E | 2 | 08/07/2012 |
| YDS1413 | *H. longipes* | China | Hubei | Dalaoling | 1671 | 31 | 3 | 51,5 | N | 110 | 55 | 4,5 | E | 2 | 08/07/2012 |
| YDS1414 | *H. longipes* | China | Hubei | Dalaoling | 1685 | 31 | 3 | 52,3 | N | 110 | 55 | 0,5 | E | 2 | 08/07/2012 |
| YDS1415 | *H. longipes* | China | Hubei | Dalaoling | 1698 | 31 | 4 | 45,7 | N | 110 | 55 | 25,7 | E | 2 | 08/07/2012 |
| YDS1416 | *H. longipes* | China | Hubei | Dalaoling | 1671 | 31 | 4 | 46,1 | N | 110 | 55 | 25,9 | E | 2 | 08/07/2012 |
| YDS1417 | *H. longipes* | China | Hubei | Dalaoling | 1671 | 31 | 4 | 46,4 | N | 110 | 55 | 25,2 | E | 2 | 08/07/2012 |
| YDS1420 | *H. longipes* | China | Hubei | Dalaoling | 1742 | 31 | 4 | 31,9 | N | 110 | 55 | 27,2 | E | 2 | 08/07/2012 |
| YDS1422 | *H. longipes* | China | Hubei | Dalaoling | 1485 | 31 | 5 | 19,9 | N | 110 | 55 | 23 | E | 2 | 08/07/2012 |
| YDS1424 | *H. longipes* | China | Hubei | Dalaoling | 1514 | 31 | 5 | 19 | N | 110 | 55 | 25,6 | E | 2 | 08/07/2012 |
| YDS1428 | *H. longipes* | China | Hubei | Dalaoling | 1903 | 31 | 5 | 3 | N | 110 | 56 | 28,3 | E | 2 | 09/07/2012 |
| YDS1432 | *H. longipes* | China | Hubei | Dalaoling | 1994 | 31 | 5 | 4,4 | N | 110 | 56 | 20,4 | E | 2 | 09/07/2012 |
| YDS1433 | *H. longipes* | China | Hubei | Dalaoling | 1984 | 31 | 5 | 3,7 | N | 110 | 56 | 21,3 | E | 2 | 09/07/2012 |
| YDS1444 | *H. longipes* | China | Hubei | Shennongjia | 1490 | 31 | 19 | 15,8 | N | 110 | 27 | 42,7 | E | 2 | 13/07/2012 |
| YDS1489 | *H. longipes* | China | Hubei | Shennongjia | 1725 | 31 | 31 | 34,4 | N | 110 | 20 | 15,4 | E | 2 | 15/07/2012 |
| YDS1490 | *H. longipes* | China | Hubei | Shennongjia | 1728 | 31 | 31 | 33,7 | N | 110 | 20 | 13,7 | E | 2 | 15/07/2012 |
| YDS1491 | *H. longipes* | China | Hubei | Shennongjia | 1787 | 31 | 31 | 34,6 | N | 110 | 20 | 15,5 | E | 2 | 15/07/2012 |
| YDS1492 | *H. longipes* | China | Hubei | Shennongjia | 1704 | 31 | 32 | 55,6 | N | 110 | 20 | 30,1 | E | 2 | 15/07/2012 |
| YDS1493 | *H. longipes* | China | Hubei | Shennongjia | 1924 | 31 | 33 | 21,7 | N | 110 | 20 | 34,1 | E | 2 | 15/07/2012 |
| YDS1494 | *H. longipes* | China | Hubei | Shennongjia | 1962 | 31 | 33 | 35,1 | N | 110 | 20 | 29,6 | E | 2 | 15/07/2012 |
| YDS1495 | *H. longipes* | China | Hubei | Shennongjia | 1961 | 31 | 33 | 35,2 | N | 110 | 20 | 29,4 | E | 2 | 15/07/2012 |
| YDS1496 | *H. longipes* | China | Hubei | Shennongjia | 1963 | 31 | 33 | 36 | N | 110 | 20 | 29 | E | 2 | 15/07/2012 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1497 | *H. longipes* | China | Hubei | Shennongjia | 2005 | 31 | 33 | 36,2 | N | 110 | 20 | 22,9 | E | 2 | 15/07/2012 |
| YDS1498 | *H. longipes* | China | Hubei | Shennongjia | 2012 | 31 | 33 | 37 | N | 110 | 20 | 21,9 | E | 2 | 15/07/2012 |
| YDS1501 | *H. longipes* | China | Hubei | Shennongjia | 2035 | 31 | 33 | 39,8 | N | 110 | 20 | 24,1 | E | 2 | 15/07/2012 |
| YDS1506 | *H. longipes* | China | Hubei | Shennongjia | 2104 | 31 | 34 | 1,1 | N | 110 | 20 | 22,8 | E | 2 | 15/07/2012 |
| YDS1509 | *H. longipes* | China | Hubei | Shennongjia | 2082 | 31 | 34 | 6,9 | N | 110 | 20 | 14,3 | E | 2 | 15/07/2012 |
| YDS1437 | *H. sargentiana* | China | Hubei | Shennongjia | 1315 | 31 | 19 | 36,3 | N | 110 | 29 | 4,4 | E | 2 | 12/07/2012 |
| YDS1438 | *H. sargentiana* | China | Hubei | Shennongjia | 1375 | 31 | 19 | 25,8 | N | 110 | 28 | 23,5 | E | 2 | 13/07/2012 |
| YDS1439 | *H. sargentiana* | China | Hubei | Shennongjia | 1381 | 31 | 19 | 23,9 | N | 110 | 28 | 24,3 | E | 2 | 13/07/2012 |
| YDS1440 | *H. sargentiana* | China | Hubei | Shennongjia | 1394 | 31 | 19 | 24,1 | N | 110 | 28 | 24,9 | E | 2 | 13/07/2012 |
