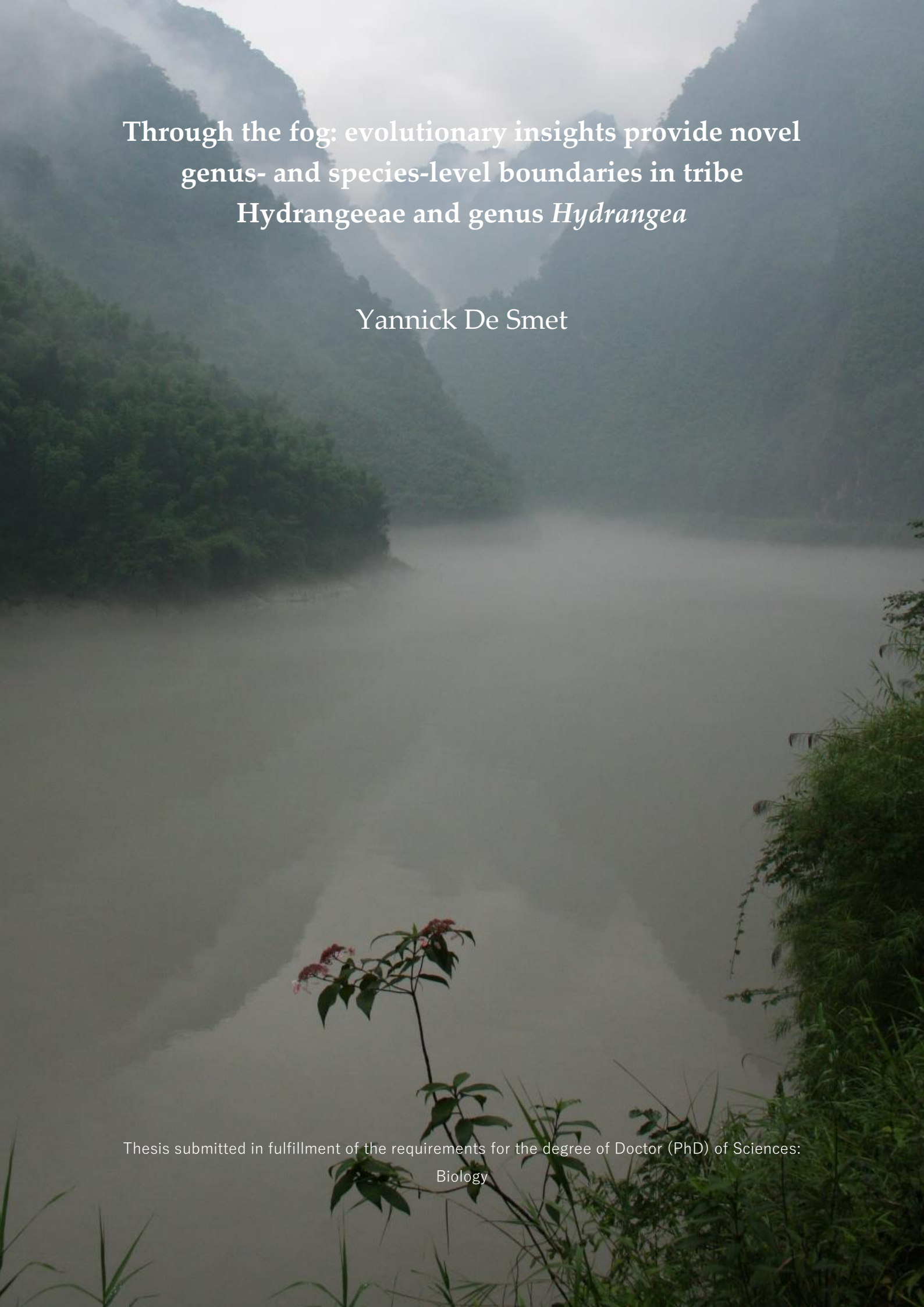

Through the fog: evolutionary insights provide novel genus- and species-level boundaries in tribe Hydrangeeae and genus *Hydrangea*

Yannick De Smet

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

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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. Photos used with permission. 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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SYSTEMATIC & EVOLUTIONARY BOTANY

Dankwoord

Het lijkt bijna een decennium geleden dat ik te horen kreeg te kunnen beginnen aan een doctoraat over *Hydrangea*. En nu, in dit reeds opvallende jaar 2020, start ik aan het schrijven van het laatste hoofdstuk; het dankwoord. Het uitvoeren en neerschrijven van een doctoraat heeft uiteraard heel wat wortels in de aarde, en zonder de bijdrage van een aantal mensen had dit werk nu zeker niet deze vorm aangenomen.

Paul, in eerste instantie waren het de lessen plantkunde in de eerste bachelor biologie die me geïnteresseerd maakten in de wondere wereld van botanische diversiteit. Daarnaast wil ik je zeker bedanken voor de vrijheid die ik had om verschillende onderzoeklijnen te volgen binnen *Hydrangea*. Het enthousiasme waarmee je elke nieuwe bevinding in *H.* sect. *Asperae* ontving werkte enorm motiverend, en is zeker een van de grote drijfveren geweest doorheen het onderzoek. Ik hoop dat de morfologische sleutel, die ik je al jaren verschuldigd was, zijn nut nog kan bewijzen.

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De interesse voor soortconcepten en algoritmes voor soortafbakening allerhande heb ik voornamelijk te danken aan de lessen van Olivier De Clerck. Bedankt voor de -soms enigmatische- inzichten wanneer ik je contacteerde met weer "vreemde" resultaten in *Hydrangea* fylogenie of soortgrenzen.

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I was able to plan two sampling expeditions within the framework of this PhD. For help during the execution of the Chinese leg of these, I would like to thank Prof. Dr. Chen Fangqing (China Three Gorges University). For the weeks we spent hunting for *Hydrangea* and exploring culture in Taiwan, I am grateful to Prof. Dr. Jer-Ming Hu (National Taiwan University) and his doctoral students who joined us in the field. Finally, I am forever grateful to Dr. Tatsuya Uemachi (The University of Shiga Prefecture) for showing us *Hydrangea* populations in beautiful Japan.

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"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 1

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 this new paradigm 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 on the most desirable scheme or characters used for the classification of all biological life. Taxonomists can range from total adherence to traditional, strictly morphology-based classifications, to purely genomic sequence-based organizations. Likewise, it could be said that some researchers prefer more "intuitive" classifications, be it based on morphology or other readily available characteristics, as opposed to purely phylogenetic organizations. Despite these differing opinions on how to create classifications, a broad consensus exists regarding the idea that classifications should be informed by evolutionary relationships. Secondly, continuing development of new tools for inferring evolutionary relationships can overturn previously widely held believes concerning relationships between taxa (e.g. morphologically similar genera which are found to be only distantly related). 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 20th 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 (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 20th 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* L., 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.

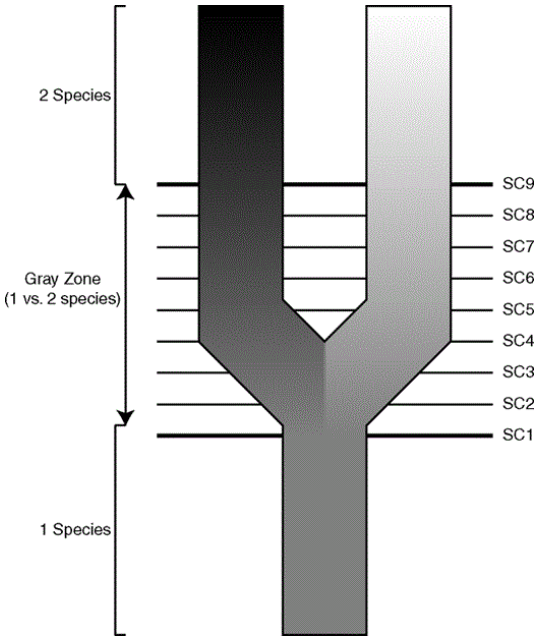

Figure 1.1: The general lineage concept of species. (after de Queiroz, 1998, 1999, 2005a). This highly simplified diagram represents a single lineage (species) splitting to form two lineages (species). The gradations in shades of gray represent the daughter lineages diverging through time, and the horizontal 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 (Syvanen et al., 1989; 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; Duminil & Di Michele, 2009). Indeed, similar morphologies can represent homoplasies (Went, 1971), 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; Sheidai, et al., 2019) 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; He et al., 2019; Weber et al., 2019). 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 sections, the broad lines of coalescent theory and the acquisition of molecular markers used in this PhD are discussed, respectively.

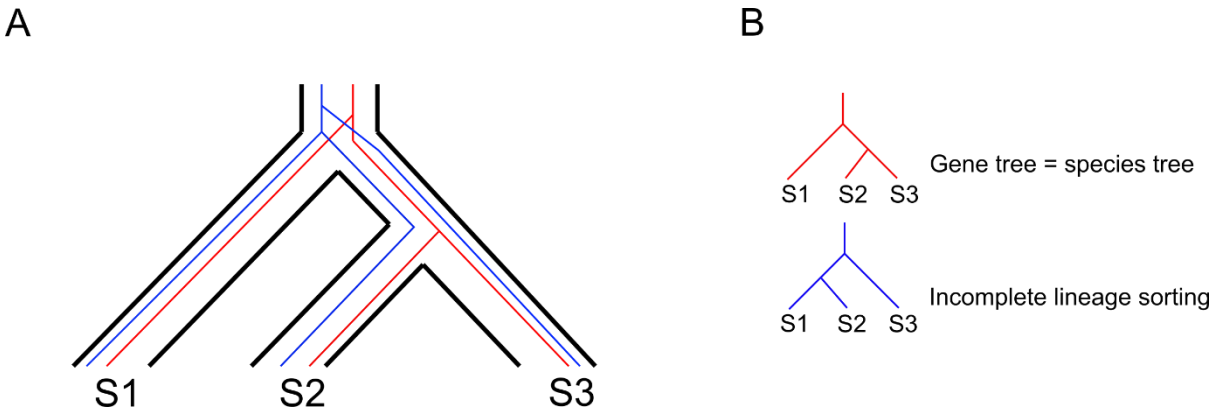

Figure 1.2: Gene tree versus species tree. The black outline in A represents the true species phylogeny, with S2 and S3 as sister taxa. The red and blue lines inside the outline represent gene trees underlying this species topology. The gene represented in red is congruent with the species, tree (B), 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.

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.

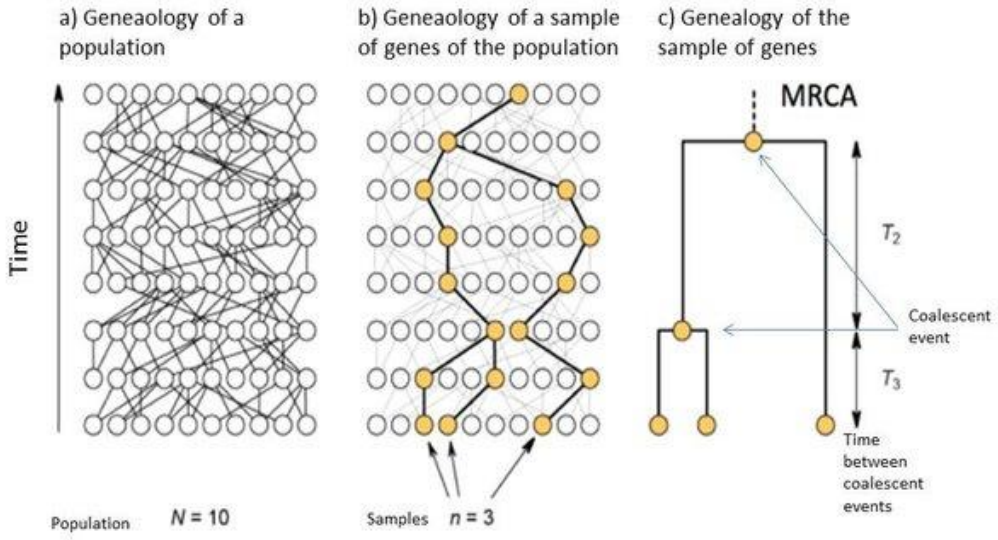

Figure 1.3: The coalescent. Neutral coalescence process running within a species tree, based on Klein (1998), Degnan & Rosenberg (2009) and Mailund (2009). Each dot represents an individual gene copy, each color a different allele, and each line connects a gene copy to its ancestor in the previous generation. 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 (Figure 1.3b). 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 (Kolaczkowski & 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 μ (mutation rate) and τ (lineage divergence time). Extending this framework with a factor Λ 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, Yang & Rannala, 2010). 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.

Hybridization and species delimitation

For vascular plants, hybridization, or the crossing of distinct species leading to the production of viable offspring, is a common occurrence in natural populations (Mallet, 2005). An increasing availability of whole genome sequences and their phylogenetic analysis (phylogenomics) has corroborated this by demonstrating the permeability of genomes in terms of gene flow across putative species barriers (Stanton et al., 2019). This situation can provide a challenge for plant species delineation using molecular lines of evidence. Indeed, gene flow can result in shared polymorphisms across divergent lineages, rendering the use of multiple appropriate molecular markers essential to recover the divergence of the studied lineages. Through this mechanism, the ability of molecular data to distinguish lineages undergoing gene flow decreases with the amount of foreign (from another, diverged lineage) genetic material introduced into the genome, a process termed introgression. When divergent lineages experience substantial gene flow, for example when persisting in sympatry (individuals of each population occur within a distance where they could interbreed), the genetic signature of their divergence can be heavily obscured by introgression. Despite being a well-known occurrence, gene flow between diverging lineages is generally not modeled in the abovementioned multilocus coalescent-based methods for phylogenetic reconstruction and species delimitation (Jackson et al., 2017). They identify independent lineages by modeling the differential sorting of ancestral alleles in isolated populations due to genetic drift, taking into account certain levels of incomplete lineage sorting (e.g. Yang & Rannala, 2010). The accuracy of these methods in detecting independent lineages has been suggested to decline when gene flow persists (Camargo et al., 2012; Jackson et al., 2017; Weber et al., 2019). Interpretation of the lineages identified by these methods can therefore be improved by information gained from methods specifically identifying gene flow (Weber et al., 2019), without a priori assignment of individuals to putative lineages (e.g. Structure, Pritchard et al., 2000). This approach of combining different methodologies or lineage identification algorithms provides more insight into the evolutionary background for each of the lineages identified, resulting in a higher possibility of detecting ongoing gene flow and hybridization. Recently, the multispecies-coalescent model implemented in the abovementioned algorithm

for species delimitation BP&P was expanded to incorporate introgression (Flouri et al., 2020). This method uses genomic sequence data to infer occurrence, timing and intensity of introgression. These and other (Jiao & Yang, 2020) expansions on the multispecies coalescent will play an important role in elucidating the role of gene flow in speciation as more phylogenomic datasets become available across the tree of life.

Monophyly vs. paraphyly

As discussed in the previous section, 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, 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.

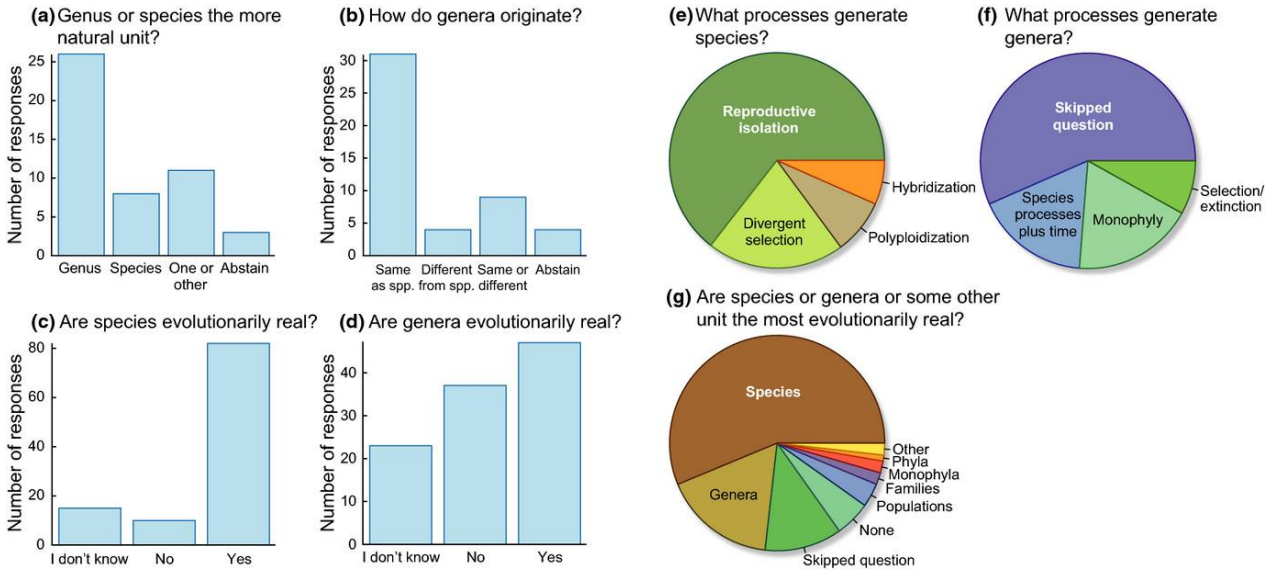

Figure 1.4: Results of surveys on views regarding taxa and their evolutionary relevance. Summary of responses (a, b) to the original Edgar Anderson (1940) survey and (c–g) the survey made by Barraclough & Humphreys (2015). In (a) 'one or other' refers to respondents who felt that sometimes genera and 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 Barraclough & Humphreys, 2015).

Related to this, the introduction of molecular phylogenetics 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, some authors have argued for the recognition of paraphyletic groups (Figure 1.5) as taxa in biological classifications during the last decades (Stuessy, 1987; Brummit, 2002, 2006; Grant, 2003; Ebach & Williams, 2004; Nordal & Stedje, 2005; Hörandl, 2006, 2007, 2010, 2014; Hörandl & Stuessy, 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 (Figure 1.5), 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 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 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 types of evolutionary entities (e.g. 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. Phylogenetic relationships 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 sensu lato (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 countering cladistic systematics is that the latter system does not account for either anagenesis or reticulate 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.

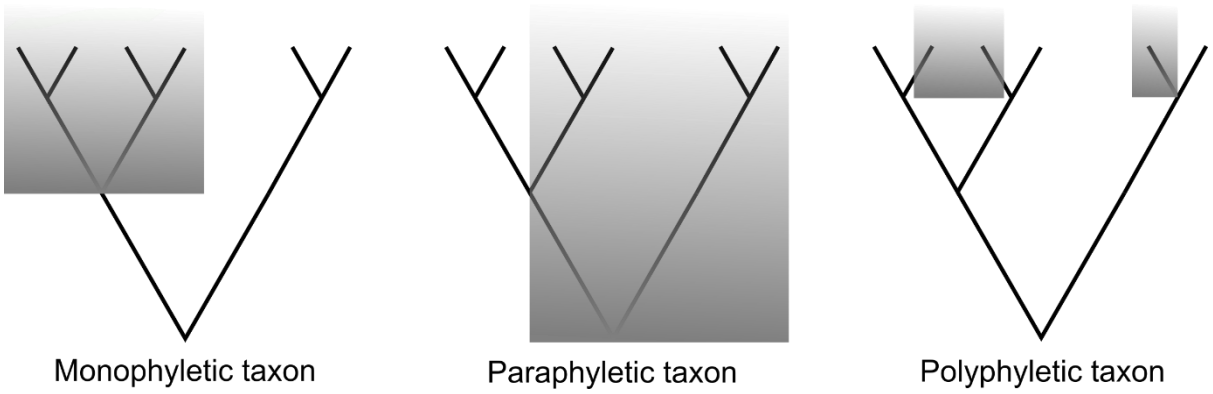

Figure 1.5: Monophyly versus paraphyly and polyphyly. A monophyletic taxon contains the most recent common ancestor of a group of organisms and all its descendants. A paraphyletic taxon includes the most recent common ancestor, but not all descendants. Finally, a polyphyletic taxon can be defined as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

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 sensu stricto (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, 2012; 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 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).

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 (Small et al., 1998; Shaw et al., 2005, 2007), and a very limited set of nuclear markers (Hughes et al., 2006) such as the ribosomal internal transcribed spacer, ITS (Baldwin et al., 1995). 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 in angiosperms, 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 (all marker abbreviations used throughout this work are summarized in Table S1.1 in Appendix 1), 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 sequences related to the maintenance of secondary structures (Nieto 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; Kao et al., 2019; Granados Mendoza 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 (e.g. Kao et al., 2019). 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* (L.) Heynh., *Populus trichocarpa* Torr. & A. Gray, *Vitis vinifera* L. and *Oryza sativa* L. 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 Dumort. by Granados Mendoza 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 polymerase chain reaction (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 suitable library preparation technique 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. Cruaud et al., 2014; Pante et al., 2015; Wagner et al., 2018; Dincă et al., 2019; Quattrini et al., 2019).

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* Thunb.. This name, however, overlapped with the already published Hortensiaceae (Martinov, 1820), based on *Hortensia opuloides* Lam., one of the many synonyms of the ornamentally important *Hydrangea macrophylla* (Thunb.) Ser., but has been conserved as the better-known name (Turland et al., 2018).

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 Juss. (Engler, 1890; Schulze-Menz, 1964; Cronquist, 1981). Other authors, however, considered the group to be closely related to the Cornaceae Brecht. Ex J. Presl., placing it in the order Cornales Link (e.g., Hutchinson, 1927; Huber, 1963; Hufford, 1992; Takhtajan, 1997). 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; Fu et al., 2017) and in a well-supported sister relationship with the Loasaceae Juss. (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 Engl. within the Hydrangeaceae. The genus *Hydrostachys* Thouars, 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 stomata, 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 branches 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 their study of Cornales evolutionary relations, 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. More recently, Magallón et al. (2015) placed Hydrostachyaceae as sister to the rest of the Cornales.

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* L.-like genera on the one hand, and *Hydrangea*-like genera on the other hand, recognizing these groups as either tribes (Hydrangeeae DC. and Philadelpheae) or subfamilies (Hydrangeoideae and Philadelphoideae). The genus *Kirengeshoma* Yatabe 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* Torr. & A. Gray and *Fendlera* Engelm. & A. Gray as sister to the rest of the Hydrangeaceae (although this relation is not supported in the study of Kim et al., 2015). 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. This table shows the different classification schemes utilized in family Hydrangeaceae. Until Hufford et al. (2001) these classifications were based solely on morphological data, while the more recent attempts include a combination of morphological and molecular evidence.

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) consist of a basal clade *Deinanthe* Maxim. + *Cardiandra* Siebold & Zucc., sister position to the "*Hydrangea* clade" (Hufford et al., 2001) which contains the ornamental genus *Hydrangea* and allied genera *Broussaisia* Gaudich., *Decumaria* L., *Dichroa* Lour., *Pileostegia* Hook f. & Thomson, *Platycrater* Siebold & Zucc. and *Schizophragma* Siebold & Zucc. The first studies providing insight into evolutionary relationships within the tribe were mainly focused at a different taxonomic level, being the elucidation of family relationships within Cornales (Morgan & Soltis, 1993; Olmstead et al., 1993; Xiang et al., 1993, 1998). Therefore, the earliest phylogenetic information for tribe Hydrangeeae is limited to a subset of the genera currently included in the tribe. 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 Mendoza 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 *Hydrangea* p.p. (pro parte). 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 Mendoza 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* L., the type species of the genus, remains unresolved. This species was recovered in different positions: being either sister to *Cardiandra* + *Deinanthe* (Samain et al., 2010), or in a grade with *H. quercifolia* W. Bartram and sister to a

clade consisting of *H*. subsect. *Calyptranthe* (Maxim.) McClintock, *H*. sect. *Cornidia* (Ruiz & pav.) Engl., the genus *Platycrater* and *H*. subsect. *Asperae* Rehder (Granados Mendoza 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; Hufford et al. 2001). Other intergeneric relationships supported in the analysis by Samain et al. (2010) are corroborated by Granados Mendoza 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 Mendoza et al. (2013) and are fully resolved.

These phylogenetic hypotheses for tribe Hydrangeeae are, at least in part, incompatible with the 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 (*H*. subsect. *Asperae*, *H*. subsect. *Americanae* (Maxim.) Engl. and *H*. subsect. *Macrophyllae* McClintock) could not be confirmed (Figure 1.6; Jacobs, 2010; Samain et al., 2010) or is rejected with high support (*H*. subsect. *Asperae* in Granados Mendoza 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.

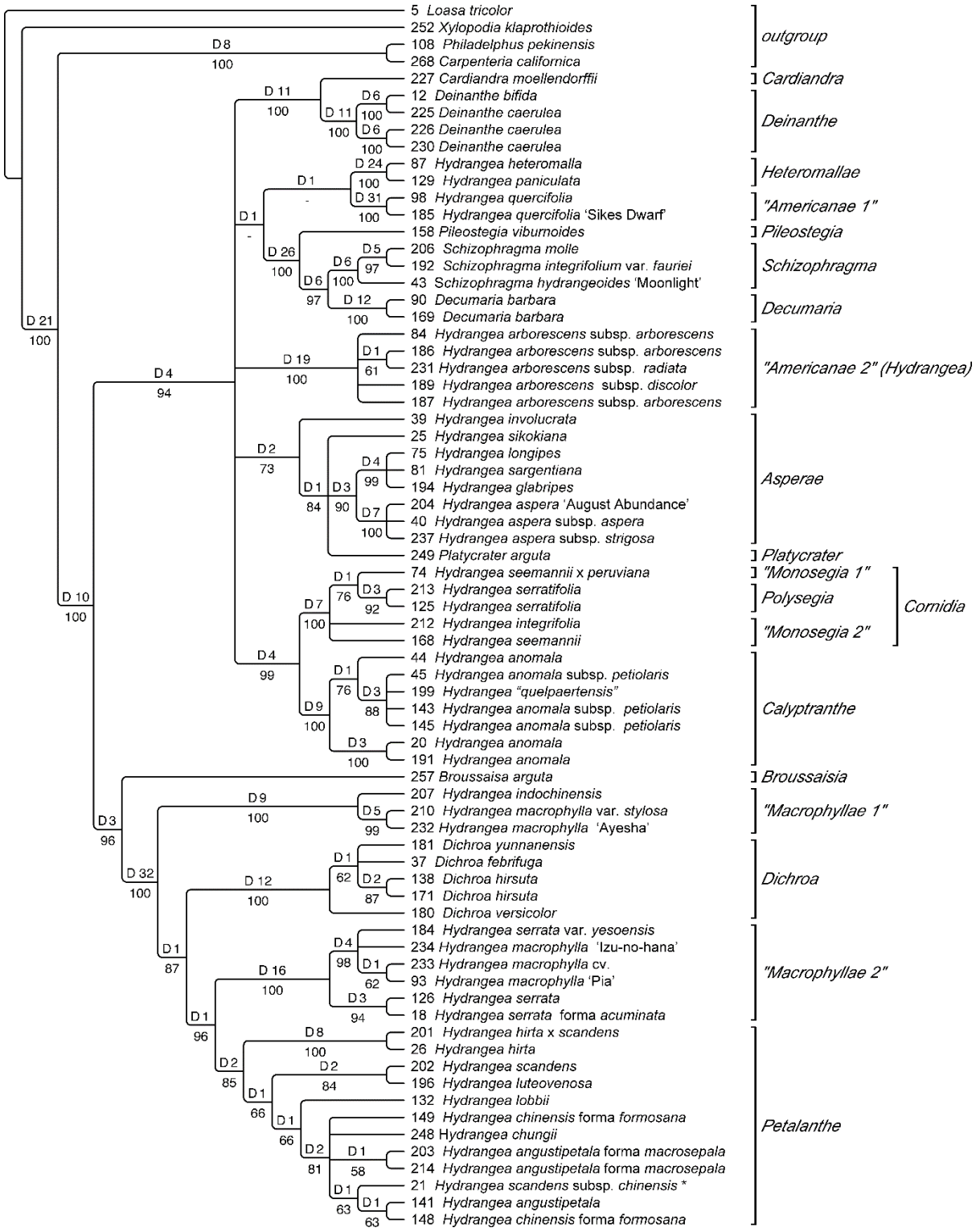

Figure 1.6: Phylogenetic hypothesis for tribe Hydrangeeae. Strict consensus tree inferred from the complete chloroplast dataset and coded length mutations (*trnK* intron, *matK* gene) by Samain et al. (2010). To evaluate nodes, the Bremer support (Decay value) has been labeled above the branch. In addition, 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 et al., 2001) form a morphologically diverse assemblage (Figure 1.7). 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 Mendoza 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* Koidz., 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 (Wong Sato & Kato, 2019). This floral dimorphism could possibly represent a synapomorphy 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 exclusively of these showy, colorful marginal flowers to which they probably owe their enormous horticultural success.

Dioecy 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 share a valvate calyx aestivation with most of the other Cornales. 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* D. Don. Merosity of floral organs is highly variable in tribe Hydrangeeae. Tetramerous, pentamerous or hexamerous perianths are common, while some exceptions exist. 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).

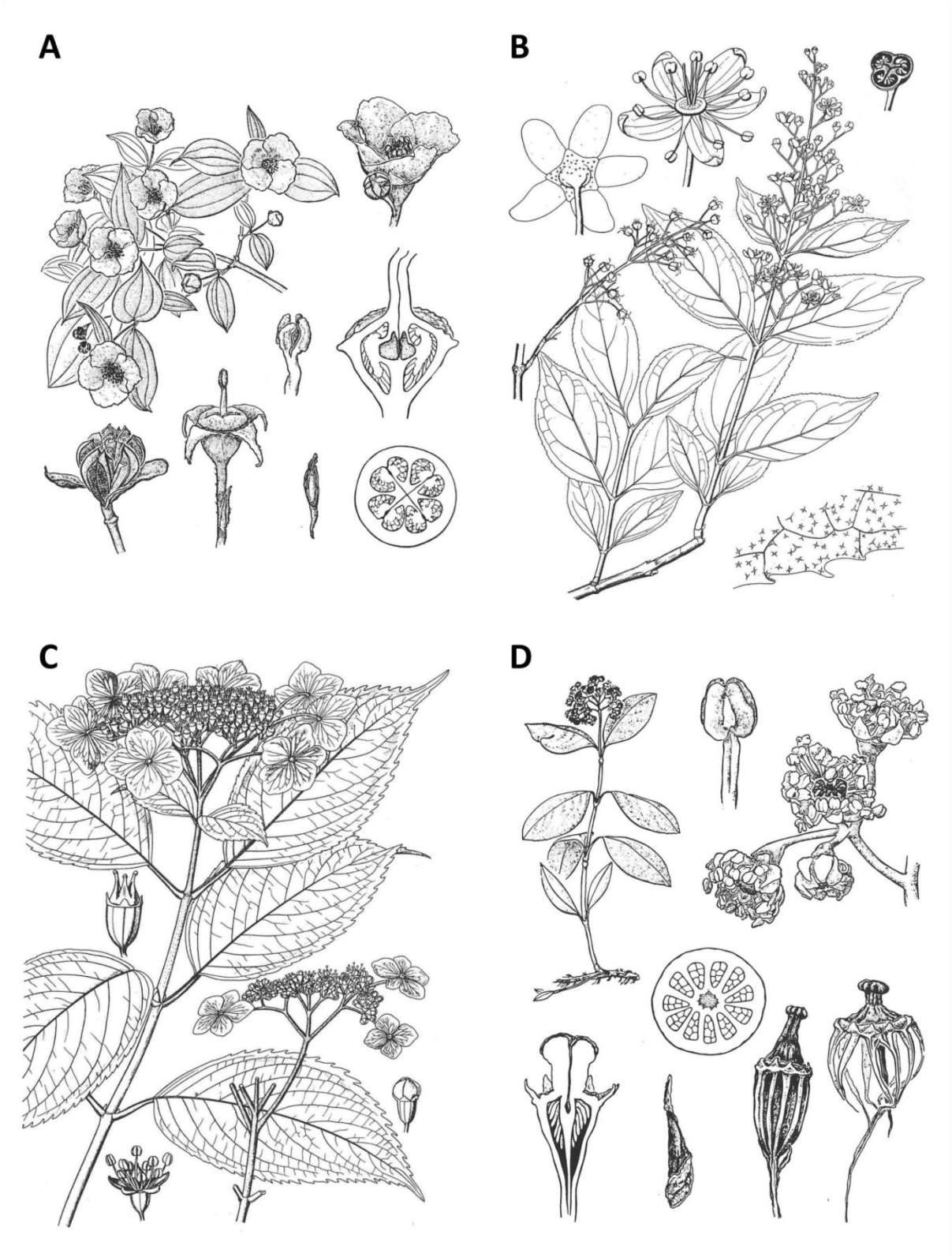

Figure 1.7: Representatives of Hydrangeaceae subfamily Hydrangeoideae. A. *Philadelphus inidorus* and B. *Deutzia paniculata* of tribe Philadelpheae. C. *Hydrangea serrata* and D. *Decumaria barbara* of tribe Hydrangeeae (Drawings adapted from Hufford, 2004).

Hydrangea aspera complex and allied taxa

Hydrangea aspera D. Don 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 *Hydrangea* series *Piptopetalae* (Maxim.), 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 *Hydrangea* 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 1.8), 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 last worldwide revision of the genus (McClintock, 1957). As such, this monograph considers only three species (Figure 1.9) within *H.* subsection *Asperae*: the morphologically variable and geographically widespread *H. aspera*, and the two Japanese species *H. involucrata* Siebold and *H. sikokiana* Maxim. 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 1.9). 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 *H.* 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 *H*. subsect. *Asperae*, with the exclusion of the Japanese endemics (*H. involucrata* and *H. sikokiana*) and *H. longifolia* Hayata.

Figure 1.8: *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).

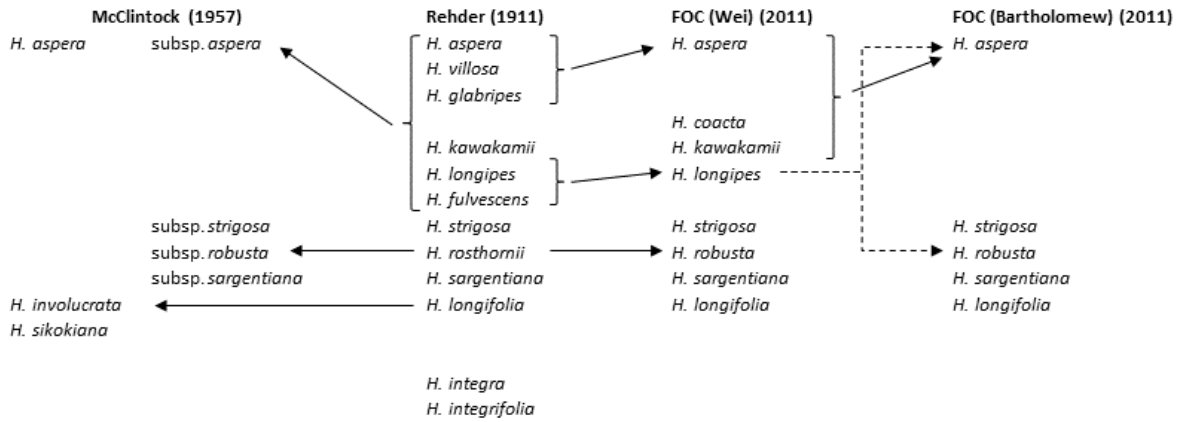

Figure 1.9: *Hydrangea aspera* species complex and allied taxa throughout revisions. The nominal taxa classified in *H.* sect. *Asperae* have been merged and split according to subsequent authors. Solid arrows indicate a merger of different taxa into a recognized species, whole dotted lines represent the splitting of a previously recognized species. Rehder (1911) placed 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 isolation. 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 characteristic for McClintock's *H*. subsect. *Cornidia*, the sister clade of *H*. 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* displays 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 *H*. 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* hybridization (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*, putatively covering a vast geographical range. Other taxa, however, have only been collected from a single population, such as *H. sargentiana* Rehder, an interesting situation from a conservationist standpoint, on which is divulged in chapter 5 (see also Appendix 5, box S5.1). Furthermore, the subsection 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* Hayata and *H. longifolia* are isolated to some degree on the island of Taiwan. Other putative species - for example, *H. aspera* (in the sense of Wei & Bartholomew, 2001), *H. robusta* Hook. f. & Thomson and *H. strigosa* Rehder - 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.

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 (Li, 2004; Isbell et al., 2017), 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 (however, see Crisci et al., 2020). 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 in conservation of *Hydrangea* biodiversity.

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* were 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, emphasis 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. This provided the necessary background for 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, collection trips to natural populations of the studied taxa was pivotal. Apart from allowing the collection of fresh samples, this provided insight into the possible threats to *Hydrangea* biodiversity. Two field trips were planned within the framework of this PhD (Figure 1.10), one to the Chinese province of Sichuan and 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

included in this thesis. In addition to personal *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 provides 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 conceptual, 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 disagreed widely on 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 combined with morphological features such as abaxial leaf pubescence (documented using scanning electron microscopy) 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 EC.*

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 potential of RADseq to generate large amounts of informative markers is harnessed for phylogenetic reconstruction and species delimitation in *Hydrangea* sect. *Asperae*. These newly acquired molecular data are utilized to evaluate the previously delimited putative species in the section, which is facilitated by using largely the same dataset as chapter 3. In addition to this comparison, the novel data provide additional lines of evidence to be used in constructing 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 EC.*

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 keeping with the general lineage concept of species, the different lines of evidence in support of each species delineation (species hypothesis), and those possibly falsifying these are expanded upon. *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, EC: Eduardo Cires Rodríguez, KB: Kenneth Bauters, MS: Marie-Stéphanie Samain, PA: Pieter Asselman, PG: Paul Goetghebeur, YDS: Yannick De Smet.

Chapter 2

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 most taxonomists, despite the ongoing debate concerning the adherence to strict monophyly in biological classifications, a new (infra)generic classification for tribe Hydrangeeae is proposed. This novel classification contains a broader circumscription of the genus *Hydrangea*, 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, and maximizing information content and familiarity, the 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 (Cornales: Hydrangeaceae). *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 documented in literature (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; Mayr & Bock, 2002; Van Wyk, 2007; Hörandl, 2010) and practicability (Brummit, 2002; Brickel et al., 2008), 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 (Donoghue & Cantino, 1988; Ebach et al., 2006). Here, some of the prominent discussion points between both schools are illustrated with the taxonomy of Hydrangeaceae tribe Hydrangeeae (Figure 2.1). 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. *Cardiandra alternifolia* (Siebold) Siebold & Zucc. E. *Deinanthe bifida* Maxim. F. *Dichroa febrifuga* Lour. G. *Pileostegia viburnoides* 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*, *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. For each of the genera in tribe Hydrangeeae the author, number of species and geographical distribution is presented.

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 chapter, 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 Mendoza et 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. Full names for all markers are presented in Table S1.1, Appendix 1. 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 *H*. sect. *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 phylogenetic hypotheses, 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 2). 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 2.3.1 (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 RAxML 7.2.8 (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).