| YDS1443 | *H. sargentiana* | China | Hubei | Shennongjia | 1412 | 31 | 19 | 23,6 | N | 110 | 28 | 25,2 | E | 2 | 13/07/2012 |
| YDS1445 | *H. sargentiana* | China | Hubei | Shennongjia | 1499 | 31 | 19 | 16,6 | N | 110 | 27 | 42,6 | E | 2 | 13/07/2012 |
| YDS1446 | *H. sargentiana* | China | Hubei | Shennongjia | 1504 | 31 | 19 | 16,3 | N | 110 | 27 | 42,1 | E | 2 | 13/07/2012 |
| YDS1447 | *H. sargentiana* | China | Hubei | Shennongjia | 1552 | 31 | 19 | 14,6 | N | 110 | 27 | 31,6 | E | 2 | 13/07/2012 |
| YDS1448 | *H. sargentiana* | China | Hubei | Shennongjia | 1532 | 31 | 19 | 16,5 | N | 110 | 27 | 34,6 | E | 2 | 13/07/2012 |
| YDS1449 | *H. sargentiana* | China | Hubei | Shennongjia | 1532 | 31 | 19 | 16,5 | N | 110 | 27 | 34,6 | E | 2 | 13/07/2012 |
| YDS1450 | *H. sargentiana* | China | Hubei | Shennongjia | 1634 | 31 | 19 | 18,1 | N | 110 | 27 | 25,3 | E | 2 | 13/07/2012 |
| YDS1451 | *H. sargentiana* | China | Hubei | Shennongjia | 1585 | 31 | 19 | 26,5 | N | 110 | 27 | 38,3 | E | 2 | 13/07/2012 |
| YDS1452 | *H. sargentiana* | China | Hubei | Shennongjia | 1598 | 31 | 19 | 27,2 | N | 110 | 27 | 37,5 | E | 2 | 13/07/2012 |
| YDS1453 | *H. sargentiana* | China | Hubei | Shennongjia | 1592 | 31 | 19 | 26,3 | N | 110 | 27 | 37,5 | E | 2 | 13/07/2012 |
| YDS1454 | *H. sargentiana* | China | Hubei | Shennongjia | 1545 | 31 | 19 | 17,8 | N | 110 | 27 | 37,3 | E | 2 | 13/07/2012 |
| YDS1468 | *H. sargentiana* | China | Hubei | Shennongjia, Nanyang | 1443 | 31 | 18 | 39 | N | 110 | 28 | 47,3 | E | 2 | 14/07/2012 |
| YDS1469 | *H. sargentiana* | China | Hubei | Shennongjia, Nanyang | 1453 | 31 | 18 | 26,9 | N | 110 | 28 | 43,8 | E | 2 | 14/07/2012 |
| YDS1470 | *H. sargentiana* | China | Hubei | Shennongjia, Nanyang | 1628 | 31 | 18 | 19,8 | N | 110 | 28 | 24,4 | E | 2 | 14/07/2012 |
| YDS1471 | *H. sargentiana* | China | Hubei | Shennongjia, Nanyang | 1644 | 31 | 18 | 18,3 | N | 110 | 28 | 21,1 | E | 2 | 14/07/2012 |
| YDS1472 | *H. sargentiana* | China | Hubei | Shennongjia, Nanyang | 1667 | 31 | 18 | 15,2 | N | 110 | 28 | 21,6 | E | 2 | 14/07/2012 |
| YDS1474 | *H. sargentiana* | China | Hubei | Shennongjia, Nanyang | 1662 | 31 | 18 | 20,2 | N | 110 | 28 | 15,9 | E | 2 | 14/07/2012 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1475 | *H. sargentiana* | China | Hubei | Shennongjia, Nanyang | | 31 | 18 | 20,3 | N | 110 | 28 | 15,8 | E | 2 | 14/07/2012 |
| YDS1673 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 927m | 33 | 56 | 50,1 | N | 134 | 24 | 5,8 | E | 3 | 07/08/2012 |
| YDS1674 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 925m | 33 | 56 | 50,6 | N | 134 | 24 | 5 | E | 3 | 07/08/2012 |
| YDS1677 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 925m | 33 | 56 | 50,1 | N | 134 | 24 | 5,9 | E | 3 | 07/08/2012 |
| YDS1678 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 928m | 33 | 56 | 50,7 | N | 134 | 24 | 5,9 | E | 3 | 07/08/2012 |
| YDS1679 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 931m | 33 | 56 | 50,5 | N | 134 | 24 | 5,4 | E | 3 | 07/08/2012 |
| YDS1687 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 1109m | 33 | 54 | 42,5 | N | 134 | 17 | 23,2 | E | 3 | 07/08/2012 |
| YDS1688 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 1140m | 33 | 54 | 42,6 | N | 134 | 17 | 21,8 | E | 3 | 07/08/2012 |
| YDS1689 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 1143m | 33 | 54 | 42 | N | 134 | 17 | 22,3 | E | 3 | 07/08/2012 |
| YDS1690 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 1140m | 33 | 54 | 42,9 | N | 134 | 17 | 22,6 | E | 3 | 07/08/2012 |
| YDS1691 | *H. sikokiana* | Japan | Tokushima prefecture | Kamikatsu | 1140m | 33 | 54 | 42,9 | N | 135 | 17 | 22,6 | E | 3 | 07/08/2012 |