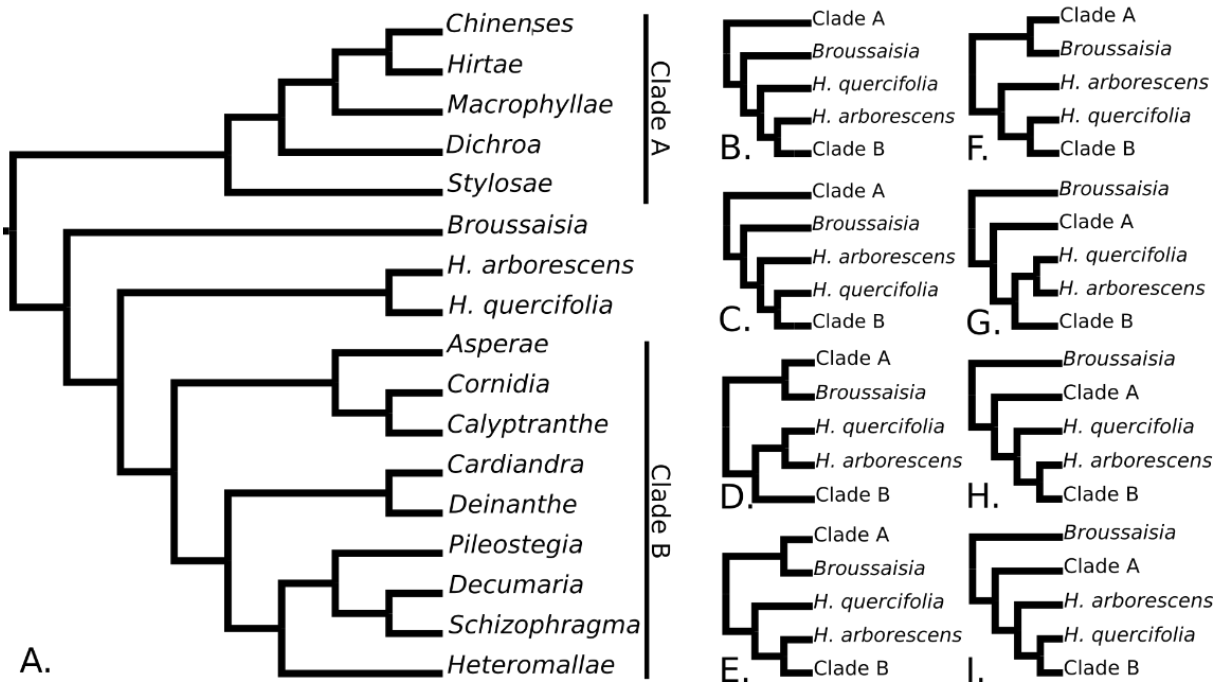

Figure 2.2: Phylogenetic hypothesis used for Bayesian hypothesis testing. A: the full tree corresponding to model M1, monophyly of all sections was constrained, as were all depicted nodes. B-I: alternative hypotheses, clade A and B are constrained as depicted in figure 2A, positions of *Broussaisia*, *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), *Hydrangea* sect. *Dichroa* is sister to a grade of the monophyletic *H.* sects. *Macrophyllae*, *Hirtae* and *Chinenses*. *Hydrangea* 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, *H*. sect. *Pileostegia* is sister to a clade containing the monophyletic *H*. sects. *Schizophragma* and *Decumaria*, while *H*. sect. *Heteromallae* is sister to this entire assemblage (PP: 0.7). *Hydrangea* section *Cardiandra* is recovered as monophyletic and in a sister relationship with a monophyletic *H*. sect. *Deinanthe*, while this assemblage is sister to the clade comprising *H*. sects. *Heteromallae-Schizophragma-Decumaria-Pileostegia*. All these sections are in turn sister to a clade containing *H*. sects. *Asperae*, *Cornidia*, *Calyptranthe* and *Platycrater arguta*. The last is phylogenetically nested within *H*. sect. *Asperae*, which in turn is sister (PP: 1) to a clade (PP: 1) containing the two highly supported monophyletic sister clades *H*. 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 *H*. 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 2). 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 2), 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 2). 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 2).

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 Figure. 2.2, 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

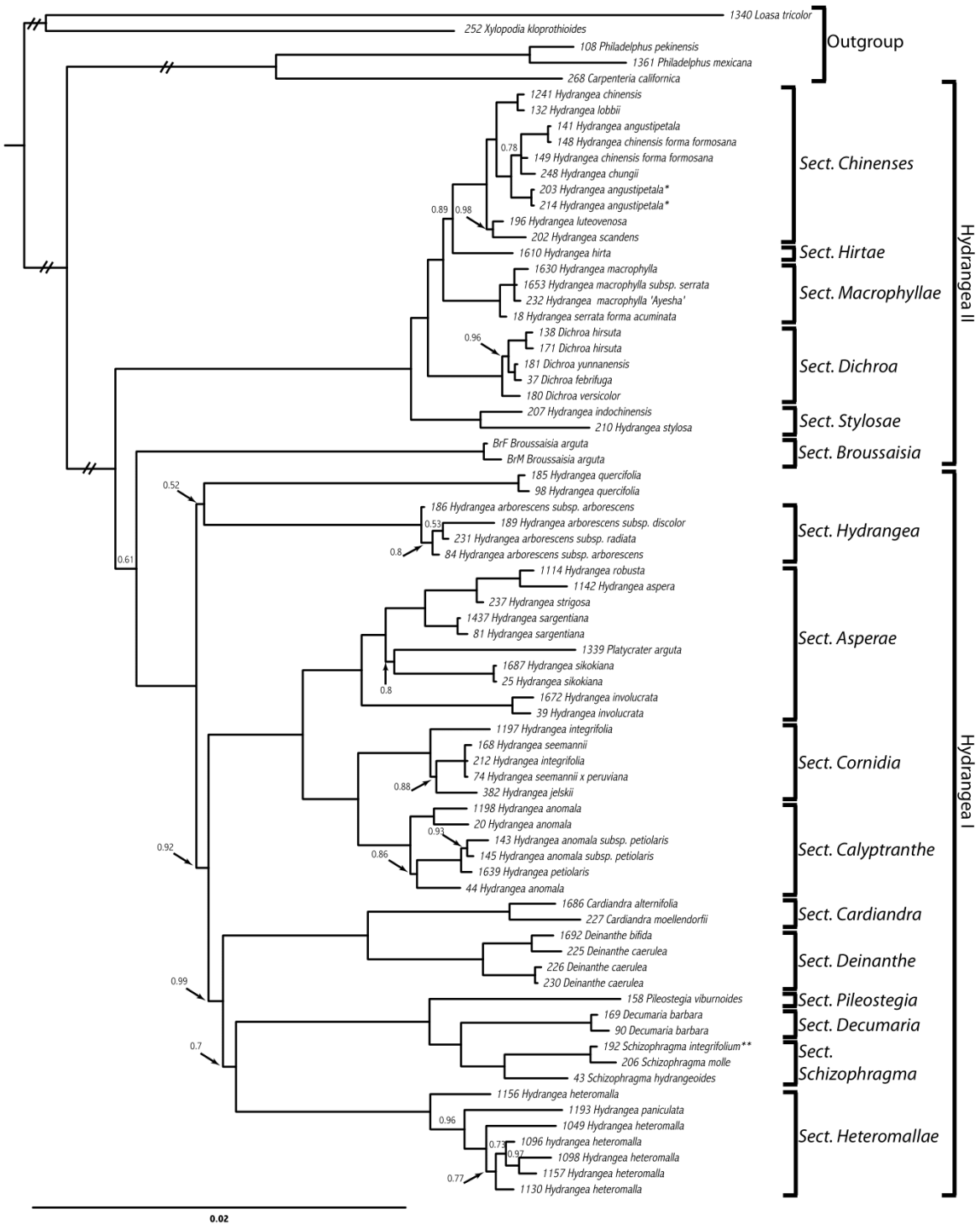

Figure 2.3: Phylogenetic hypothesis based on plastid dataset. The 50% majority rule consensus tree based on the combined plastid dataset with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. Section names according to the new infrageneric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

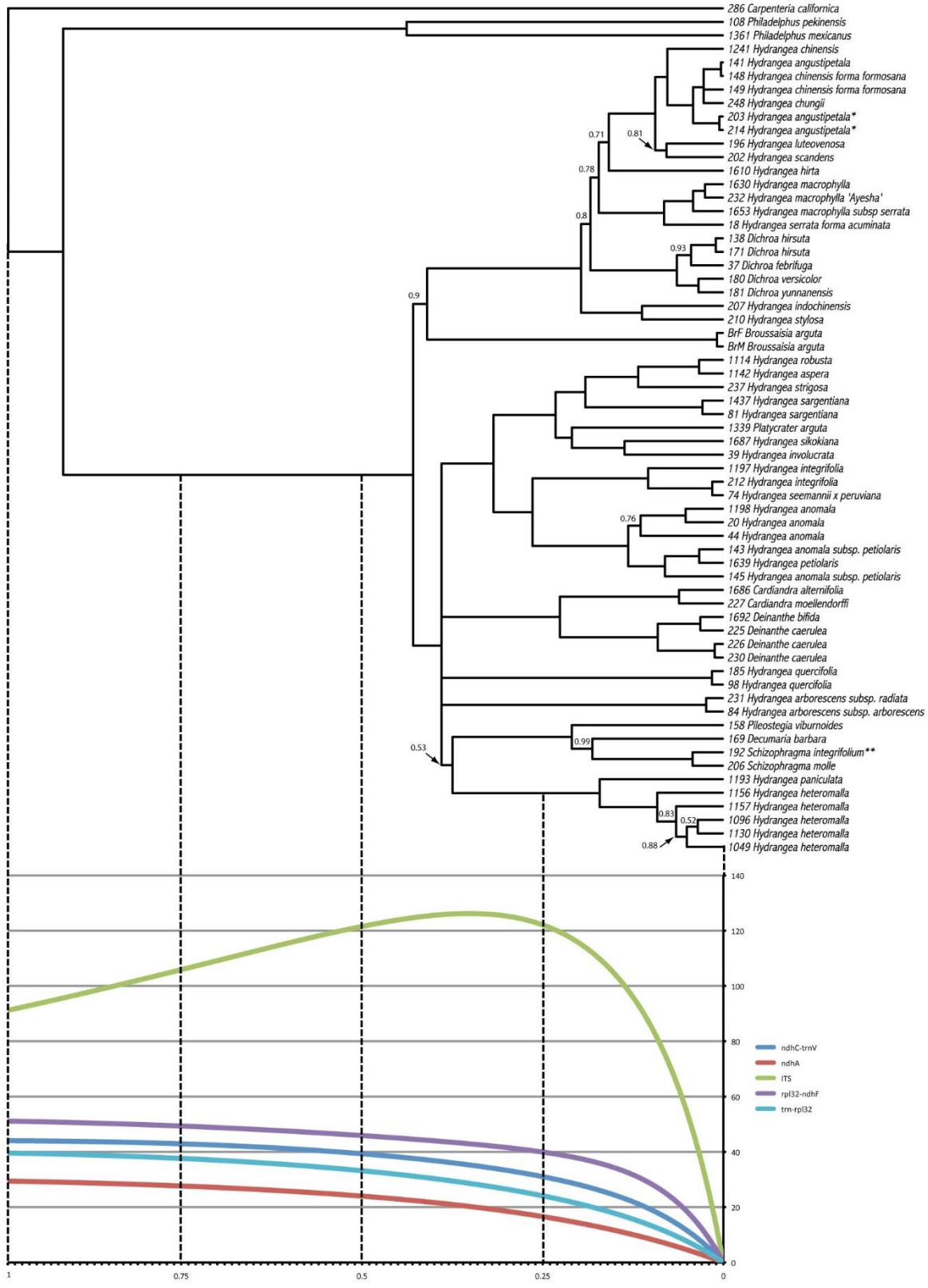

Figure 2.4: Phylogenetic informativeness plot. Net phylogenetic informativeness across time for all four sequenced regions, plotted against the ultrametric phylogenetic tree based on ITS and plastid sequences, excluding indel data. Posterior probabilities for drawn branches only displayed if below 1.

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 2) 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 *H.* sect. *Asperae* (plus *Platycrater*) as sister to the sister *H.* 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 2). 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 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 desired morphological homogeneity of the resulting taxa. For example, in order to retain the genus *Platycrater*, McClintock's *H.* 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 number 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 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 *H*. sect. *Asperae* in order to avoid the creation of a polyphyletic *H*. sect. *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 2, 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 3

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 (*H. sargentiana* and *H. longipes*), 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.

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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 sensitive 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 sequences 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.

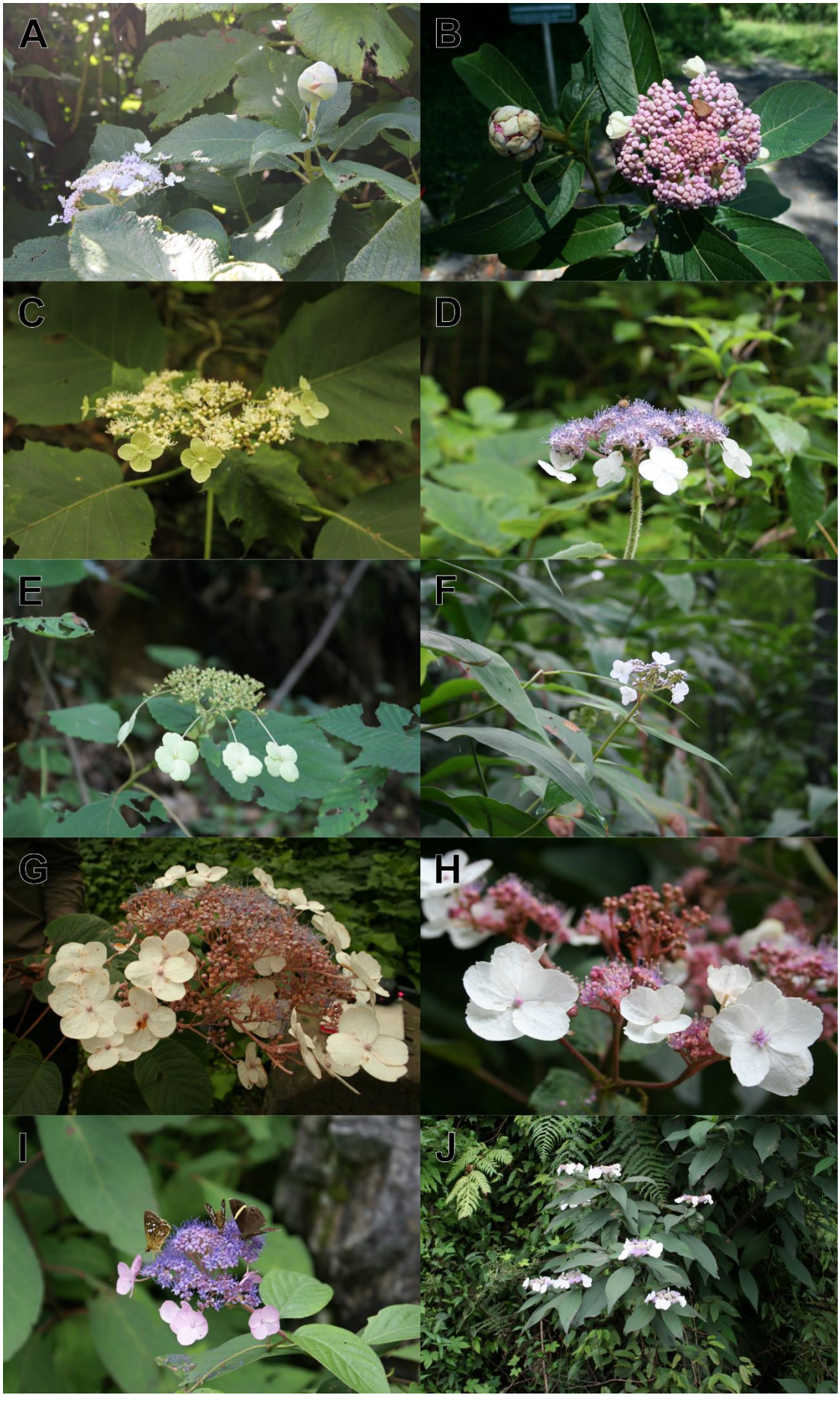
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Figure 3.1 (previous page): General morphology for representatives of *H*. sect. Asperae. A: *Hydrangea involucrata* (Japan), B: *H. longifolia* (Taiwan), C: H. *sikokiana* (Japan), D: *H. sargentiana* (Hubei, China), E: *H. longipes* (Hubei, China), 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 for 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, 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 (with the exception of *H. kawakamii* Hayata) representatives of *H.* sect. *Asperae* as separate species, owing to their distinct morphology (Figure 3.1: 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 *H.* sect. *Asperae* occur in sympatry while others remain strongly geographically isolated, such as *H. kawakamii*, 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 *Hydrangea* sect. *Asperae*. However, two cytogenetic studies (Cerbah et al., 2001; Mortreau et al., 2010) have demonstrated variation in the genomic organization among members of *H*. sect. *Asperae*. While most *Hydrangea* species present a chromosome number of 2n = 36, most members of *H*. 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 *Hydrangea* 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 *H*. 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 *H*. sect. *Asperae* morphotypes. Moreover, the variation found in this character is objectively documented using scanning electron microscopy.

Material and methods

Taxon sampling and initial morphological identification

This study included 29 specimens identified as representatives of *Hydrangea* sect. *Asperae* and one species from its sister clade *H.* sect. *Cornidia* as outgroup. Most of these specimens were collected in China (provinces of Sichuan and Hubei), Taiwan 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 by morphological comparison 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 preferred, 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 *H.* 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. Three 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). Full names for all markers are presented in Table S1.1, Appendix 1. 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 wilsoniana* Wangerin, *C. officinalis* Siebold & Zucc., and *Philadelphus incanus* Koehne generated by Zhang et al. (2011), and are specific for *Hydrangea* sect. *Asperae*. For a list of primer sequences see Table S3.2 in Appendix 3. 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 (Löytynoja & Goldman, 2005). All newly generated sequences were deposited in the European Nucleotide Archive (ENA, Table S3.1 in Appendix 3).

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 2.0 (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 (θ) and root age (τ_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 α/β and variance α/β^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. For *Hydrangea platyarguta*, none of the nuclear markers could be amplified, despite designing several custom primers. This taxon was therefore excluded from the study. Single gene trees for chloroplast and nuclear markers agree on topology of the deeper branches. Specimens identified as *H. longifolia* Hayata and *H. involucrata* form a well-supported clade in all gene trees (Figures S3.1-S3.8 in Appendix 3), which is sister to a larger clade containing all other representatives of *H*. 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 *Hydrangea* sect. *Asperae* compared to the single gene trees (Figure 3.2). The topological placement for the Japanese species (*H. involucrata*, *H. sikokiana*) 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).

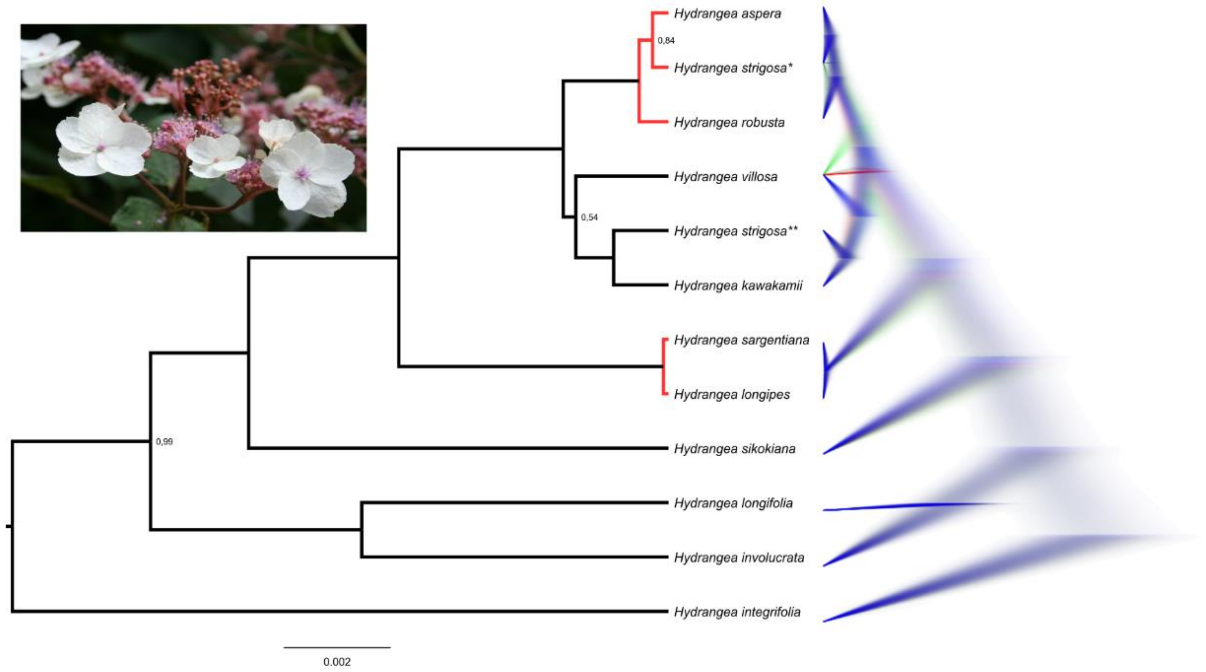


Figure 3.2: Maximum clade credibility species tree inferred from *BEAST (left) and cloudogram (right). In this tree, only posterior probabilities lower than 1 are displayed, and nodes with speciation probabilities lower than 1 (as inferred in BP&P) in any of the prior combinations are shown in red. Therefore, nominal taxa connected to these red branches are considered conspecific in a conservative interpretation of the BP&P results. The cloudogram 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. *Hydrangea strigosa**: Sichuan lineage, *Hydrangea strigosa***: Hubei lineage. The inflorescence depicted is that of *H. kawakamii*.

Bayesian species delimitation

Bayesian species delimitation results for *Hydrangea* 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 τ and θ 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 τ and θ. 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

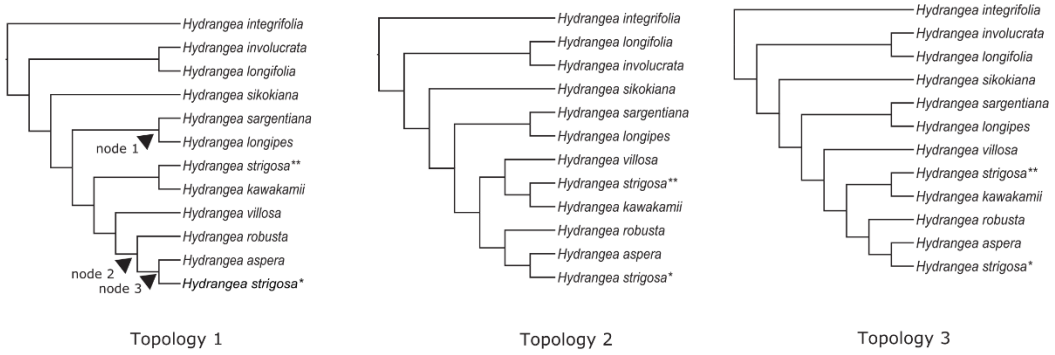


Figure 3.3: Summary of posterior speciation probabilities calculated by BP&P. The figure shows posterior probabilities for different combinations of: priors for tau and theta, guide tree topology, algorithm 0 or 1. All other nodes in the tree consistently scored a posterior probability of 1 for every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

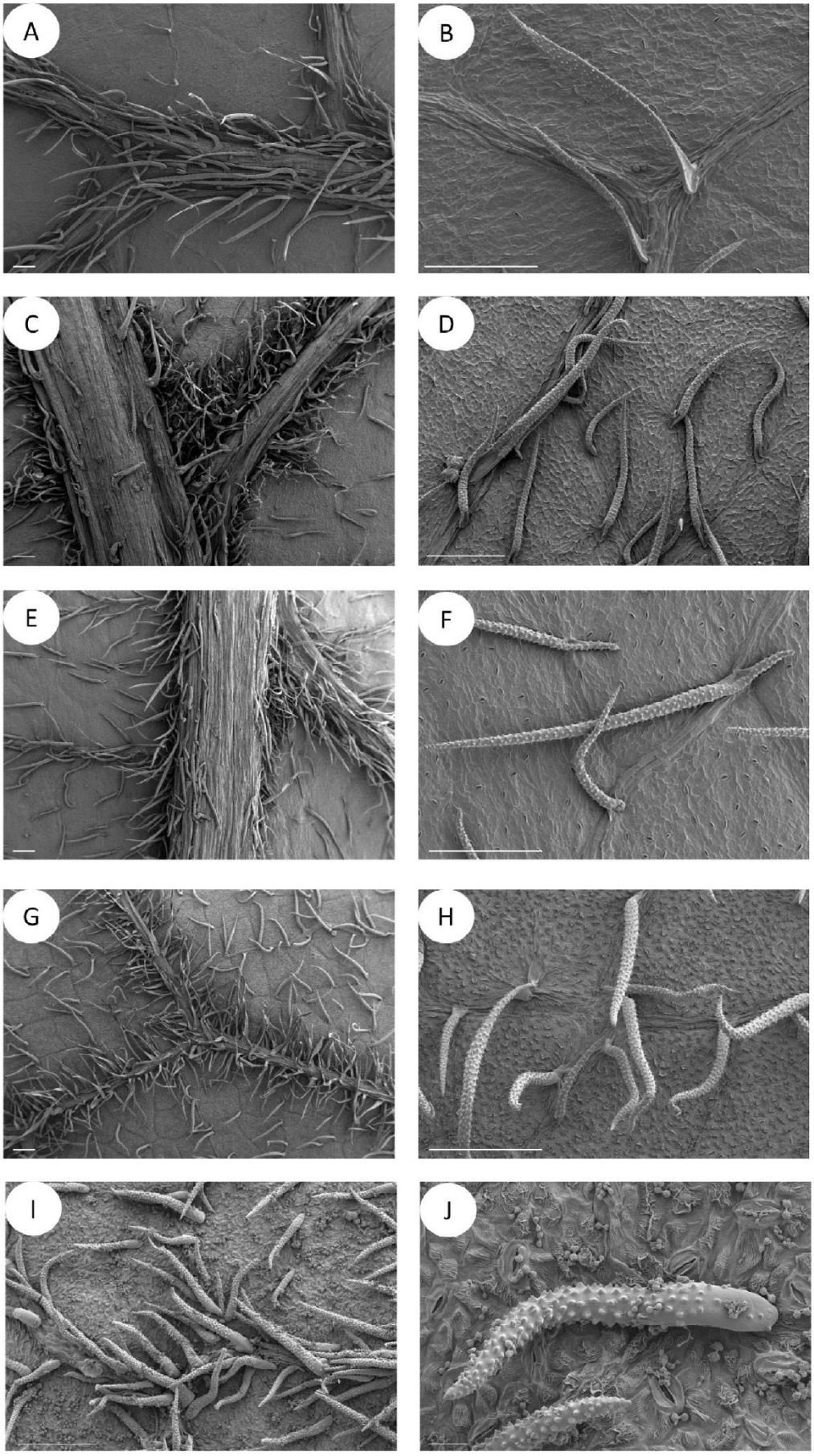
types coincide with the types described in protologues and previous revisions of *Hydrangea* sect. *Asperae*. Besides variation in the morphology of the trichomes, differences in the ornamentation of the leaf surface were observed, more specifically, in the presence or absence of white papillae.

For nominal taxa *Hydrangea 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. 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, *Hydrangea strigosa* (Figure 3.4: Q & R) and *H. robusta* (Figure 3.4: I & J) exhibit small appressed hairs on their lower leaf surface. 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 autapomorphic type of trichomes. In *Hydrangea 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).

In addition to 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 *Hydrangea strigosa* (both Sichuan and Hubei populations) (Figure 3.4: Q & R), lending the abaxial surface of fresh leaves a whitish habitus. Less conspicuous papillae are preset in *H. kawakamii* (Figure 3.4: G & H).


A
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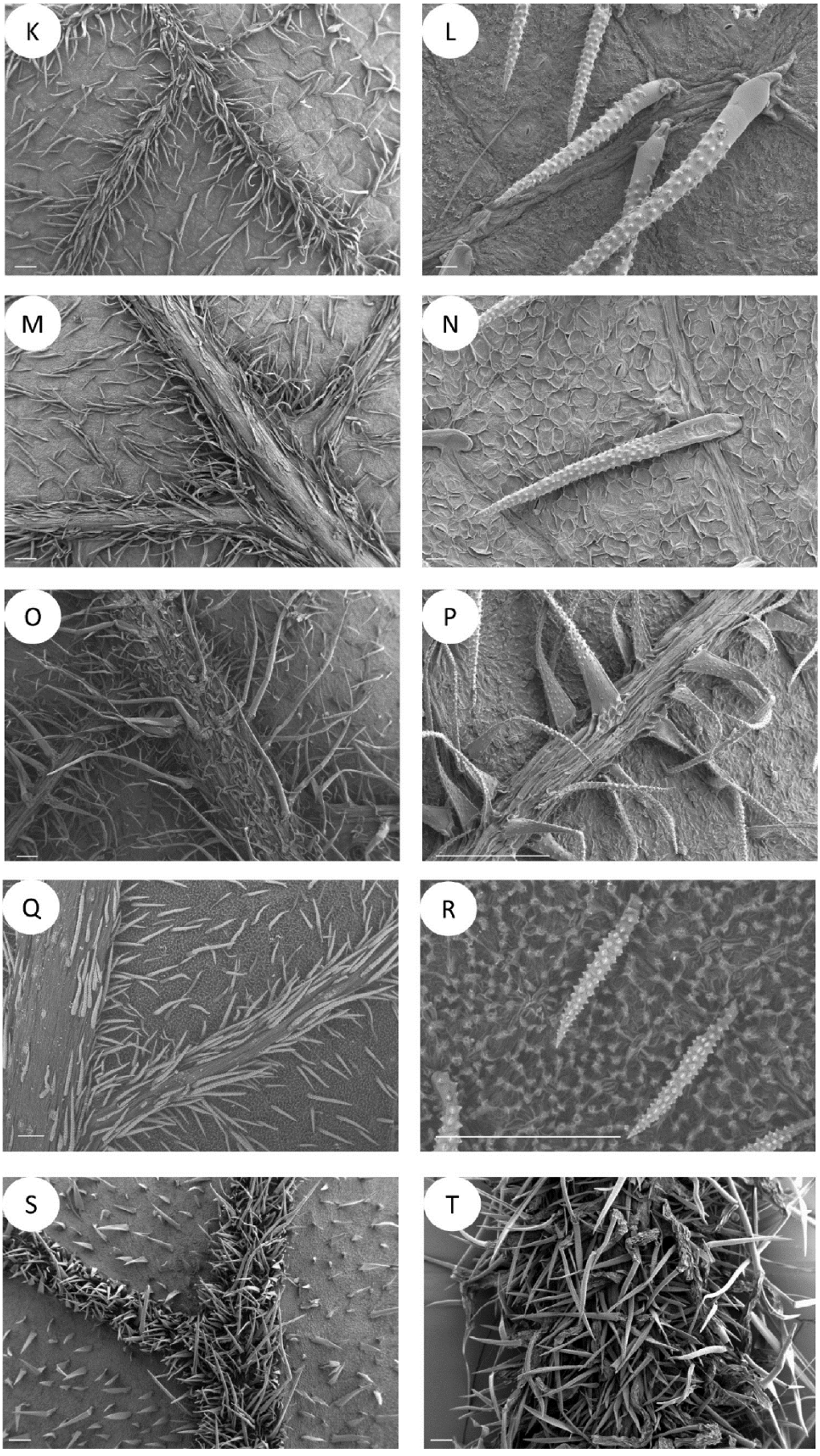
K
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Figure 3.4 (two previous pages): Scanning electron micrographs for the abaxial leaf surface of *H.* sect. *Asperae* representatives. The left column displays general overviews, while the right column presents details of the typical trichomes for 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 200 μm in A, B, C, D, E, F, G, H, I, K, M, O, P, Q, R, and 20 μm 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. While 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 *Hydrangea* 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, most are morphologically clearly identifiable based on clear-cut diagnostic characters, such as abaxial leaf pubescence (e.g. Figure 3.4). Lineage 8 being the exception, combining multiple types of indumentum.

Results from the coalescent analyses and gene trees suggest *Hydrangea 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 *H*. sect. *Asperae* is possibly the driving factor behind this pronounced divergence.

Within the *Hydrangea 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 morphological diagnosability 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 *Hydrangea 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 indumentum 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 *H*. 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*, also occurring 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, 1957; 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 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 they 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 4

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 difficult to interpret 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 for integrating molecular and morphological data will aid in stabilizing species boundaries. Since only a limited amount of molecular studies into the subject are available, additional polymorphic molecular markers should be acquired. 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.

Adapted from: De Smet, Y., Cires, E., De Clerck, O., Goetghebeur, P., Wanke, S., Samain, M.-S. Genome wide RADseq data resolves phylogeny and species boundaries in the *Hydrangea aspera* species complex. Submitted to *Molecular Phylogenetics and Evolution*.

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 sufficiently variable markers, available for a large dataset of non-model organisms. Reduced representation libraries sequencing (e.g. Restriction site associated DNA sequencing or 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. Likewise, species delimitation of recently diverged species, or genetically uniform species complexes requires large amounts of polymorphic markers. These have been acquired using RADseq, achieving better resolution in identifying species boundaries in snails (Razkin et al., 2016), lizards (Nieto-Montes de Oca et al., 2017), insects (Dincă et al., 2019) and corals (Quattrini et al., 2019) compared to Sanger based-markers.

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 taxa generated by this splitting approach have been criticized (Samain et al., 2019), and will not be followed in the current study.

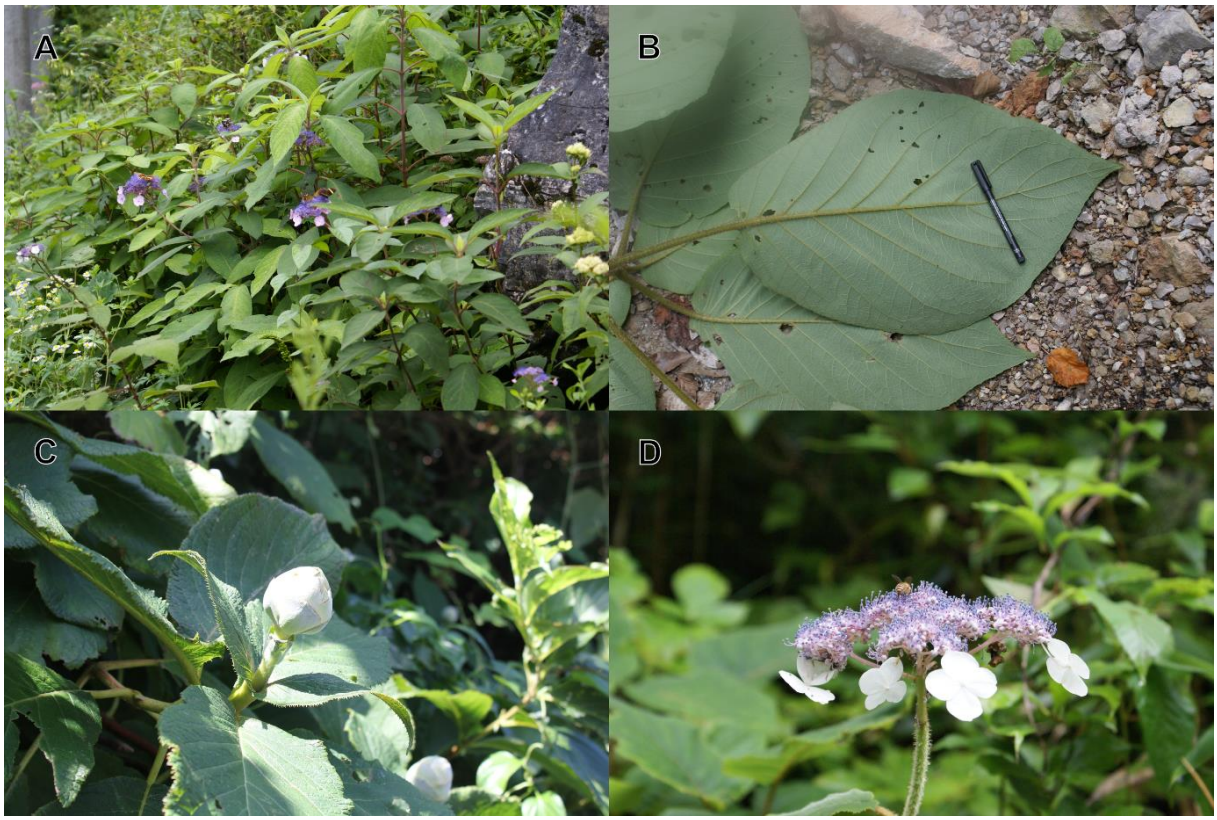

Figure 4.1: *Hydrangea* sect. *Asperae* morphology. A: General habitus of *H. aspera*. B: Leaf size of *H. sargentiana*. C: young inflorescence of *H. longifolia*, with enveloping involucral bracts. D: Inflorescence of *H. sargentiana*, showing the characteristic fleshy hairs on the peduncle.

Evolutionary relationships in the predominately east and southeast Asian *Hydrangea* section *Asperae* (Rehder) Y. De Smet & Samain are relatively well-known. 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), resolution within the group was gradually increased. The evolutionary position of *H. villosa* Rehder, however, as well as the exact relationships within the *H. aspera* D. Don - *H. robusta* Hook. f & Thomson - *H. strigosa* Rehder group remain unresolved. In addition, the section 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. Given their renown as ornamental species, even subtle morphological variation was often considered sufficient to warrant description of new taxa in *Hydrangea*, as this could be of interest to collectors and breeders. In doing this, authors of novel species

implicitly adopt a morphology-based criterion for species delimitation (similar to the phenetic species criterion, Sokal & Crovello, 1970), but do not address other potential sources of evidence, which could corroborate or reject the hypothesized species boundaries. 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 *H.* sect. *Asperae* is the *H. aspera* species complex (Figure 4.1). This group consists of several nominal taxa, excluding all Japanese and some Taiwanese members of the section. Different revisions have allocated these taxa to different numbers of recognized species (Rehder, 1911; McClintock, 1957; Wei & Bartholomew, 2001) depending on the weight assigned to certain morphological characters. In her classic revision 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, with lanceolate to ovate leaves, are distinguished by a strigose (*H. aspera* subsp. *strigosa*) or velutinous (*H. aspera* subsp. *aspera*) leaf indumentum. 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 reinstating nominal taxa from synonymy based on distinct morphological characteristics. An example of the latter is found in *Hydrangea longipes* Franch., which is differentiated from other *H.* 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*. Within 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 should 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 represents an explicitly documented line of evidence for the interpretation of species boundaries. Even contradictions between species delimitation algorithms can contribute to the understanding of the biological processes driving speciation. This approach identified three groups of nominal taxa in *H*. sect. *Asperae* (De Smet et al., 2017). The first group displays general agreement among all lines of evidence examined, and consists of nominal taxa *H. involucrata* Siebold, *H. longifolia* Hayata, *H. sikokiana* Maxim., *H. kawakamii* Hayata and *H. villosa*. Specimens in the second group could clearly be identified as separate nominal taxa, based on diverging morphology, but were found to be genetically uniform, based on the eight sampled markers. For this group, containing *H. sargentiana* Rehder 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 consists of two separate lineages, occurring in two disjunct regions (De Smet et al., 2017). One of them (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, as the species occur in sympatry throughout Sichuan. Interestingly, all three morphotypes generally occur along an altitudinal gradient, with intermediate forms connecting morphologically discrete 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. Testing of additional suitable 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.

In the current study, the potential of RAD sequencing to generate high amounts of informative markers is employed to improve insights into diversification and species boundaries of *H*.

sect. *Asperae*. These markers will be employed to elucidate the position of *H. villosa* within the *H. aspera* complex, as well as the exact relationships among *H. aspera*, *H. robusta* and *H. strigosa* complex. At the species level, the RADseq markers will be used to test the species hypotheses proposed in the previous study utilizing single copy nuclear markers. If the lack of genetic divergence between morphotypes is an artefact of the low copy nuclear markers used, a large amount of RADseq markers should provide better resolution. On the other hand, lack of variation in the RADseq markers could confirm the absence of genetic divergence between morphotypes. 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 *H.* sect. *Asperae*, and one accession of the related genus *Philadelphus* (Table S4.1 in Appendix 4) as outgroup, collected at type locations where possible (Figure 4.2). Allocation of specimens to nominal taxa follows the aforementioned authors. Because of the failure to amplify any of the nuclear markers used in De Smet et al. (2017) for *H. platyarguta*, combined with the divergent nature of this taxon (see long branch in De Smet et al., 2015a) it was excluded from the present study. This level of divergence could cause the loss of shared restriction sites, and therefore homologues alleles with the other taxa. 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 500-750 bp 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 250 bp 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 (< 15 bp), 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 artefact 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).