| YDS1030 | *H. strigosa* | China | Sichuan | Lesha | 385 | 29 | 35 | 27,1 | N | 103 | 36 | 42,3 | E | 1 | 24/07/2011 |
| YDS1032 | *H. strigosa* | China | Sichuan | Emei Shan | 541 | 29 | 34 | 6,4 | N | 103 | 26 | 22,4 | E | 1 | 24/07/2011 |
| YDS1033 | *H. strigosa* | China | Sichuan | Emei Shan | 567 | 29 | 34 | 7,6 | N | 103 | 26 | 22,6 | E | 1 | 24/07/2011 |
| YDS1035 | *H. strigosa* | China | Sichuan | Emei Shan | 548 | 29 | 34 | 7,3 | N | 103 | 26 | 22,4 | E | 1 | 24/07/2011 |
| YDS1036 | *H. strigosa* | China | Sichuan | Emei Shan | 551 | 29 | 34 | 7,3 | N | 103 | 26 | 22,3 | E | 1 | 24/07/2011 |
| YDS1057 | *H. strigosa* | China | Sichuan | Emei Shan | 873 | 29 | 29 | 23,4 | N | 103 | 22 | 34,2 | E | 1 | 24/07/2011 |
| YDS1061 | *H. strigosa* | China | Sichuan | Jinkouhe | 301 | 29 | 17 | 10,9 | N | 103 | 8 | 27,8 | E | 1 | 24/07/2011 |
| YDS1062 | *H. strigosa* | China | Sichuan | | 924 | 29 | 19 | 5,3 | N | 103 | 5 | 47,7 | E | 1 | 24/07/2011 |
| YDS1064 | *H. strigosa* | China | Sichuan | | 941 | 29 | 19 | 59,2 | N | 103 | 5 | 36,5 | E | 1 | 24/07/2011 |
| YDS1065 | *H. strigosa* | China | Sichuan | | 943 | 29 | 19 | 58,9 | N | 103 | 5 | 36,4 | E | 1 | 24/07/2011 |
| YDS1066 | *H. strigosa* | China | Sichuan | Wawu Shan | 874 | 29 | 39 | 15,6 | N | 103 | 9 | 49,1 | E | 1 | 25/07/2011 |
| YDS1067 | *H. strigosa* | China | Sichuan | Wawu Shan | 888 | 29 | 39 | 18,4 | N | 103 | 9 | 50,9 | E | 1 | 25/07/2011 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1068 | *H. strigosa* | China | Sichuan | Wawu Shan | 895 | 29 | 39 | 20,4 | N | 103 | 9 | 49,9 | E | 1 | 25/07/2011 |
| YDS1069 | *H. strigosa* | China | Sichuan | Wawu Shan | 875 | 29 | 39 | 15,4 | N | 103 | 9 | 49,8 | E | 1 | 25/07/2011 |
| YDS1070 | *H. strigosa* | China | Sichuan | Wawu Shan | 919 | 29 | 39 | 20,1 | N | 103 | 10 | 14,4 | E | 1 | 25/07/2011 |
| YDS1071 | *H. strigosa* | China | Sichuan | Wawu Shan | 1116 | 29 | 38 | 7,1 | N | 103 | 4 | 15 | E | 1 | 25/07/2011 |
| YDS1073 | *H. strigosa* | China | Sichuan | Wawu Shan | 1107 | 29 | 37 | 48,7 | N | 103 | 2 | 40,2 | E | 1 | 25/07/2011 |
| YDS1074 | *H. strigosa* | China | Sichuan | Wawu Shan | 1108 | 29 | 37 | 48,8 | N | 103 | 2 | 40,5 | E | 1 | 25/07/2011 |
| YDS1075 | *H. strigosa* | China | Sichuan | Wawu Shan | 1110 | 29 | 37 | 50 | N | 103 | 2 | 39,7 | E | 1 | 25/07/2011 |
| YDS1076 | *H. strigosa* | China | Sichuan | Wawu Shan | 1088 | 29 | 40 | 18,3 | N | 103 | 2 | 16 | E | 1 | 25/07/2011 |
| YDS1077 | *H. strigosa* | China | Sichuan | Wawu Shan | 1089 | 29 | 40 | 17,9 | N | 103 | 2 | 15,9 | E | 1 | 25/07/2011 |
| YDS1078 | *H. strigosa* | China | Sichuan | Wawu Shan | 1205 | 29 | 41 | 13,8 | N | 102 | 59 | 7,7 | E | 1 | 25/07/2011 |
| YDS1080 | *H. strigosa* | China | Sichuan | Wawu Shan | | 29 | 41 | 14,8 | N | 102 | 59 | 6,9 | E | 1 | 25/07/2011 |
| YDS1099 | *H. strigosa* | China | Sichuan | Yingping | 962 | 29 | 50 | 3,8 | N | 102 | 43 | 16,3 | E | 1 | 27/07/2011 |
| YDS1100 | *H. strigosa* | China | Sichuan | Yingping | 924 | 29 | 50 | 1,1 | N | 102 | 43 | 5,5 | E | 1 | 27/07/2011 |
| YDS1102 | *H. strigosa* | China | Sichuan | Niba Shan | 911 | 29 | 49 | 47,6 | N | 102 | 41 | 45 | E | 1 | 27/07/2011 |
| YDS1103 | *H. strigosa* | China | Sichuan | Niba Shan | 987 | 29 | 48 | 40,7 | N | 102 | 40 | 48,6 | E | 1 | 27/07/2011 |
| YDS1104 | *H. strigosa* | China | Sichuan | Niba Shan | 1277 | 29 | 44 | 22,9 | N | 102 | 37 | 35,4 | E | 1 | 27/07/2011 |
| YDS1105 | *H. strigosa* | China | Sichuan | Niba Shan | 1284 | 29 | 44 | 22,3 | N | 102 | 37 | 34,4 | E | 1 | 27/07/2011 |