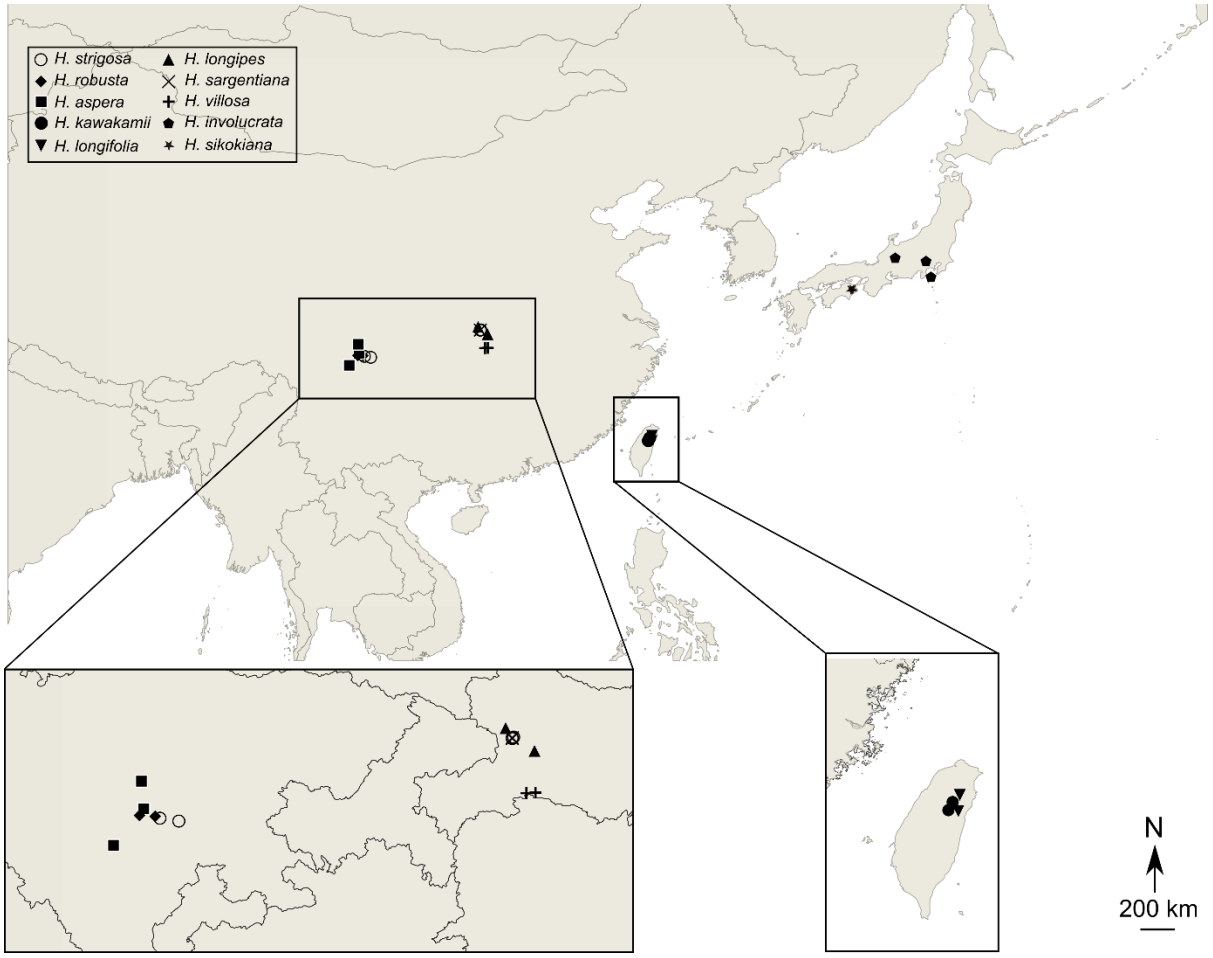

Figure 4.2: Distribution map. Geographic distribution of the samples used in the present study. All samples represent species of *Hydrangea* sect. *Asperae*. This image was generated using QGIS 3.12.1. Orientation and scale indicated in bottom right corner.

Ustacks-SiLiX analyses

The resulting 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 (Bouckaert et al., 2019). 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 (Bouckaert et al., 2019), 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 v.1.6.2 (Drummond &

Rambaut, 2007). The resulting trees were summarized as a Maximum Clade Credibility Tree in TreeAnnotator v1.6.2 (Drummond & Rambaut, 2007).

Phylogenetic inference: species tree

Species trees were estimated with *BEAST v2.6.0 (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 log files 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 as detailed above. 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).

Bayesian species delimitation

Bayesian species delimitation was conducted using the program BP&P v. 4.1.4 (Yang, 2015; Flouri et al., 2018), which 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. 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 BP&P runs. 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. The population size parameters (θs) are assigned the inverse-gamma prior IG(3, 0.002). The divergence time at the root of the species tree (τ0) 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, 250 bp × 2 read length for a total of 3,9 Gb. Processing and demultiplexing the dataset 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 (Appendix 4, Table S4.2).

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 alignments, 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 4, 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 for the relationships surrounding *H.*

aspera (Figure 4.3). The rest of the topology is generally 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 *H.* sect. *Asperae*. This larger clade consists of *H. sikokiana*, which is recovered (PP: 1) as sister to all other taxa in the Ustacks dataset; its position in the SNP topology is poorly supported, but is not in contradiction to the position as sister to the remaining ingroup species in the RADa tree. Both datasets recover *H. sargentiana* and *H. longipes* as sister to the rest of the Chinese portion 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 of them (PP: 1 in Ustacks dataset, PP: 0.96 in SNP dataset) contains *H. strigosa* (Hubei lineage) and *H. kawakamii* with one of two samples identified as the nominal taxon *H. strigosa* (Sichuan lineage). The second clade (PP: 0.93 in Ustacks dataset, PP: 0.81 in SNP dataset) contains the other *H. strigosa* (Sichuan lineage) specimen, as well as 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.

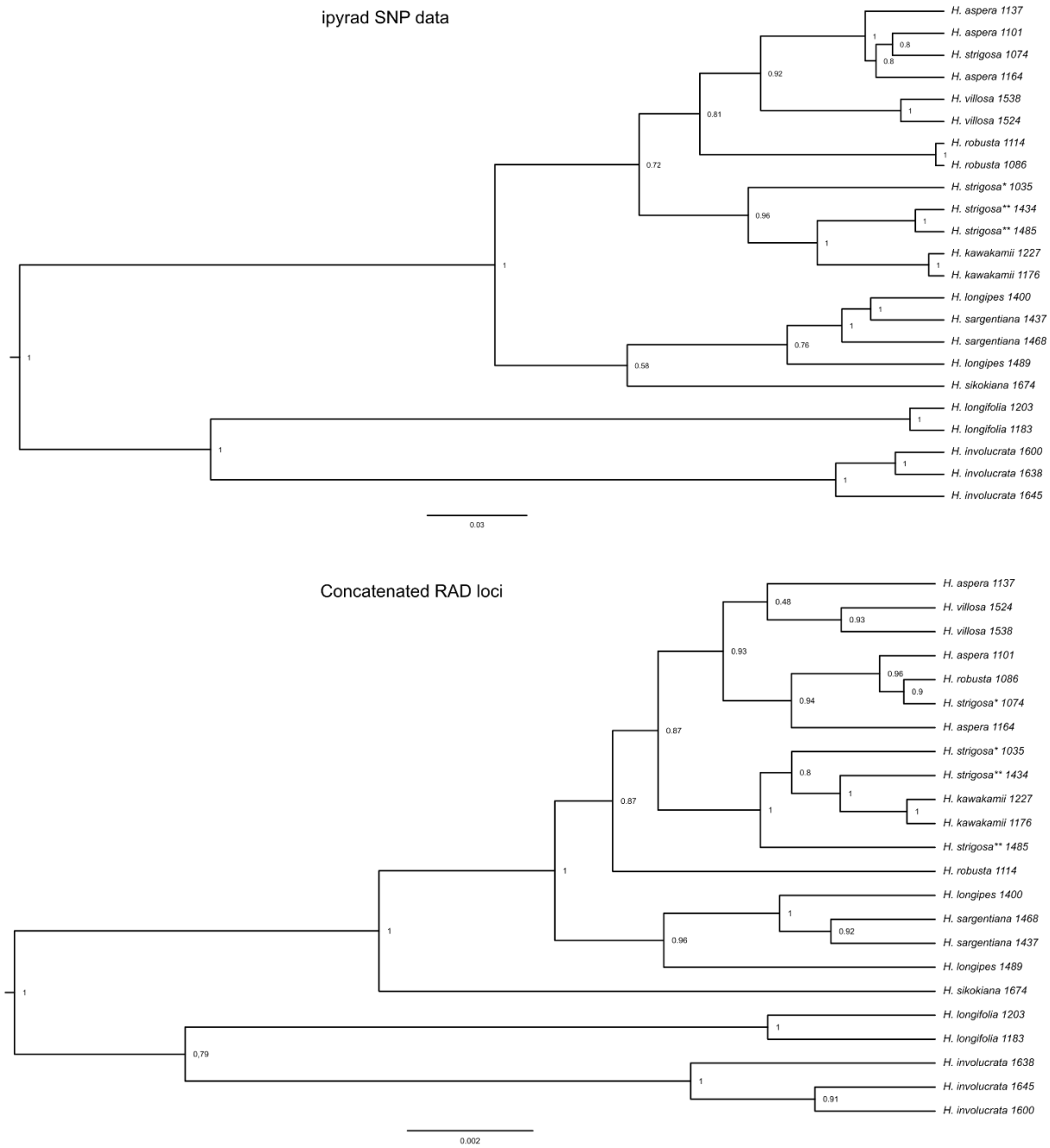

Figure 4.3: Concatenated gene trees. Maximum clade credibility trees generated for the SNP dataset resulting from the ipyrad analysis, and the concatenated dataset (RADa) generated from the seven alignments containing all sampled species resulting from the Ustacks/SiliX analysis. Values indicated at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

Bayesian species tree reconstruction

No well-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 RADscn 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 *Hydrangea 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*.

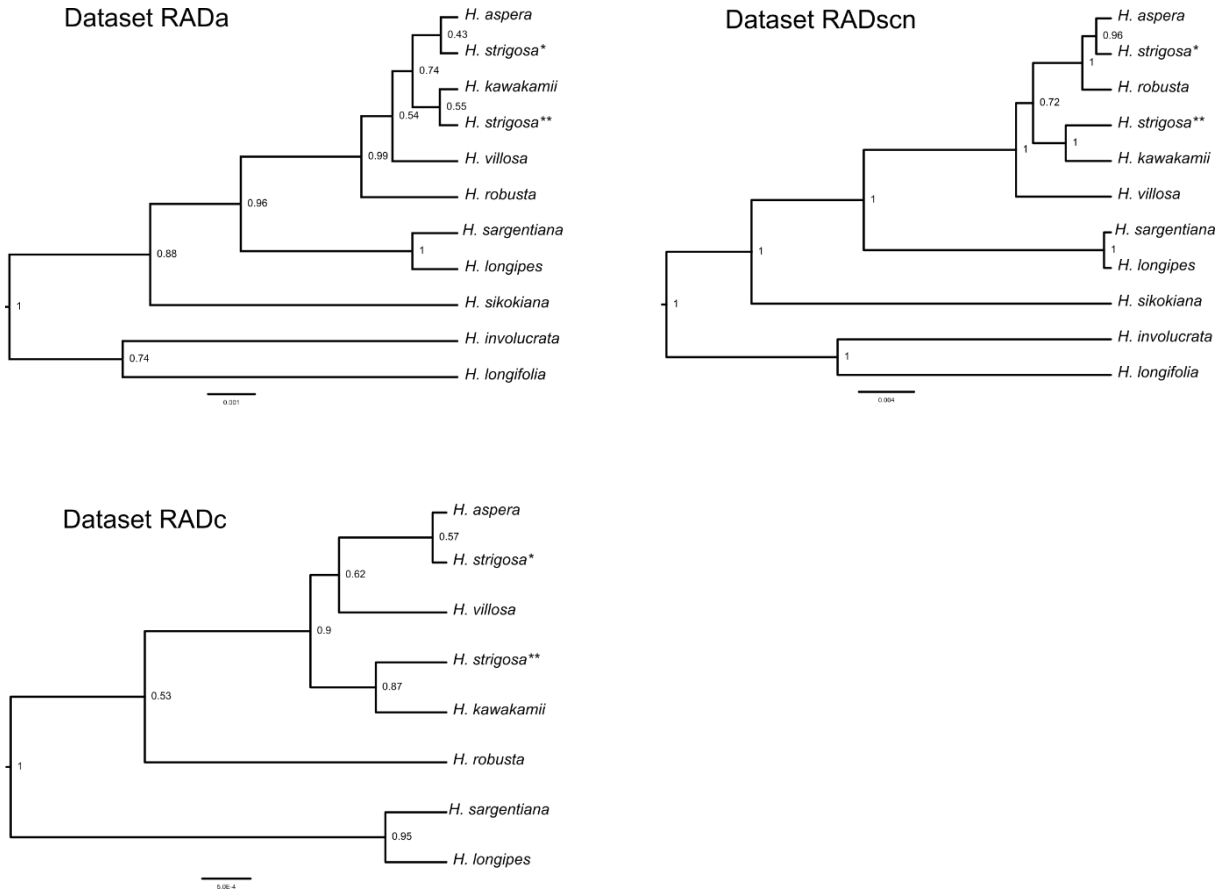

Figure 4.4: Species trees. Maximum clade credibility species trees inferred from *BEAST runs for datasets RADa, RADscn and RADc. Posterior probabilities are depicted along the respective nodes. *Hydrangea strigosa* is split up into the Sichuan lineage (*) and Hubei lineage (**).

Bayesian species delimitation in BP&P

The results for Bayesian species delimitation with BP&P 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 *Hydrangea 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 BP&P 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 *Hydrangea 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 BP&P 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

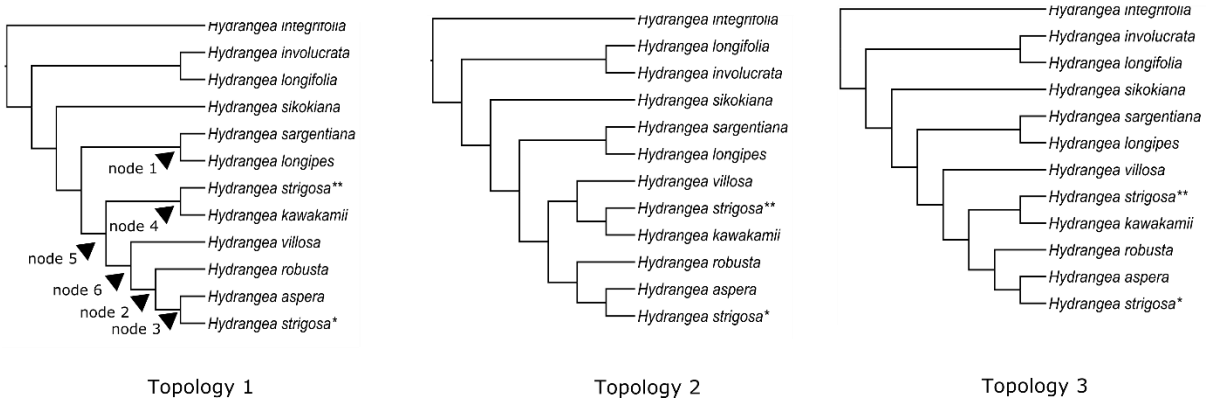

Figure 4.5: BP&P results. Summary of posterior speciation probabilities calculated by BP&P for different combinations of guide tree topology (as depicted below) and dataset used in the analysis. Posterior speciation probabilities are given for each of the nodes who did not consistently garner a PP of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

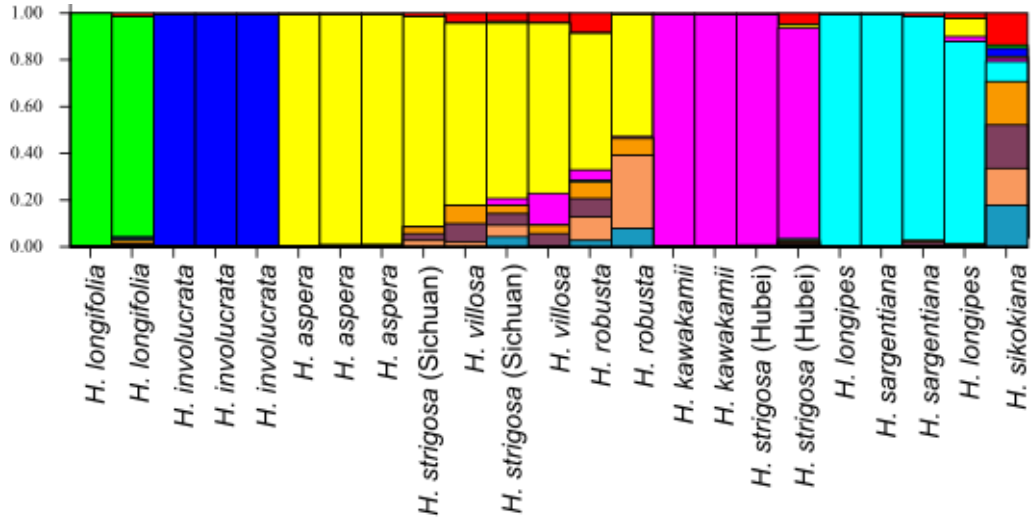

Figure 4.6: Structure bar plot. Results of the STRUCTURE analysis for the SNP dataset generated by the ipyrad pipeline. Depicted here is the distribution for the most likely K value (K = 11), as determined by the delta K approach (Evanno et al., 2005). Each bar represents a single individual, for which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

Discussion

Species delimitation in *Hydrangea* 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 *H.* 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 *H.* 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; chapter 3) and morphology-based revisions (McClintock, 1947; Wei & Bartholomew, 2001), therefore creating evidence-based, stable species hypotheses within *H.* sect. *Asperae*.

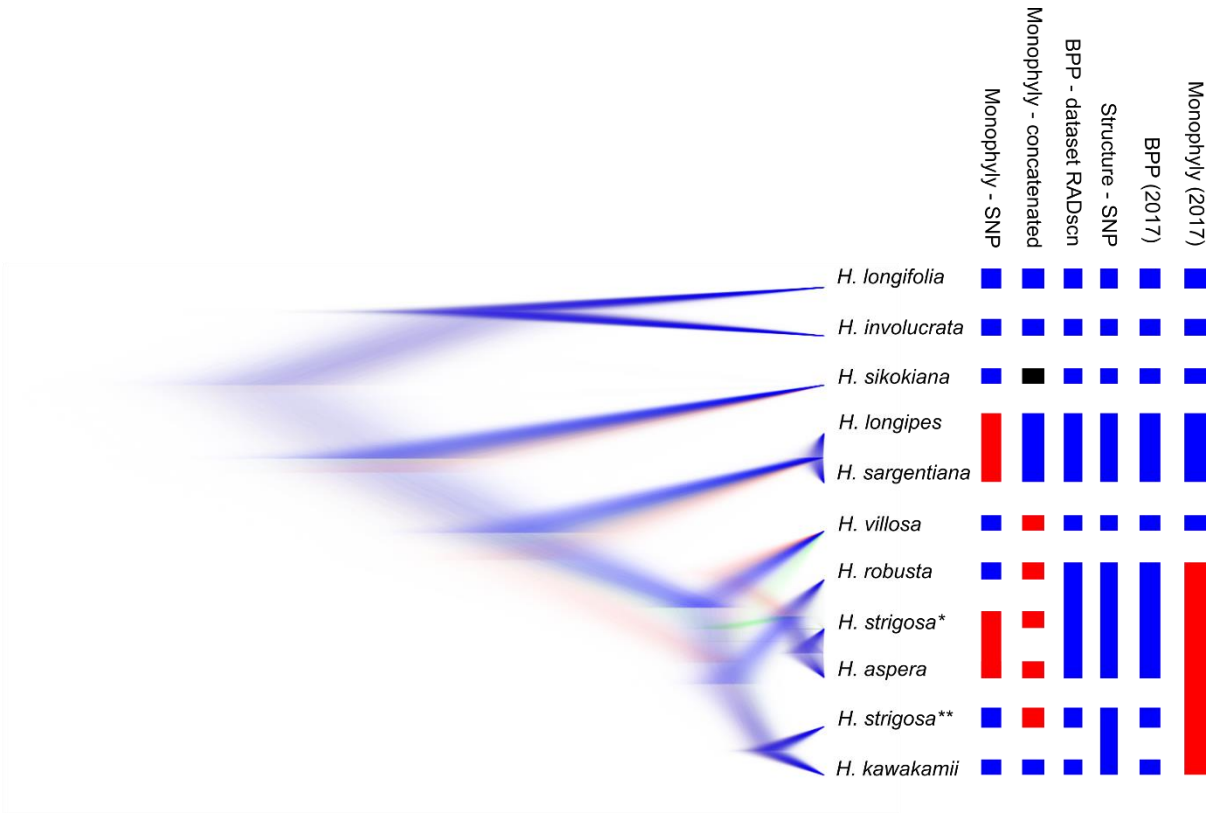

Figure 4.7: Comparison of different operational criteria. The cloudogram (left) represents the posterior distribution of species trees inferred in the five independent *BEAST runs. Different colors represent different topologies, in which the blue topology coincides with the MCC tree. Higher color density 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 *Hydrangea 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 *Hydrangea involucrata* and *H. sikokiana*, and the Taiwanese endemic *H. longifolia*. Young inflorescences of *H. involucrata* and *H. longifolia* are unique within *H.* 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 *H.* 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 their distinctiveness and molecular divergence from the rest of the section, in agreement with previous studies (De

Smet et al. 2017). The evidence presented 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 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, branches 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 these taxa could be due to their recent divergence, or ongoing gene flow. This renders the *H. sargentiana* – *H. longipes* complex an interesting case for the study of speciation in the face of gene flow. Extensive sampling of both morphotypes, combined with a genome wide sequencing effort could reveal genomic regions contributing to this kind of speciation. Similar research has been

successful in *Heliconius* Kluk. butterflies (Nadeau et al., 2012). This case perfectly exemplifies the advantages of comparing multiple lines of evidence for species delimitation. 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 (morphology and geographic distribution), 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 BP&P) can be observed for nominal taxa *H. villosa* and *H. kawakamii*. Absence of pronounced 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 distinguishable from *H. aspera*. Characters employed traditionally for diagnosing species in *H.* 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, in sympatry, genetic divergence between these three taxa has not

yet been detected (De Smet et al., 2017), suggesting incomplete reproductive isolation. Furthermore, sympatric populations morphologically similar to the type specimens (or populations in allopatry) 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 lineage 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 each nominal taxon as 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 location 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 *Hydrangea* sect. *Asperae*

The combined analyses of RADseq data and previously sequenced traditional markers improved support for two nodes in the *H.* 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). However, the phylogenetic position of *H. villosa*

among two larger clades, one containing *H. aspera*, *H. robusta* and *H. strigosa* (Sichuan lineage) and another one containing *H. kawakamii* and *H. strigosa* (Hubei lineage) remains unsupported (PP: 0,72). Likewise, the phylogenetic analysis based on 1475 SNPs could not recover the relationship between *H. villosa* and these two 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 *H*. 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 including whole-genome or gene duplications, and 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 *H*. sect. *Asperae*. Indeed, variations in chromosome number have been described (Funamoto & Tanaka, 1988; Cerbah et al., 2001; Mortreau et al., 2010) 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 4), 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 could potentially be ascribed to uneven quality and quantity of 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 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. Future studies in *H*. sect. *Asperae* could circumvent at least part of the difficulties described above by adopting more targeted NGS-based approaches in acquiring sequence data. One such option is target enrichment through custom designed probes, which has been shown to be effective in elucidating plant phylogenetic relationships (Mandel et al., 2014), and species radiations (Nicholls et al., 2015). The challenge here will be developing a probe set for a sufficient number of nuclear markers informative at and below the species level (Hollingsworth et al., 2016). However, increasing availability of transcriptomes and genomic data for *Hydrangea* (Chen et al., 2015; Rinehart et al., 2018), as well as universal probe sets (Johnson et al., 2018) could remedy this.

Conclusion

The current study is the first to apply RADseq to species delimitation and phylogenetic reconstruction in the genus *Hydrangea*. Despite low and uneven sequencing coverage across the individual samples, these new data were able to solidify several insights in *H*. 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 call for the merging of the latter two nominal taxa into a single species (*H. aspera*). 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 5

Taxonomic treatment of *Hydrangea* sect. *Asperae*

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

Carl Linnaeus (1707-1778)

Abstract

The predominantly southeast Asian *Hydrangea* section *Asperae* has seen several shifts in species boundaries, mainly focused around the *H. aspera* complex (all species formerly classified in McClintock's *H. aspera*). Within this complex, different interpretations of morphological variation have led authors to recognize between one and nine putative species. Recent studies utilized both traditional Sanger sequencing-based and RADseq-based markers to elucidate genetic diversity within the section. Both data types agreed on the recognition of several distinct genetic lineages in the *H. aspera* complex, some of which could be characterized by discrete morphological characters, such as leaf indumentum. Integrating these new data within the framework of an explicitly defined species concept (general lineage concept of species) allows for the postulation of evidence-driven species boundaries within the section. Species delineated in this way can therefore be understood as hypotheses, corroborated by diverse lines of evidence. For *H.* section *Asperae*, ten species were recognized, for which the supporting evidence is presented here. All of these taxa coincide with published names, being: *H. sikokiana*, *H. involucrata*, *H. longifolia*, *H. sargentiana*, *H. longipes*, *H. villosa*, *H. kawakamii*, *H. strigosa*, *H. aspera* and *H. platyarguta*. Therefore, no new species were described for the section, although the circumscription of several nominal taxa changed to include other previously published names. The nominal taxon *H. robusta*, included in previous molecular studies in the group was merged with *H. aspera*, as insufficient evidence could be gathered for its retention as species. The widespread *H. strigosa* was found to contain two distinct genetic lineages, with only one (situated in Hubei, China) considered here to be linked to the type specimen. The other evolutionary lineage contained both *H. strigosa* and *H. aspera* morphotypes. It is hypothesized here that this is a consequence of interbreeding between these two species when they occur in sympatry.

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

Introduction

Taxonomy and systematics of the Hydrangeaceae tribe Hydrangeeae DC. 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). The first suggestions of the polyphyletic nature of the genus *Hydrangea* L. (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 & Lumawag, 2019), others proposed morphology-based alternatives, not reflecting evolutionary relationships (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 sections, congruent with supported clades retrieved in the nuclear and chloroplast based phylogenetic hypotheses. One of these sections, *H.* sect. *Asperae* (Rehder) Y. De Smet & Samain, contains small to larger deciduous shrubs, with the largest diversity centered in mainland China and Japan (Figure 5.1). Several taxa exhibit wide distributions, occurring throughout Nepal, India and south to central China (*H. aspera* D. Don), while others are highly localized in their distribution (*H. sargentiana* Rehder, *H. villosa* Rehder). 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 (Wong Sato & Kato, 2019). Since the merging of several genera into *Hydrangea*, *H*. sect. *Asperae* contains one species exhibiting an aberrant morphology, *H. platyarguta* Y. De Smet & C. Granados. This taxon develops larger flowers compared to the other representatives, showing a multitude (>25) of yellow stamens, and developing apically poricidal capsules. Marginal flowers are conspicuous for having connate sepals, forming a deep cup. 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 revisions (Rehder, 1911; Chun, 1954; McClintock, 1957; Wei, 1991; Wei & Bartholomew). Authors generally agree on the species status for the two Japanese representatives of *H.* section *Asperae* (*H. involucrata* Siebold and *H. sikokiana* Maxim.), and the Taiwanese *H. longifolia* Hayata. The remaining nominal taxa in the section constitute the *H. aspera* species complex. This excludes *H. integrifolia* Hayata and *H. integra* Hayata, which Rehder (1911) included in his *H*. subsect. *Asperae*, but were later relegated to the current section Cornidia. This move is supported by both morphological (stellate pubescence, involucral bracts in young inflorescences) and molecular (Samain et al., 2010) evidence. Interpretation of morphological variation found in the *H. aspera* species complex accounts for most of the discrepancies in species numbers between revisions of the section (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, species delineation 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) studied both molecular and morphological variation among and within putative species. Diagnostic value was found in the abaxial leaf indumentum, where different types of trichomes were objectively documented using scanning electron microscopy (SEM). Based on eight molecular markers, the same study found support for eight genetic lineages within section *Asperae*. These lineages were later corroborated in different analyses based on RADseq markers acquired for the same dataset (De Smet et al., submitted; chapter 4). Integrating all these lines of evidence for species recognition within the framework of the general lineage concept of species (de Queiroz, 1999), showed that one genetic lineage could be split up into two recently diverged, or interbreeding species (*H. longipes* Franch. and *H. sargentiana*), while another contained a spectrum of morphotypes connecting two previously recognized taxa (*H. aspera* and *H. robusta* Hook. f. & Thomson). Therefore, previous studies proposed to recognize nine independently evolving lineages (in addition to *H. platyarguta*) within *H*. section *Asperae*. These coincide with the nominal nominal taxa *H. involucrata*, *H. sikokiana*, *H. longifolia*, *H. villosa*, *H. kawakamii* Hayata, *H. sargentiana*, *H. longipes*, *H. strigosa* Rehder (only the populations based in Hubei, China), *H. aspera*.

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.

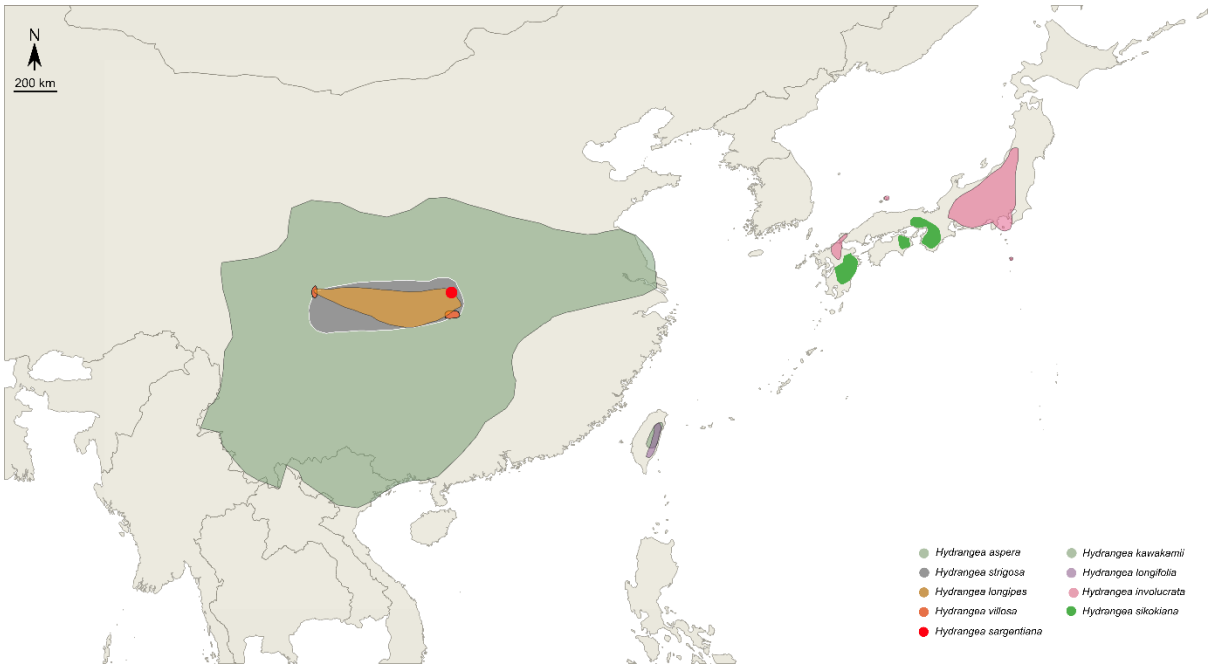

Figure 5.1: Distribution of *Hydrangea* sect. *Asperae* in Mainland China, Japan and Taiwan. Distribution of the specimens used in this study and previous chapters. Personal collections, herbarium material and material grown in garden (wild collected) are included. Specimens labeled as *H. aspera* collected in Nepal and India are not depicted. Figure generated using QGIS 3.12.1. Orientation and scale indicated in top left corner.

Materials and methods

Morphological variability of published taxa was studied in herbarium specimens (including type material) on loan from herbaria (AAU, CAS, E, G, GB, GENT, K, MICH, S, US, WU) (abbreviations according to Thiers, 2016), 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 5 (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 planned and executed. Collection trips were made in the Chinese provinces of Sichuan and Hubei, the island of Taiwan and Japan, as these were identified as hotspots for *H.* sect. *Asperae* diversity. During fieldwork, each collection consisted of fresh leaves collected in silica gel, herbarium specimens of leaves and inflorescences (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 5 (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, with 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 in the study of morphological variability of 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 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 (*Hydrangea involucrata*, *H. sikokiana, H. villosa*). On the other hand, when identified lineages concur with the descriptions in Wei & Bartholomew (2001) these descriptions were adopted (*H. aspera*, *H. strigosa*, *H. kawakamii*, *H. sargentiana*), or expanded on when not all characteristics were sufficiently described (*H. longipes*, *H. longifolia*).

For each of the species described, a short summary of the conservation status is given. These descriptions are based on the assessment of the taxa by IUCN (International Union for Conservation of Nature) Red List (IUCN, 2020), or personal observations when no such assessment was available. All of the assessments made in this chapter have been communicated to IUCN, with the goal of adding several of the species discussed to the Red List.

Representative sequences useful in molecular identification of the species described here are presented when available. As an important note, single sequence species identification represents an oversimplification of the species concept adhered to in this work, as well as the evolutionary reality of the entities delimited here. The representative sequences are therefore provided as a means of rapid identification of the described taxa, not as basis for species delimitation, which should always encompass a wider array of lines of evidence. This is illustrated by the inability to provide single sequence identification for the *Hydrangea aspera* species complex.

Taxonomic treatment of *Hydrangea* section *Asperae*

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

Platycrater Siebold & Zucc., Fl. Jap. 1: 62. 1837-1838.

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.

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

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

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

General morphology. 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.

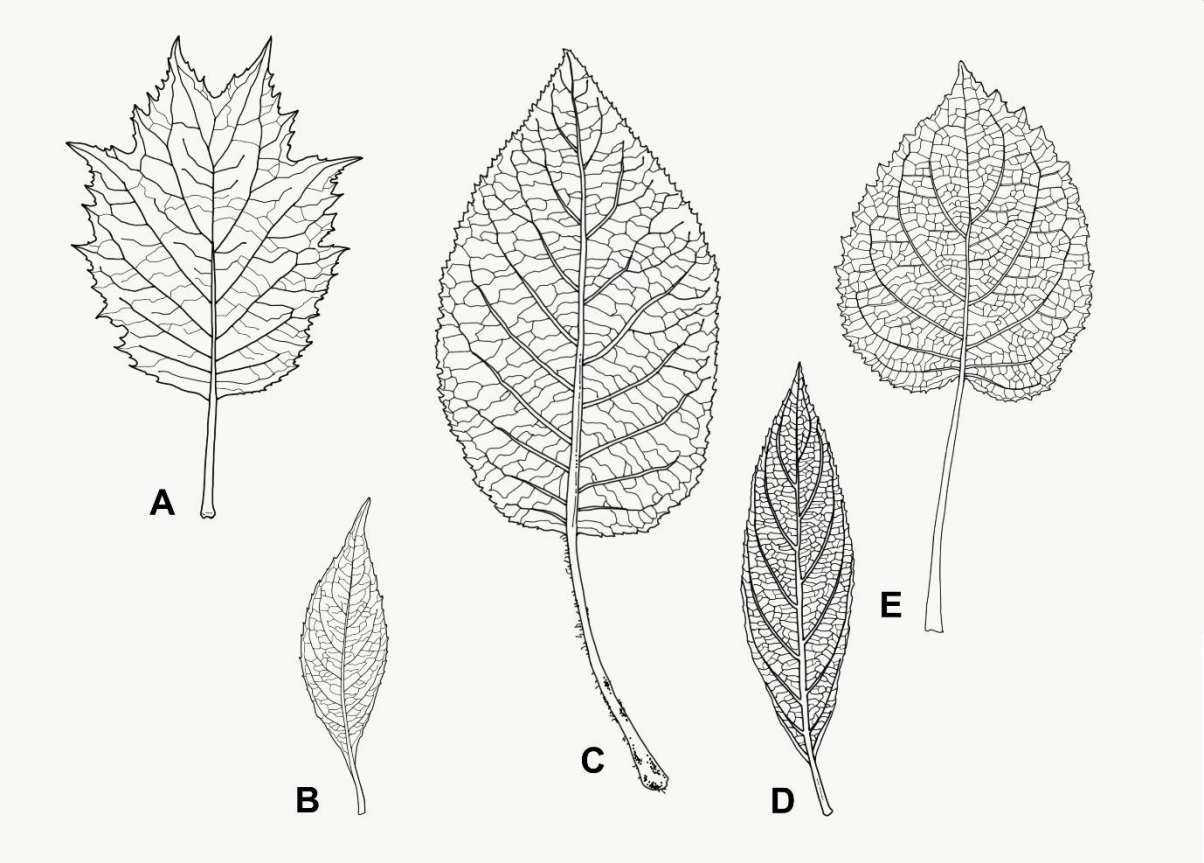

Figure 5.2: Leaf shape diversity in *Hydrangea* sect. *Asperae*. Several distinct leaf shapes exist in the section, with the pinnately lobed leaves of *H. sikokiana* (A) and the slightly decurrent lamina of *H. platyarguta* (B) being easily recognizable. Other taxa, however, can show a continuous variation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

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

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. Fertile flowers few and large, 4- and 5-merous, with sepals well-developed, petals white, stamens numerous, forming a central yellow mass; marginal flowers with 3-4 enlarged sepals completely united into a cup-like structure. .....***H. platyarguta***
2b. Sepals poorly developed, often present as small teeth only; enlarged sepals of marginal flowers not united, not forming a cup-like structure.....3

3a. Immature inflorescence globose, completely enveloped by large and broadly ovate to rounded, pale involucral bracts (Figure 5.3A), long-lasting, and eventually leaving large scars at the base of the inflorescence when fully grown. 4
3b. Immature inflorescence with ovate-lanceolate bracts, not completely enveloping the immature inflorescence, deciduous and leaving no noticeable scars at the base of fully developed inflorescences. 5

4a. Petioles, branchlets and abaxial leaf surface exhibiting appressed, two-branched T-hairs (Figure 5.3B). Leaves lanceolate. (Endemic to Taiwan.) .....***H. longifolia***
4b. Petioles, branchlets and abaxial leaf surface with appressed, simple hairs. Leaves ovate. (Endemic to Japan)..... ***H. involucrata***

5a. Petioles, branchlets and abaxial midveins of leaves covered with thick fleshy hairs (Figure 5.3C) terminating in thinner apex. Hairs greenish translucent with dark brown apex when fresh, brownish yellow in dried specimens.....***H. sargentiana***
5b. Petioles, branchlets and abaxial midveins with simple, non-branching hairs or glabrous. 6

6a. Petioles long and slender (Figure 5.2). Leaf lamina membranous, with tufts of white hairs visible to the naked eye present in the axils of secondary veins on abaxial surface .....
.....***H. longipes***
6b. Petioles always thick, short or long. Leaf lamina papery, no such white tufts present.....7

7a. Abaxial leaf surface covered with appressed hairs and white papillae, granting a whitish aspect to the leaf (Figure 5.3D). In some specimens, the abaxial surface presents a purplish

color when fresh (Figure 5.3E), fading to dull green or brown in herbarium specimens.....
.....***H. strigosa***

7b. No such papillae present, abaxial leaf surface with villous indumentum, hairs erect or appressed. Abaxial leaf surface light to darker green, retaining color or darkening to brown in herbarium specimens..... 8

8a. Petioles with short white to grayish appressed hairs or glabrous. Younger branches terete to noticeably 4-angled. ..... ***H. aspera***

8b. Petioles with erect hairs, either long and conspicuous or short, densely pubescent. Younger branches terete..... 9

9a. 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 elliptical to obovate-lanceolate. Seeds with longitudinal veins only. (Endemic to China).. .....***H. villosa***

9b. Petioles and peduncles densely short pubescent, erect hairs yellowish to white in fresh specimens, darkening in herbarium specimens. Branchlets glabrescent, in some specimens with short erect hairs on young branchlets, never with long and villous hairs. Leaves oblong-ovate to elliptical. Seeds with smaller, transverse veins between longitudinal veins. (Endemic to Taiwan). .....***H. kawakamii***