| YDS1107 | *H. strigosa* | China | Sichuan | Niba Shan | 1292 | 29 | 44 | 18,7 | N | 102 | 37 | 34 | E | 1 | 27/07/2011 |
| YDS1119 | *H. strigosa* | China | Sichuan | Erlang Shan | 887 | 30 | 5 | 20,7 | N | 102 | 41 | 32,2 | E | 1 | 31/07/2011 |
| YDS1121 | *H. strigosa* | China | Sichuan | Erlang Shan | 978 | 30 | 2 | 36,7 | N | 102 | 35 | 52 | E | 1 | 31/07/2011 |
| YDS1122 | *H. strigosa* | China | Sichuan | Erlang Shan | 973 | 30 | 2 | 21,7 | N | 102 | 35 | 11,1 | E | 1 | 31/07/2011 |
| YDS1123 | *H. strigosa* | China | Sichuan | Erlang Shan | 974 | 30 | 2 | 21,7 | N | 102 | 35 | 10,3 | E | 1 | 31/07/2011 |
| YDS1125 | *H. strigosa* | China | Sichuan | Erlang Shan | 972 | 30 | 2 | 22 | N | 102 | 35 | 9,2 | E | 1 | 31/07/2011 |
| YDS1126 | *H. strigosa* | China | Sichuan | Erlang Shan | 1107 | 30 | 0 | 8,4 | N | 102 | 28 | 49,6 | E | 1 | 31/07/2011 |
| YDS1127 | *H. strigosa* | China | Sichuan | Erlang Shan | 1107 | 30 | 0 | 8,5 | N | 102 | 28 | 45,7 | E | 1 | 31/07/2011 |
| YDS1128 | *H. strigosa* | China | Sichuan | Erlang Shan | 1197 | 29 | 59 | 25,6 | N | 102 | 26 | 47 | E | 1 | 31/07/2011 |
| YDS1145 | *H. strigosa* | China | Sichuan | Hailuogou Glacier Park | 2014 | 29 | 36 | 12,5 | N | 102 | 4 | 23,3 | E | 1 | 03/08/2011 |
| YDS1147 | *H. strigosa* | China | Sichuan | Lingguan | 825 | 30 | 16 | 55,4 | N | 102 | 50 | 50,68 | E | 1 | 03/08/2011 |
| YDS1150 | *H. strigosa* | China | Sichuan | Baoxing | 1120 | 30 | 25 | 11,9 | N | 102 | 50 | 24,9 | E | 1 | 03/08/2011 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1151 | *H. strigosa* | China | Sichuan | Yanjing | 1494 | 30 | 33 | 27,6 | N | 102 | 53 | 47,8 | E | 1 | 03/08/2011 |
| YDS1152 | *H. strigosa* | China | Sichuan | Yanjing | 1493 | 30 | 33 | 27,6 | N | 102 | 53 | 47,5 | E | 1 | 03/08/2011 |
| YDS1153 | *H. strigosa* | China | Sichuan | Yanjing | 1492 | 30 | 33 | 27,7 | N | 102 | 53 | 47,2 | E | 1 | 03/08/2011 |
| YDS1160 | *H. strigosa* | China | Sichuan | Tongla Shan | 1128 | 30 | 23 | 41,8 | N | 102 | 47 | 20,2 | E | 1 | 04/08/2011 |
| YDS1161 | *H. strigosa* | China | Sichuan | Ming Li | 1074 | 30 | 25 | 15,8 | N | 102 | 44 | 59,8 | E | 1 | 04/08/2011 |
| YDS1162 | *H. strigosa* | China | Sichuan | Ming Li | 1078 | 30 | 25 | 16 | N | 102 | 44 | 58,4 | E | 1 | 04/08/2011 |
| YDS1434 | *H. strigosa* | China | Hubei | Shennongjia | 1331 | 31 | 19 | 38,4 | N | 110 | 29 | 3,2 | E | 2 | 12/07/2012 |
| YDS1435 | *H. strigosa* | China | Hubei | Shennongjia | 1335 | 31 | 19 | 31,3 | N | 110 | 29 | 1,5 | E | 2 | 12/07/2012 |
| YDS1436 | *H. strigosa* | China | Hubei | Shennongjia | | 31 | 19 | 38,4 | N | 110 | 28 | 57,4 | E | 2 | 12/07/2012 |
| YDS1441 | *H. strigosa* | China | Hubei | Shennongjia | 1396 | 31 | 19 | 24,2 | N | 110 | 28 | 24,9 | E | 2 | 13/07/2012 |
| YDS1442 | *H. strigosa* | China | Hubei | Shennongjia | 1409 | 31 | 19 | 21,6 | N | 110 | 28 | 20,5 | E | 2 | 13/07/2012 |
| YDS1456 | *H. strigosa* | China | Hubei | Shennongjia | 1373 | 31 | 18 | 50,1 | N | 110 | 28 | 52,3 | E | 2 | 14/07/2012 |
| YDS1457 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1350 | 31 | 18 | 49,4 | N | 110 | 28 | 53,5 | E | 2 | 14/07/2012 |
| YDS1458 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1356 | 31 | 18 | 49,3 | N | 110 | 28 | 54,6 | E | 2 | 14/07/2012 |
| YDS1459 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1357 | 31 | 18 | 49,4 | N | 110 | 28 | 55,5 | E | 2 | 14/07/2012 |
| YDS1460 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1398 | 31 | 18 | 50,4 | N | 110 | 29 | 5,5 | E | 2 | 14/07/2012 |
| YDS1461 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1401 | 31 | 18 | 50,2 | N | 110 | 29 | 5,8 | E | 2 | 14/07/2012 |
| YDS1462 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1403 | 31 | 18 | 50,3 | N | 110 | 29 | 4,7 | E | 2 | 14/07/2012 |
| YDS1463 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1404 | 31 | 18 | 49,8 | N | 110 | 29 | 3,7 | E | 2 | 14/07/2012 |
| YDS1464 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1411 | 31 | 18 | 47,1 | N | 110 | 29 | 2,3 | E | 2 | 14/07/2012 |
| YDS1465 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1418 | 31 | 18 | 42,3 | N | 110 | 28 | 59,4 | E | 2 | 14/07/2012 |
| YDS1466 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1433 | 31 | 18 | 43,2 | N | 110 | 28 | 52,4 | E | 2 | 14/07/2012 |
| YDS1467 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1438 | 31 | 18 | 43,7 | N | 110 | 28 | 49,9 | E | 2 | 14/07/2012 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1473 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1678 | 31 | 18 | 13 | N | 110 | 28 | 25,8 | E | 2 | 14/07/2012 |
| YDS1476 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1227 | 31 | 20 | 18,2 | N | 110 | 29 | 24,7 | E | 2 | 14/07/2012 |
| YDS1477 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1162 | 31 | 19 | 56,2 | N | 110 | 29 | 24,5 | E | 2 | 14/07/2012 |
| YDS1478 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1151 | 31 | 20 | 2 | N | 110 | 29 | 28 | E | 2 | 14/07/2012 |
| YDS1479 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1086 | 31 | 20 | 8,7 | N | 110 | 29 | 35,7 | E | 2 | 14/07/2012 |
| YDS1480 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 1085 | 31 | 20 | 8,1 | N | 110 | 29 | 35,9 | E | 2 | 14/07/2012 |
| YDS1482 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 773 | 31 | 20 | 47,9 | N | 110 | 30 | 19 | E | 2 | 14/07/2012 |
| YDS1483 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 775 | 31 | 20 | 48,2 | N | 110 | 30 | 18,9 | E | 2 | 14/07/2012 |
| YDS1484 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 762 | 31 | 20 | 52,7 | N | 110 | 30 | 15,9 | E | 2 | 14/07/2012 |
| YDS1485 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 740 | 31 | 20 | 32 | N | 110 | 30 | 32,8 | E | 2 | 14/07/2012 |
| YDS1486 | *H. strigosa* | China | Hubei | Shennongjia, Nanyang | 710 | 31 | 20 | 39,1 | N | 110 | 31 | 28,7 | E | 2 | 14/07/2012 |
| YDS1512 | *H. strigosa* | China | Hubei | Muyuping | 1439 | 31 | 27 | 42,4 | N | 110 | 24 | 13,6 | E | 2 | 16/07/2012 |
| YDS1513 | *H. strigosa* | China | Hubei | Muyuping | | 31 | 27 | 11,8 | N | 110 | 24 | 2,9 | E | 2 | 16/07/2012 |
| YDS1514 | *H. strigosa* | China | Hubei | Muyuping | 1193 | 31 | 27 | 10 | N | 110 | 24 | 3,5 | E | 2 | 16/07/2012 |
| YDS1515 | *H. strigosa* | China | Hubei | Muyuping | 1194 | 31 | 27 | 10,1 | N | 110 | 24 | 3,4 | E | 2 | 16/07/2012 |
| YDS1516 | *H. strigosa* | China | Hubei | Muyuping | 1141 | 31 | 26 | 47,7 | N | 110 | 25 | 10 | E | 2 | 16/07/2012 |
| YDS1517 | *H. strigosa* | China | Hubei | Muyuping | 1140 | 31 | 26 | 47 | N | 110 | 25 | 10,4 | E | 2 | 16/07/2012 |
| YDS1518 | *H. strigosa* | China | Hubei | Muyuping | 1112 | 31 | 26 | 20,3 | N | 110 | 25 | 22,6 | E | 2 | 16/07/2012 |
| YDS1519 | *H. strigosa* | China | Hubei | Muyuping | 1102 | 31 | 26 | 27,9 | N | 110 | 25 | 34,4 | E | 2 | 16/07/2012 |
| YDS1522 | *H. strigosa* | China | Hubei | Wufeng county | 882 | 30 | 10 | 5,7 | N | 110 | 58 | 5,5 | E | 2 | 20/07/2012 |
| YDS1523 | *H. strigosa* | China | Hubei | Wufeng county | 882 | 30 | 10 | 5,8 | N | 110 | 58 | 5,4 | E | 2 | 20/07/2012 |
| YDS1530 | *H. strigosa* | China | Hubei | Wufeng county | | 30 | 10 | 20,4 | N | 110 | 57 | 25,1 | E | 2 | 20/07/2012 |