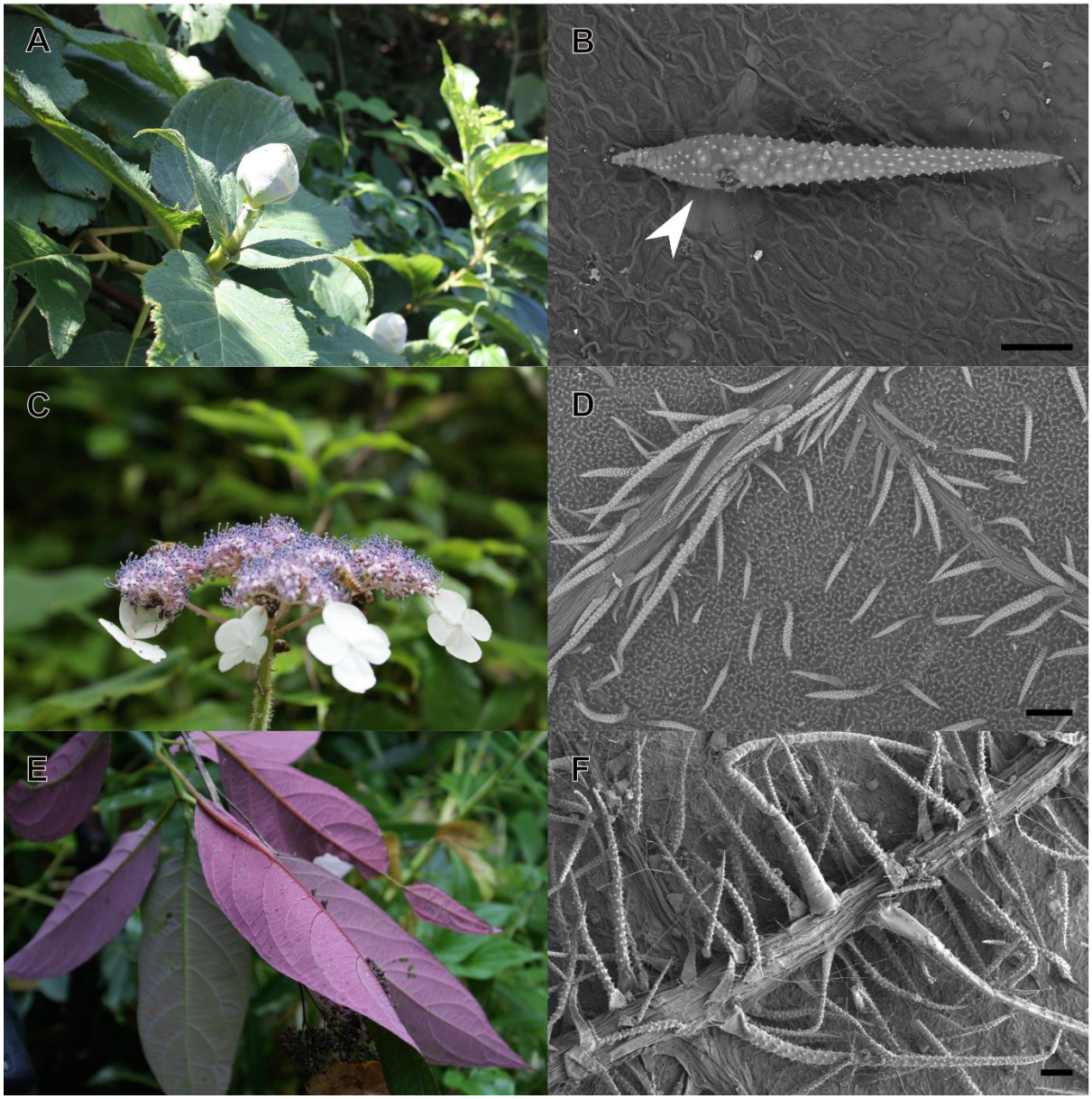

Figure 5.3: Diagnostic characters for several taxa of *Hydrangea* sect. *Asperae*. A: Young inflorescence of *H. involucrata*, showing the involucral bracts enveloping the young inflorescence. B: Scanning Electron Micrograph of branching hairs on the abaxial leaf surface of *H. longifolia*. White arrow indicates the position of the connection between leaf surface and hair. Both branches are parallel to the leaf surface, with one considerably shorter than the other. 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 color 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. Scale bars in the Scanning Electron Micrographs represent 100 μm.

Taxonomic treatment of the species in *Hydrangea* sect. *Asperae*

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

Type information. Tanaka 475 collected in Japan, Honshu Island, Wakayama and Mie prefectures (former Kii province) (LE).

Synonyms. Platycrater sikokiana (Maxim.) H. Ohba & S. Akiyama.

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

Representative sequences. LT838927, LT838928. ITS sequences available in EMBL (European Molecular Biology Laboratory) nucleotide sequence database.

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. 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. Styles 2.1-1.5 mm long. Capsule apex truncate, 2-3 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 in the section 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).

Conservation status. Not listed on the IUCN Red List. Populations assessed during field work were not observed to experience any major threats and no significant future threats have been identified. This species is however endemic to a limited area of Japan.

2. ***Hydrangea involucrata*** Siebold. Nova Acta Physico-Medica Academiae Caesareae Leopoldino-Carolinae Naturae Curiosorum 14(2): 691. 1829.

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

Synonyms. *Platycrater involucrata* (Siebold) H. Ohba & S. Akiyama.

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

Representative sequences. LT838924, LT838925, LT838926. ITS sequences available in EMBL nucleotide sequence database.

Morphological description. Shrubs small, 1-2 m high. Branchlets, peduncles and petioles covered in appressed, simple 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. Inflorescences corymbose cymes, 10-15 cm 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. Styles 2 or 3, 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 striately 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 are an important character for differentiating the inflorescences from those present in the *H. aspera* species complex. *Hydrangea involucrata* 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* Hayashi), but none of the analyses in this work have been able to corroborate this distinction. 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).

Conservation status. Not listed on the IUCN Red List. Populations assessed during field work were not observed to experience any major threats and no significant future threats have been identified. Widely distributed throughout Japan.

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.

Representative sequences. LT838922, LT838923. ITS sequences available in EMBL nucleotide sequence database.

Description. Shrubs 1-3 m high. Branchlets, petioles, leaf blades, and inflorescences densely covered with appressed, simple and 2-branched hairs. The 2-branched hairs with a short and longer branch, both parallel to the leaf surface. 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 striately 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 of 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 differs 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).

Conservation status. Not listed on the IUCN Red List. Populations assessed during field work were not observed to experience any major threats and no significant future threats have been identified. This species is endemic to Taiwan, where certain populations are already protected by their occurrence in nature reserves.

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).

Representative sequences. It is not possible to unequivocally identify this species based on a single molecular marker (Chapter 3 and 4).

Description. Shrubs 2-3 m high. Branchlets, petioles, and peduncles with dense, brownish, semitranslucent, long, apically forked, basally swollen and fleshy 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. 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. All available molecular data show no or very limited genetic divergence from the closely related *H. longipes*. Forming a clade with the latter, sister to the rest of the *H. aspera* complex.

Operational criteria. No genetic support was recovered for separation from *H. longipes*. However, divergent morphology and geographical distribution are sufficient to recognize both taxa at the species level.

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). All available molecular data show no or very little divergence between *Hydrangea sargentiana* and the morphologically very different *H. longipes*. The latter lacks the distinct fleshy trichomes, has erect hairs forming visible white tufts in the axils of secondary leaf veins (abaxial surface), and much thinner petioles and peduncles. Furthermore, *H. sargentiana* has a very limited geographical distribution, with the only known population located in the Chinese province of Hubei, Xingshan Xian. 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. aspera*, lacking the characteristic fleshy trichomes (e.g. the specimen labeled *H. sargentiana*, collected by Kirkham, Flanagan, Howick & McNamara as SICH1801, see Appendix 5 (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 (Appendix 5, Box S5.1) 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).

Conservation status. Not yet listed on the IUCN Red List, data will be submitted in order to list this species. Only a single population known, occurring in a protected nature reserve. The

rarity and population size for this species make it necessary to implement extra conservation efforts, starting with listing on the IUCN Red List.

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 (holotype & isotype: 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.

Representative sequences. It is not possible to unequivocally identify this species based on a single molecular marker (Chapter 3 and 4).

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 with long erect hairs. 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. 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 striately veined.

Relationships. All available molecular data show no or very little genetic divergence from the closely related *H. sargentiana*. Forming a clade with the latter, sister to the rest of the *H. aspera* complex.

Operational criteria. No genetic support was recovered for separation from *H. sargentiana*. However, divergent morphology and geographical distribution are sufficient to recognize both taxa at the species level.

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*. These tufts consist of large amounts of erect villous hairs. As depicted in chapters 3 and 4 (De Smet et al., 2017), this species is genetically highly similar to *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 and parts of Sichuan, while only a single population of *H. sargentiana* is known) represent enough evidence to consider both morphotypes as independent evolutionary lines (De Smet et al., 2017; chapters 3 and 4). The name *H. longipes* was described independently by Franchet (1885) and Hemsley (1887). However, no morphological distinction between the type specimens for both nominal taxa could warrant the recognition of two species. The small differences in leaf shape and pubescence quoted by the authors 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 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).

Conservation status. Not listed on the IUCN Red List. Populations assessed during field work were not observed to experience any major threats and no significant future threats have been identified. Large populations and moderate to wide geographical distribution.

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).

Representative sequences. It is not possible to unequivocally identify this species based on a single molecular marker (Chapter 3 and 4).

Description. Shrubs 1-3m high. Branchlets, petioles, leaf blades and peduncles covered with 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 and longer, 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 striately veined.

Relationships. None of the phylogenetic studies focusing on *H.* sect. *Asperae* were able to 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* 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 brownish 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).

Conservation status. Not listed on the IUCN Red List. Populations assessed during field work were not observed to experience any major threats and no significant future threats have been identified. Large populations, geographical distribution rather limited. More research on occurrence of this species is needed to assess possible threats to its conservation.

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).

Representative sequences. It is not possible to unequivocally identify this species based on a single molecular marker (Chapter 3 and 4).

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 long erect hairs and small inconspicuous papillae, 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 striately 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 (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.

Conservation status. Not listed on the IUCN Red List. Populations assessed during field work were not observed to experience any major threats and no significant future threats have been identified. This species is however endemic to a limited area of Taiwan.

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. (holotype: A!, isotypes 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).

Representative sequences. It is not possible to unequivocally identify this species based on a single molecular marker (Chapter 3 and 4).

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, 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 striately veined.

Relationships. The Hubei lineage, which is linked to the type location and connected to 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*.

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*, 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, molecular-based species delimitation methods fail to distinguish both discrete morphotypes. Furthermore, when occurring in sympatry (as is the case in Sichuan) morphological intermediates between the *H. aspera* and *H. strigosa* morphology occur. Interestingly

morphology of specimens varies along an altitudinal gradient. Specimens referable to the type of *H. strigosa* occur at lower altitudes (500-1200 m), while those referable to the type of *H. aspera* grow at higher altitudes (900-2800 m). Populations of morphotypes clearly ascribable to each of these nominal taxa occur, nevertheless a plethora of intermediate forms have been observed. 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).

Conservation status. Not listed on the IUCN Red List. Populations assessed during field work were not observed to experience any major threats and no significant future threats have been identified. Large populations with wide distribution.

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).

Representative sequences. It is not possible to unequivocally identify this species based on a single molecular marker (Chapter 3 and 4).

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. Styles 2 or 3, spreading to recurved, 1-2 mm. Capsules with apex truncate, 3-5mm. seeds fusiform, winged at both ends, 0,4-0,5 mm; striately veined.

Relationships. Closely related to *H. strigosa* (Sichuan lineage), part of an unresolved polytomy 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 taxon *H. aspera* represents an intricate species complex. The species as recognized here encompasses several previously recognized taxa. These are not supported as species due to lack of molecular divergence, and morphological diagnosability. Indeed, these

previously published taxa are based on differences in leaf shape and pubescence, a character which is shown to vary along altitudinal range of the species (900-2800 m).

Further population level studies are required in order to elucidate the morphological variation within *H. aspera*. One hypothesis consists of the taxon containing several previously diverged lineages, the boundaries between which have been eroded by heavy gene flow and introgression. Possible support for this can be found in the study by Cerbah et al (2001), where individuals identified as *H. aspera* and *H. robusta* showed differing chromosome numbers (2n =36 and 2n = 34, respectively). Another hypothesis invokes phenotypic plasticity of a single evolutionary lineage, caused by differing conditions at increasing altitude. Lacking a 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).

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)

Conservation status. Listed by ICUN Red List as Least Concern with following justification: This tree species has a very wide distribution, large population, is not currently experiencing any major threats and no significant future threats have been identified.

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

Type information. Lectotype: Siebold (L0104524) collected in Japan (L!).

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

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

Representative sequences. Insufficient molecular data is available for this species in order to present representative sequences.

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 *H*. 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, *H*. sect. *Asperae* should include this morphologically unique species. Further research is required to confirm the exact relationships between *H. platyarguta* and the other taxa contained in the section. This might be complicated by the high level of molecular divergence between the taxa, as illustrated by 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 *H*. 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.

Conservation status. Not listed on the IUCN Red List. The rarity and population size for this species make it necessary to implement extra conservation efforts, starting with listing on the IUCN Red List.

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Chapter 6

General discussion

The overarching goal of this doctoral thesis can be summarized as increasing the evolutionary understanding of the genus *Hydrangea* L. 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* Y. De Smet & Samain 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 DC. 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* Gaudich., *Cardiandra* Siebold & Zucc., *Decumaria* L., *Deinanthe* Maxim., *Dichroa* Lour., *Pileostegia* Hook f. & Thomson, *Platycrater* Siebold & Zucc. and *Schizophragma* Siebold & Zucc.), 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; Platnick, 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 evolutionary position of *H. arborescens* L., the type of 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.

Acceptance of the 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 the proposed *Hydrangea* classification (Lin & Chung, 2017; Sodusta & Lumawag, 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 *Hydrangea* s.s. sections *Stylosae* Y. De Smet & Samain 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 evolutionary uninformative 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* Hook. f. & Thomson (part of *H.* sect. *Stylosae*) would be more closely related to *Hortensia chinensis* (Maxim.) H. Ohba & S. Akiyama (*Hydrangea chinensis* Maxim. in *H.* sect. *Chinenses* in the classification presented in chapter 2) than to *Hydrangea arborescens.* This proposal, however, did find limited support (Huang et al., 2018).

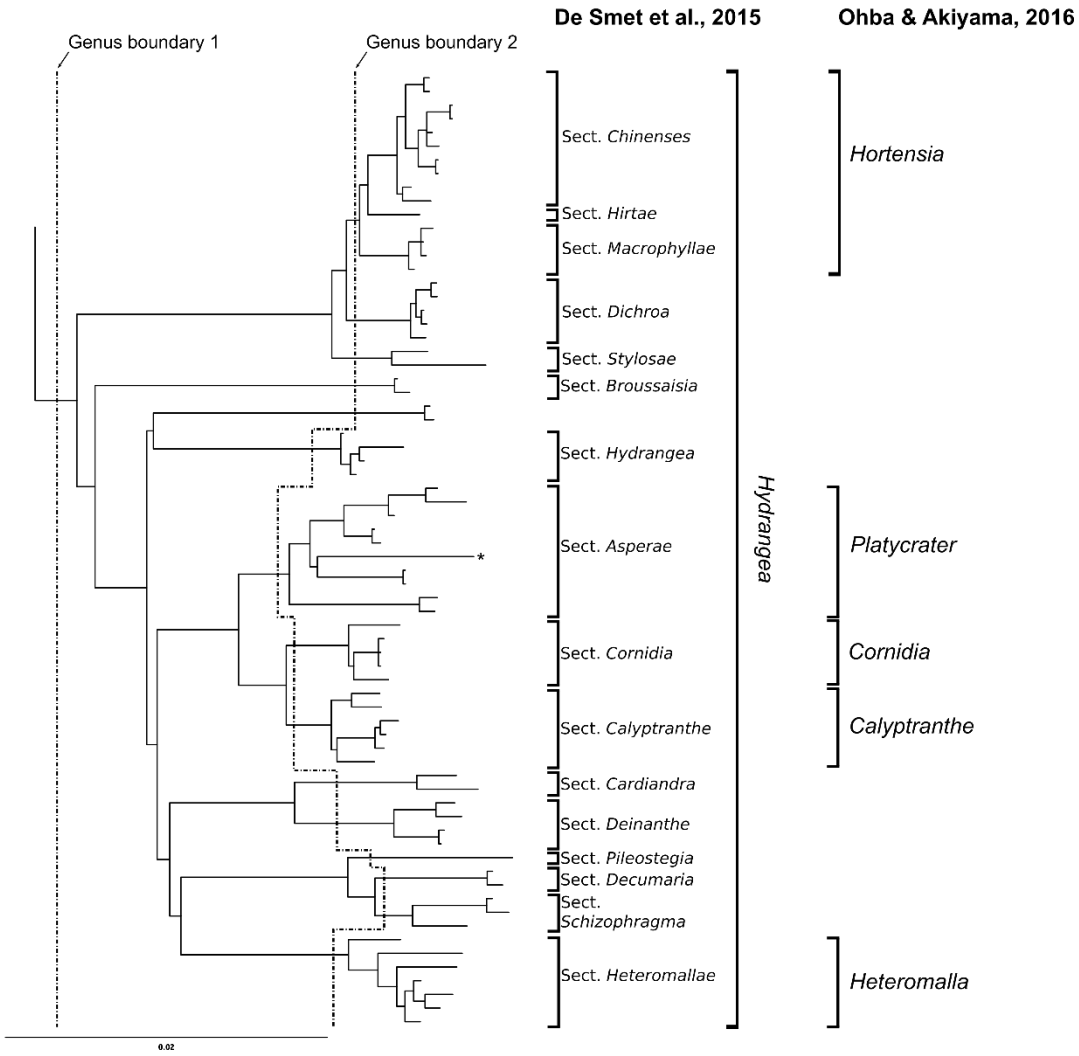

Figure 6.1: Classifications for tribe Hydrangeeae as proposed by De Smet et al. (2015a) and Ohba & Akiyama (2016). Names in the right part of the figure indicate the named taxa in both classifications, compared to the phylogenetic hypothesis inferred by De Smet et al. (2015a). The dotted lines across the phylogenetic tree indicate two different hypotheses regarding the resolution of a polyphyletic *Hydrangea*. Genus boundary 1 coincides with the scheme proposed in De Smet et al. (2015a). Genus boundary 2 represents an alternative way of delimiting monophyletic genera in tribe Hydrangeeae. Some of the genera delimited in this way coincide with those proposed by Ohba & Akiyama (2016), while also recognizing additional monophyletic entities as genera (see for example *H.* sect. *Cardiandra*). The asterisk indicates the position of *H. platyarguta*. The full topology, including support values and tip labels, is presented as Figure 2.3.

Challenges in creating an evolutionary informative classification

Under the current nomenclatural code (Turland et al., 2018; but also in previous versions), authors of new classifications are under no obligation to explicitly define the evolutionary relationships of novel taxa. This allows for the creation of monophyletic, paraphyletic and even polyphyletic taxa at all hierarchical levels of classification (Lebuhn, 2012). As detailed in

the introduction (Chapter 1), a general acceptance of the evolutionary world view has seen a drive towards evolutionary informative classifications and taxa. Within this framework a broad acceptance of exclusively describing monophyletic taxa exists, while polyphyletic taxa are deemed unacceptable in any classification. At higher taxonomic levels, a limited group of authors have made a case for the acceptance of paraphyletic taxa (Sosef, 1997, Hörandl, 2006, Hörandl & Stuessy, 2010), which found little traction in the broader systematic community. In the present work, paraphyletic taxa are not accepted at the supraspecific level, in agreement with the arguments made by Lebuhn (2012). If the aim is to create a classification reflecting evolutionary relationships, the taxa created in the currently available ranked system of classification should be in line with the best available estimates of the tree of life. Furthermore, since the Linnaean system of classification lacks the possibility to specify the nature of each described taxon, adherence to monophyly provides implicit genealogical information in a classification scheme. For example, when all genera in a certain family are monophyletic, this provides insights into the way their constituting taxa relate to each other. With the acceptance of paraphyletic genera, this is no longer the case. Certain members of a paraphyletic rest group will be more closely related to members of the genus nested within the paraphyletic group. Since this information is not explicitly mentioned in Linnaean classifications, both paraphyletic and monophyletic groups will appear side by side, although they represent different evolutionary entities.