| YDS1531 | *H. strigosa* | China | Hubei | Wufeng county | 968 | 30 | 10 | 20,3 | N | 110 | 57 | 25,1 | E | 2 | 20/07/2012 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| YDS1546 | *H. strigosa* | China | Hubei | Wufeng county | 1137 | 30 | 10 | 54,1 | N | 110 | 43 | 5,1 | E | 2 | 20/07/2012 |
| YDS1547 | *H. strigosa* | China | Hubei | Wufeng county | 687 | 30 | 12 | 47,2 | N | 110 | 37 | 39,4 | E | 2 | 21/07/2012 |
| YDS1552 | *H. strigosa* | China | Hubei | Wufeng county | 942 | 30 | 11 | 28,7 | N | 110 | 33 | 25,4 | E | 2 | 21/07/2012 |
| YDS1553 | *H. strigosa* | China | Hubei | Changyang city | 1112 | 30 | 41 | 7 | N | 110 | 33 | 19,5 | E | 2 | 22/07/2012 |
| YDS1554 | *H. strigosa* | China | Hubei | Changyang city | 1114 | 30 | 41 | 7 | N | 110 | 33 | 19,9 | E | 2 | 22/07/2012 |
| YDS1555 | *H. strigosa* | China | Hubei | Changyang city | 1119 | 30 | 41 | 7,5 | N | 110 | 33 | 17,9 | E | 2 | 22/07/2012 |
| YDS1520 | *H. villosa* | China | Hubei | Wufeng county | 906 | 30 | 10 | 5 | N | 110 | 58 | 3,6 | E | 2 | 20/07/2012 |
| YDS1521 | *H. villosa* | China | Hubei | Wufeng county | 884 | 30 | 10 | 5 | N | 110 | 58 | 3,6 | E | 2 | 20/07/2012 |
| YDS1524 | *H. villosa* | China | Hubei | Wufeng county | 882 | 30 | 10 | 6,1 | N | 110 | 58 | 8,4 | E | 2 | 20/07/2012 |
| YDS1525 | *H. villosa* | China | Hubei | Wufeng county | 884 | 30 | 10 | 5,2 | N | 110 | 58 | 4,8 | E | 2 | 20/07/2012 |
| YDS1527 | *H. villosa* | China | Hubei | Wufeng county | 960 | 30 | 10 | 20,6 | N | 110 | 57 | 27,2 | E | 2 | 20/07/2012 |
| YDS1528 | *H. villosa* | China | Hubei | Wufeng county | 960 | 30 | 10 | 20,1 | N | 110 | 57 | 27,2 | E | 2 | 20/07/2012 |
| YDS1529 | *H. villosa* | China | Hubei | Wufeng county | 967 | 30 | 10 | 20,2 | N | 110 | 57 | 25,1 | E | 2 | 20/07/2012 |
| YDS1532 | *H. villosa* | China | Hubei | Wufeng county | 969 | 30 | 10 | 20,4 | N | 110 | 57 | 25,1 | E | 2 | 20/07/2012 |
| YDS1533 | *H. villosa* | China | Hubei | Wufeng county | 971 | 30 | 10 | 20 | N | 110 | 57 | 25,9 | E | 2 | 20/07/2012 |
| YDS1534 | *H. villosa* | China | Hubei | Wufeng county | 1001 | 30 | 9 | 49,1 | N | 110 | 48 | 27,1 | E | 2 | 20/07/2012 |
| YDS1535 | *H. villosa* | China | Hubei | Wufeng county | 1004 | 30 | 9 | 45,2 | N | 110 | 48 | 11,8 | E | 2 | 20/07/2012 |
| YDS1536 | *H. villosa* | China | Hubei | Wufeng county | 1017 | 30 | 9 | 38,4 | N | 110 | 47 | 45,3 | E | 2 | 20/07/2012 |
| YDS1537 | *H. villosa* | China | Hubei | Wufeng county | 1043 | 30 | 9 | 30,9 | N | 110 | 46 | 55,6 | E | 2 | 20/07/2012 |
| YDS1538 | *H. villosa* | China | Hubei | Wufeng county | 1055 | 30 | 9 | 31,3 | N | 110 | 46 | 54,6 | E | 2 | 20/07/2012 |
| YDS1539 | *H. villosa* | China | Hubei | Wufeng county | 1056 | 30 | 9 | 31,6 | N | 110 | 46 | 54,9 | E | 2 | 20/07/2012 |
| YDS1540 | *H. villosa* | China | Hubei | Wufeng county | 1090 | 30 | 9 | 20,8 | N | 110 | 45 | 56,6 | E | 2 | 20/07/2012 |
| YDS1541 | *H. villosa* | China | Hubei | Wufeng county | 1089 | 30 | 9 | 21,1 | N | 110 | 45 | 56,6 | E | 2 | 20/07/2012 |
| YDS1544 | *H. villosa* | China | Hubei | Wufeng county | 1134 | 30 | 10 | 53,7 | N | 110 | 43 | 6,3 | E | 2 | 20/07/2012 |
| YDS1545 | *H. villosa* | China | Hubei | Wufeng county | 1136 | 30 | 10 | 54,2 | N | 110 | 43 | 5 | E | 2 | 20/07/2012 |
| YDS1548 | *H. villosa* | China | Hubei | Wufeng county | 712 | 30 | 12 | 33 | N | 110 | 37 | 14,8 | E | 2 | 21/07/2012 |
| YDS1549 | *H. villosa* | China | Hubei | Wufeng county | 1100 | 30 | 11 | 32,9 | N | 110 | 32 | 39,4 | E | 2 | 21/07/2012 |
| YDS1550 | *H. villosa* | China | Hubei | Wufeng county | 1102 | 30 | 11 | 33,6 | N | 110 | 32 | 39,5 | E | 2 | 21/07/2012 |
| YDS1551 | *H. villosa* | China | Hubei | Wufeng county | 1105 | 30 | 11 | 34,2 | N | 110 | 32 | 39,3 | E | 2 | 21/07/2012 |

# Curriculum Vitae

# YANNICK DE SMET

Curriculum Vitae | 2020

## PERSONALIA

Bornem, 28.12.1987
+32 (0)474 645 149
yvp.desmet@gmail.com

Brugsevaart 42
9030 Mariakerke
Belgium

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## EDUCATION

**Ghent University – FWO predoctoral mandate in Biology**
October 2010 – December 2020

**Ghent University – Master of Science in Biology**
October 2008 – July 2010 | *graduated summa cum laude*

**Ghent University – Bachelor of Science in Biology**
October 2005 – July 2008 | *graduated summa cum laude*

**OLVP Bornem – Techniek Wetenschappen**
September 2001 – July 2005

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## Dissertations

**Master Thesis**
2009-2010 | Promotors: Prof. Dr. Paul Goetghebeur & Dr. Marie-stéphanie Samain
"Soort zkt. Grens, *Epimedium* (Berberidaceae) Soorten Zonder Grenzen." | Awarded the "Gabriël de Waele" prize

**Bachelor Thesis**
2008 | Promotors: Prof. Dr. Olivier de Clerck & Prof. Dr. Anne Willems
"Isolatie en karakterisering van endosymbiontische bacteriën in groenwieren."

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## Professional experiences

**Ghent University – Research group Spermatophytes – PhD student**
October 2010 – 2014 | FWO-mandate |
http://www.spermatophytes.ugent.be/page3/page9/page41/index.html
Fieldwork, extensive lab experience, education

**Royal Museum for Central Africa – Entomology – Molecular Biologist**
July 2015 – April 2018 | http://www.africamuseum.be/home
Fieldwork, extensive lab experience

**FPS Health, Food Chain Safety and Environment – Registration of Pesticides – Expert in Efficacy**

April 2018 – January 2019
Efficacy evaluation of new plant protection products, writing official reports

**FPS Health, Food Chain Safety and Environment – Cell Species – Inspector EUTR legislation**
January 2019 – Present
Legislation, Conservation, Law enforcement, International meetings and contacts

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## SKILLS

**Laboratory work**
Designing and testing novel methods for: DNA extractions, PCR amplifications.
Experienced in cloning, growing cell cultures.
Gel electrophoresis, operating e-gel and PCR product extraction.
Operating and maintaining ABI capillary sequencer.
Managing and planning sequencing projects.

**Next generation sequencing**
Acquiring high quality DNA.
Preparing RAD-seq and Illumina MiSeq libraries.
Interpreting and processing NGS data, from raw sequences to publishable data.

**Fieldwork**
Independently planning and executing sampling in inaccessible areas of Asia and Africa.
Contacting and negotiating with foreign institutions, both from a distance and locally.

**Data processing**
Processing and maintaining large amounts of (sequence) data.
Author of several scientific papers in peer-reviewed journals.
Preparing presentations and lessons for both specialized and non-specialized audiences.
Preparing official reports, following strict regulatory guidelines.

**Enforcing European law**
Interpreting legal documents and legislation (Belgian, EU and non-EU).
Auditing larger and smaller businesses.
Writing injunctions, communicating with public prosecutors and lawyers.

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## SOFTWARE

**Windows | Mac OS X | Linux** (ubuntu)
**Microsoft Office** (Word, Excel, PowerPoint, Acces) | **Open Office** (Writer, Calc, Impress)
**Scripting and coding** (experienced in Bash-scripting, basics of perl and R)
**Phylogenetic reconstruction** (MrBayes, RaxML, BEAST and *BEAST, STEM, PAUP, MEGA)
**Population genetics** (STRUCTURE, STRUCTURAMA, BP&P. basics of ARLEQUIN, MIGRATE and MESQUITE)
**processing NGS data** (CLC bio, STACKS, BLASTN, SiLiX, pyRAD)

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## LANGUAGES

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| Dutch: | mother tongue |
| English: | excellent |
| French: | good |
| German: | basic knowledge |

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## CERTIFICATES AND COURSES

**Survival Chinese part 1** – Universitair centrum voor talenonderwijs – 2010 (1 semester).

**Getting started with HPC** – Ghent University Doctoral Schools – 2012 (1 week).

**Computational molecular evolution** – European Molecular Biology Organization, Greece – 2012 (10 days).

**Next generation sequencing workshop** – Botanical Garden Edinburgh, Scotland – 2012 (3 days).

**Auditor/Lead Auditor Kwaliteitsmanagementsysteem ISO 9001:2015**

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## SCIENTIFIC OUTPUT

A1 Publications:

**De Smet, Y**., Goetghebeur, P., Wanke, S., Asselman, P., Samain, M. S. (2011) Additional evidence for recent divergence of Chinese *Epimedium* (Berberidaceae) derived from AFLP, chloroplast and nuclear data supplemented with characterisation of leaflet pubescence. *Plant Ecology and Evolution* 145 (1): 73-87.

Cires, E., **De Smet, Y**., Cuesta, C., Goetghebeur, P., Sharrock, S., Gibbs, D., Oldfield, S., Kramer, A., Samain, M. S. (2013) Gap analyses to support ex situ conservation of genetic diversity in *Magnolia*, a flagship group. *Biodiversity and conservation* 22 (3): 567-590.

**De Smet, Y**., Granados Mendoza, C., Wanke, S., Goetghebeur, P., Samain, M.S. (2015). Molecular phylogenetics and new (infra)generic classification to alleviate polyphyly in tribe *Hydrangeeae* (Cornales: Hydrangeaceae). *Taxon* 64 (4).

Granados, C.M., Naumann, J., Samain, M.S., Goetghebeur, P., **De Smet, Y**., Wanke, S. (2015) A genome-scale mining strategy for recovering novel rapidly-evolving nuclear single-copy genes for addressing shallow-scale phylogenetics in *Hydrangea. BMC Evolutionary Biology* 15 (1).

**De Smet, Y**., Tatsuya, U., Granados, C. M., Wanke, S., Goetghebeur, P., Samain, M.S. (2015) Coalescent species delimitation in Hydrangea sect. Asperae (Hydrangeaceae) evaluates traditionally defined morphotypes. *Molecular Phylogenetics and Evolution* 114: 415-425.

Sonet, G., **De Smet, Y.**, Tang, M., Virgilio, M., Young, A.D., Skevington, J.H., Mengual, X., Backeljau, T., Liu, S., Zhou, X., De Meyer, M., Jordaens, K. (2019) First mitochondrial genomes of five hoverfly species of the genus *Eristalinus* (Diptera: Syrphidae). *Genome* 62(10): 677-687.

P1 Publications:

**De Smet, Y.,** Larridon, I., Bauters, K., Goetghebeur, P., Samain, M.S. (2015) Re-discovering *Hydrangea sargentiana*, a taxon in need of conservation action. *Acta Horticulturae* 1087: 221-224.

Presentations:

Boundary conflicts: *Epimedium* (Berberidaceae), species without boundaries? *Poster presentation*. Young Botanists' Forum, 2010, Belgium.

Applying the General Lineage Concept of Species to Asian Hydrangea. *Poster presentation*. Annual Meeting on Plant Ecology and Evolution 1, 2012, Belgium.

Know your limits: the importance of species and generic boundaries for conservation. *Oral presentation*. 3rd Science in Botanic Gardens Conference, 2014, Gran Canaria.

Biodiversity research and plant breeding, a mutually beneficial relationship. *Oral presentation*. 25th International EUCARPIA Symposium Section Ornamentals: CROSSING BORDERS, 2015, Belgium.

Manuscripts in preparation:

**De Smet, Y**., Cires Rodríguez, E., Goetghebeur, P., Wanke, S., Samain, M.-S. (submitted to Heredity) Genome wide RADseq data resolves phylogeny and species boundaries in the *Hydrangea aspera* species complex

**De Smet, Y.**, Jordaens, K. Multilocus phylogeny and species delimitation in the hoverfly genera *Eristalinus* and *Eristalodes.*

## VARIA

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|---|---|
| 2003-2005: | **Executive Commitee** NPO Jeugdhuis Kadee. |
| 2005-2007: | 2 terms as elected member of the **Board**, NPO jeugdhuis Kadee |
| 2019-present: | **Vice-President** of the Belgian Historical European Martial Arts federation (combat sports federation) |

## REFERENCES

Ghent University – Department Biology – Research Group Spermatophytes

Prof. Dr. Em. Paul Goetghebeur

Tel.: +32 (0)9 264 50 55 | E-mail: Paul.Goetghebeur@Ugent.be

Website: www.spermatophytes.ugent.be

Royal Museum for Central Africa – Section Entomology

Dr. Kurt Jordaens

Tel.: +32 (0)2 769 5373 | E-mail: kurt.jordaens@africamuseum.be

Website : http://www.africamuseum.be/research/biology/invertebrates