During the establishment of a novel classification for tribe Hydrangeeae, the issue of paraphyletic taxa was not encountered. Indeed, the main discrepancy between the traditional classification and inferred phylogenetic hypothesis resided in the polyphyletic nature of *Hydrangea*. Nevertheless, arguments similar to those presented in defense of paraphyletic taxa have been brought up in appeals for generic segregation (Ohba & Akiyama, 2016) of tribe Hydrangeeae (e.g. morphological recognizability, convenience for end-users of taxonomy). Interestingly, no arguments are being made to preserve a polyphyletic *Hydrangea*. Instead, Ohba & Akiyama (2016) base their classification on the phylogenetic hypothesis presented in De Smet et al. (2015a), only explicitly defining monophyletic genera (but see implicit consequences of these segregations for *Hydrangea* in Figure 6.1). In this regard, both rivaling classifications for tribe Hydrangeeae agree in their underlying goal, creating an evolutionary relevant classification, only containing natural entities (monophyletic groups). The rejection

of the generic concept proposed in De Smet et al. (2015a) by Ohba & Akiyama (2016) can be conceptualized as disagreement on which level of nested clades (see doted lines in Figure 6.1) are attributed the genus status.

The abovementioned disagreement illustrated another challenge faced when reconciling the Linnaean classification system containing named hierarchical ranks with phylogenetic hypotheses consisting of nested clades. Rapid increase in availability of phylogenetic hypotheses across the tree of life is resolving a number of nested clades far greater than the number of named ranks available in the Linnaean classification system (Mishler, 2009). Therefore, when it is accepted that named ranks above the species level should represent clades in the tree of life, the assignment of these ranks (e.g. genus or family) to a certain level of nested clades becomes arbitrary and artificial (Figure 6.1). As an important consideration, although the assignment of their rank is artificial, the clades identified through these names will still represent real, natural entities, given that they are monophyletic groups in the tree of life (Mishler, 2010). Applied to taxonomic revisions, subsequent authors studying the same phylogenetic hypothesis could attach the genus level to different levels of nested clades. This is illustrated in Figure 6.1 by the two alternative levels of genus boundaries represented by dotted lines. The resulting genera would represent real entities, the conflict between subsequent revisions firmly lodged in the realm of semantics: the level of nested clades which should be identified as the genus level.

In order to represent a real, evolutionary entity (Mishler, 2010), the current work has opted to attach the genus name *Hydrangea* to a monophyletic clade, encompassing all taxa previously classified in *Hydrangea* s.s., *Broussaisia*, *Cardiandra*, *Decumaria*, *Dichroa*, *Deinanthe*, *Schizophragma*, *Pileostegia* and *Platycrater*. The alternative of splitting this group up into several genera could be considered as equally evolutionary relevant, if all resulting genera represent real entities, i.e. monophyletic groups. This differs from the approach taken by Ohba & Akiyama (2016), where only several of the morphologically identifiable sections are segregated as genera. Nevertheless, the recognition of a larger *Hydrangea*, encompassing all abovementioned morphologically distinct groups presents several advantages over recognizing a plethora of smaller, albeit monophyletic genera. The most important advantage to be gained is related to taxonomic stability. Despite representing the best estimation of

evolutionary relationships within tribe Hydrangeeae, the phylogenetic hypothesis presented in Figure 2.3 exhibits limited support for the position of several taxa. Resolving the affinities of these taxa might influence the monophyly of closely related groups, which is especially important in the case of *H. arborescens*, the type of genus *Hydrangea*. The proposed broader circumscription of *Hydrangea* is robust in the face of such changes. In this way, taxonomy in the group remains stable, which can have an important influence on other fields relying on stable names, such as conservation. A second advantage to the chosen resolution to *Hydrangea* polyphyly is the retention of the name *Hydrangea* for several well-known garden ornamentals. Splitting into smaller monophyletic groups would attach the name *Hydrangea* to the clade containing *H. arborescens*. According to the clades inferred in Figure 2.3, one of the more well-known ornamentals in the group, *H. macrophylla* (Thunb.) Ser., would be relegated to a different genus, since it does not share a clade with the type of *Hydrangea* at the nested clade level linked to the named rank "genus" in the tree.

Another challenge to strict application of monophyletic clades in tribe Hydrangeeae is the abovementioned status of *Hydrangea* and allied taxa as well-known garden ornamentals. The number of end-users of taxonomy (e.g. breeders, horticulturalists, policy makers and enforcers) affected by taxonomic changes made in the name of scientific rigor will be larger in such popular groups, compared to groups with limited use. End-users could prefer non-phylogenetic classification systems, potentially focusing on pollination syndrome, morphological recognizability or breeding system. This dichotomy in the goals for classifications have led even the fiercest proponents of strictly phylogenetic systems (see for example concluding remarks in Lebuhn, 2012) to assert the inevitability of multiple systems persisting in parallel.

This can be illustrated in tribe Hydrangeeae. Despite the wide acceptance of the need to resolve polyphyletic *Hydrangea*, none of the possible solutions within a strict phylogenetic classification will lead to the retention of all well-known names. Splitting *Hydrangea* will see the need to create several new genera, resulting in arguably the most famous ornamental *H. macrophylla* no longer residing within the genus *Hydrangea*. Alternatively, merging all genera in the tribe into a larger *Hydrangea* loses the easily recognizable names of the morphologically diverse satellite genera at the genus level. By this, another, related challenge is pinpointed. In

the current classification system, the genus name is part of the species binomial. This particular property of Linnaean classifications potentially complicates acceptance of nomenclatural changes by end users at the genus level. The genus name is part of the "handle" used to refer to species in different disciplines, spanning from horticulture over conservation policy to population ecology. Relegating recognizable names which are no longer deemed suitable at the genus level to another rank of classification, as was attempted through section names in tribe Hydrangeeae, does not seem a viable strategy to promote acceptance because of adherence to binomials. Even though the entity represented by this handle (the species) will not change when genera are brought in line with evolutionary relationships, acceptance remains difficult.

Given the observed challenges, the current nomenclatural codes seem ill-equipped to accommodate a strict adherence to monophyletic taxa. Proposals have been made to adapt nomenclatural codes to name phylogenetic taxa (Barkley et al., 2004). Alternatively, completely new, rank-free systems of classifications have been proposed (e.g. PhyloCode; de Queiroz & Gauthier, 1990, 1992) based entirely on phylogenetic hypotheses. Although this novel approach has merit as being rooted in a rigorous evolutionary worldview, its acceptance might deepen the divide between evolutionary systematics and end-users of taxonomy (but see Mishler, 2010).

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

Owing to different interpretations of the taxonomic relevance for several morphological 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 could be ascribed to the lack of alternative lines of evidence, as well as the absence of reference to the species concept adhered to. Explicit adherence to a species concept can provide information on what data was used to infer species boundaries in a group. Alternatively, not defining the species concept, uncertainty remains regarding: the biological properties of the entity delimited, the level of divergence and type of data necessary to describe new species related to those already described and the objectivity of the boundaries inferred.

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.

Table 6.1: species recognized within *Hydrangea* section *Asperae* by different revisions. Both authors of the Flora of China (FOC) (Wei & Bartholomew, 2001), 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) (2001)	FOC (Bartholomew) (2001)	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

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). Genetically diverged lineages in the focal section were identified using different methods. In the first study (De Smet et al., 2017; chapter 3), four chloroplast (*trnV-ndhC* IGS, *rpl32-ndhF* IGS, *trnL-rpl32* IGS and *ndhA* intron) and four nuclear regions (*TIF3H1*, *SMC1*-44, *SMC1*-22, ITS) were used to identify divergent lineages by reciprocal monophyly and coalescent Bayesian species delimitation (Yang & Rannala, 2010). The eight lineages identified by the latter method chiefly coincided with eleven nominal taxa described in *H.* sect. *Asperae*. Two nominal taxa (*H. sargentiana* Rehder and *H. longipes* Franch.) were lumped in one genetic lineage, while another (*H. strigosa* Rehder) was split into two lineages, one of which contained two other nominal taxa (*H. aspera* D. Don and *H. robusta* Hook. f. & Thomson). In order to assess the validity of these genetic lineages, additional molecular markers (RADseq markers) were acquired for a highly congruent dataset. Applying coalescent Bayesian species delimitation, these new data supported the same eight genetic

lineages, increasing confidence in their inference (Figure 4.7). Similar results were obtained from analyzing RADseq SNP data for reciprocal monophyly and population structure (STRUCTURE, Pritchard et al., 2000).

Through combining the various molecular data-based species delimitations with morphological observations, geographical distribution and previously published data (e.g. Cerbah et al., 2001), well-supported species boundaries could be hypothesized within *H.* sect. *Asperae*. In a taxonomic treatment of the section (chapter 5), supporting evidence for each recognized species is explicitly outlined, along with the rationale to split or lump previously published nominal taxa. By providing this type of data with each species delineation, future research can start from an objective basis from which to describe new species in the group. Furthermore, in order to alter the species boundaries in *H*. sect *Asperae*, evidence will be needed to refute decisions proposed here, increasing taxonomic stability in the section. The following paragraphs provide a condensed description of the evolutionary lineages recognized in *H*. sect. *Asperae*, and how different types of data corroborate them.

Receiving support as an independently evolving lineage from all tested operational criteria are the Japanese taxa *H. involucrata* Siebold and *H. sikokiana* Maxim., as well as the Taiwanese *H. longifolia* Hayata. 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. In addition, each of these species is morphologically distinct from each other recognized taxon in the section.

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 evolutionary lineages. 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 morphological variation within the genus, as it is currently known from a single, relatively small population (De Smet et al., 2015b; Appendix 5, box S5.1).

Nominal taxa *Hydrangea villosa* Rehder and *H. kawakamii* Hayata 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 *Hydrangea aspera*, *H. robusta* and *H. strigosa*, available genetic and morphological data provide conflicting insights. A genetic lineage containing morphotypes attributable to all three nominal taxa is recovered in both molecular studies. Nevertheless, a genetically distinct lineage containing only specimens with the *H. strigosa* morphotype (collected in Hubei, China) is also recovered in both studies. Combining the available data led to the hypothesis that *H. strigosa* constitutes a separate species when occurring in allopatry (such as in Hubei) from other closely related species. However, when occurring in sympatry (as is the case in Sichuan) it intermixes with the closely related *H. aspera* (which contains the related *H. robusta*). The latter is evidenced by the presence of specimens exhibiting an intermediate morphology, as well as shared molecular variation (Figure 4.6). The species *H. aspera* on the other hand, remains in need of further study, as it encompasses two different chromosome numbers (Cerbah et al., 2001). Part of the issues remaining with the interpretation of the *H. aspera* – *H. robusta* – *H. strigosa* species complex, as it occurs in Sichuan stem from challenges related to reconciling modern taxonomic insights with traditional taxonomy, as discussed further in the next paragraph.

Challenges in reconciliating traditional taxonomy 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, integrating these new techniques into species delimitation, and their formal description according to traditional Linnaean binomial nomenclature has been problematic. During the

current study, several of these areas of conflict were encountered, as outlined in the following paragraphs.

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. Leaché & Fujita, 2010; Bauer et al., 2011; Fujita & Leaché, 2011), since morphology-based diagnoses are still required by all 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 contains morphotypes attributable to *H. aspera*, and is hypothesized to be the result of gene flow between closely related taxa. The taxonomic treatment of *H.* sect. *Asperae* provided in this work attempts to alleviate this conflict, by describing both morphological and molecular divergence between all recognized taxa.

A second conflict relevant to the present study is related to assigning sampled individuals to nominal taxa within the Linnaean nomenclatural framework. Since published names are 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. As a consequence, the formally published binomial which needs to be applied to the lineage remains uncertain to a degree. Conversely, if the goal is to evaluate the validity of a given nominal taxon using molecular data, specimens need to be obtained pertaining to this taxon with a high degree of certainty. This certainty is dependent on morphological divergence in the putative species under study. In *H.* sect. *Asperae*, several morphospecies 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, and after studying the type specimen, none of the collected specimens could be appointed to this nominal taxon. Furthermore, herbarium specimens identified as *H. coacta* C.F. Wei did not differ sufficiently or consistently with specimens referable to *H. villosa* or *H. aspera*. 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. aspera* is based primarily on morphological similarity.

The abovementioned issues associated with linking genetic lineages to published names could be alleviated by the availability of type specimen sequence data for systematic research, as is sometimes the case in fungi (e.g. De Crop et al., 2017). In plant systematics, DNA extraction from older, dried specimens can be challenging, rendering availability of type specimen sequences a utopia more often than not. 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. For other taxa, it was possible to collect fresh material at or close to type locations, strengthening the link between the acquired sequence data and the formally published names. 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* Rehder, which is located in a region which is currently inaccessible. 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* species complex exemplifies the difficulties associated with acquiring sequence data reliably linked to nominal taxa, and possible repercussions for species delimitation. It

was possible to collect *H. strigosa* specimens in two regions, one of which contains the type location. At the other location however, *H. strigosa* occurs in sympatry with *H. aspera*, a closely related species with which it potentially hybridizes. For *H. aspera*, specimens complying morphologically with the descriptions and type specimens were gathered only at the location where it is sympatric with *H. strigosa*. The same is true for *H. robusta*, a species now synonymized with *H. aspera*. Collecting at the type location for both *H. aspera* and *H. robusta* was beyond the scope of the current PhD. It is therefore impossible to rule out whether *H. aspera* and *H. robusta* form independent lineages when occurring in allopatry.

Another possible complicating factor in species delimitation research for ornamental plants lies in their artificial selection and crossing. This illustrates the importance of acquiring plant material from natural populations, preferably independent from such artificially selected entities. If this is not possible, garden accessions which are believed to not be the result of artificial crossing could be used (Uemachi et al., 2014), with the necessary reservations. For *H.* sect. *Asperae*, the material used in this work was mainly collected at sites sufficiently isolated from breeding operations. For *H. macrophylla*, one of the most popular ornamentals in the group, this could nevertheless proof problematic. Indeed, the mop-head morphotype, with inflorescences predominately exhibiting the showy, enlarged flowers, have been seen to escape from gardens and nurseries. These often artificially selected or even crossbred (Reed et al., 2001; Uemachi et al., 2014; Alexander, 2017) individuals can spread their genetic material throughout natural populations, confounding speciation research.

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. The success of future studies attempting to increase support and resolution at this level will depend on the availability of suitable 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, different variants of RAD sequencing markers have been successfully adopted to resolve phylogenetic relationships in taxa where Sanger based 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 potentially be informative on the genus-scale, if the primers were adapted. In addition, molecular markers providing resolution across the tribe Hydrangeeae could contribute to the remaining uncertainties regarding evolutionary relationships within *H*. sect. *Asperae*. In Chapter 3 and 4, *H. platyarguta* could not be included in the phylogenetic analyses. Custom primers designed specifically for the section could not amplify the targeted markers in this species, possibly due to its divergent nature (see long branch in Figure 6.1). However, markers with a wider applicability across the tribe could recover information regarding the position of *H. platyarguta* within *H*. section *Asperae*.

Combining different types of data for species delimitation in *H.* sect. *Asperae* has uncovered an interesting conflict between morphological and genetic divergence in the *H. sargentiana* – *H. longipes* species pair. The limited genetic distance between *H. sargentiana* and *H. longipes* provides a stark contrast with their morphology, especially since this divergence is upheld in

sympatry. Future studies could address the biological mechanism by which these two taxa reached the seemingly contradictory state of morphological divergence while remaining genetically close. A possible hypothesis is divergence, with rapid deviation in morphology, potentially coupled with introgression between incipient species. Analogous studies in *Anopheles* Meigen mosquitos (Fontaine et al., 2015) and *Heliconius* Kluk. butterflies (Edelman et al., 2019), have shown introgression following rapid species radiation to overwrite genetic signatures of the original bifurcation event. However, if divergent selection is still acting upon several loci in each of the diverging lineages, these loci and closely linked genomic regions will remain indicative of the lineages' divergence. This situation has been documented in the genus *Littorina* Férussac (Wilding et al., 2001), and has been studied extensively in *Heliconius* butterflies (e.g. Beltrán et al., 2002; Nadeau et al., 2012). Identifying these islands of divergence in hybridizing taxa such as *H. sargentiana* and *H. longipes* can greatly contribute to the understanding of potential selection pressures at work to maintain distinct taxa (or morphotypes) despite ongoing gene flow. A similar research line could be envisioned for the morphologically variable *H. aspera*. Molecular data provided in this work shows potential introgression when *H. aspera* occurs in sympatry with *H. strigosa.* Genetic characterization at the population level of the continuous morphological variation along altitudinal clines in this taxon is necessary to address this potential species complex. However, the first step towards better understanding of this species would be to sample its type location, and compare the genetic material acquired there with the samples available from China.

Addressing the species complexes centered around *Hydrangea aspera* and *H. sargentiana – H. longipes* would require suitable molecular markers. Parallel to the remaining issues at the tribe-level, these research questions could be addressed using one of the available variations on RADseq. Similar attempts in the *H. macrophylla - H. serrata* complex provided sufficient resolution at the population-level (Wu & Alexander, 2019). However, given the issues encountered using this methodology at and below species level (Chapter 4), targeted sequence capture (reviewed in: Lemmon & Lemmon, 2013) could be more promising. Advantages over RADseq variations include higher rate of success in low quality samples, more straightforward orthology assessment and higher per locus information content (reviewed in: Harvey et al., 2016). Since both orthology assessment and unequal coverage provided issues in *H.* sect. *Asperae*, sequence capture could provide a more informative dataset. At the level of

interest in this study group, the Hyb-Seq (Weitemier et al., 2014) method, combining targeted sequence capture, high-throughput sequencing and genome skimming has shown promising results for other plant groups (Dodsworth et al., 2019; but see Forrest et al., 2019). Since this method was capable of resolving rapid recent radiations in the neotropical *Burmeistera* H. Karst. & triana complex (Bagley et al., 2020), it holds potential for the resolution of the species complexes in *H.* sect. *Asperae*. This method would however require access to transcriptomes or genomic data for the section, or closely related taxa. These genomic resources could be mined for known single-copy nuclear genes previously identified in other angiosperm genomes (De Smet et al., 2013). In addition to species delimitation, Hyb-Seq has been shown to be applicable in resolving phylogenetic relationships where traditional markers fail, based on sets of single copy nuclear markers. A pilot study in the New World sages (*Salvia* subgenus *Calosphace* Benth. (Epling)) found increased resolution and support across their phylogenetic hypothesis compared to previous studies (Fragoso-Martínez et al., 2017). Similar approaches could be applied to tribe Hydrangeeae, in order to resolve the evolutionary placement of *H. arborescens* and *Broussaisia*.

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 sampling of taxa classified in the Hydrangeaceae tribe Hydrangeeae was 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 align Hydrangeeae systematics 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 *Hydrangea* 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* Y. De Smet & C. Granados. 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

Several representatives of family Hydrangeaceae are well-known for being popular garden ornamentals. Perhaps foremost among these is *Hydrangea*, a genus in which the Research Group Spermatophytes, Ghent University, accrued valuable experience over the course of several studies. Through this earlier work, it became apparent that the group could provide interesting case studies regarding delimitation of both genera and species. The pursuit of these research lines became the basis for the current Doctoral Thesis.

The Cornales family Hydrangeaceae contains two tribes, Philadelpheae and Hydrangeeae. The latter consists of the genus *Hydrangea* 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, 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* sect. *Hydrangea* and *Hydrangea* sect. *Cornidia*, while this bifurcation is not supported by any of the available phylogenetic evidence. Furthermore, evolutionary cohesion for several subsections (*H.* subsect. *Asperae*, *H.* subsect. *Americanae* and *H.* 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 was 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, leaf indumentum, 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

Verschillende vertegenwoordigers van de familie Hydrangeaceae genieten bekendheid omwille van hun populariteit als sierplant. Misschien wel de bekendste is *Hydrangea*, een genus waarvoor de Onderzoeksgroep Zaadplanten, Universiteit Gent, een aanzienlijke ervaring uitbouwde doorheen verschillende studies. Door dit eerdere werk werd het duidelijk dat de groep een aantal interessante case studies aangaande afbakening van genera en soorten omvatte. Het nastreven van deze onderzoekslijnen vormde de basis voor de huidige doctoraatsthesis.

De familie Hydrangeaceae omvat twee tribus: Philadelpheae en Hydrangeeae. Deze laatste bestaat uit het genus *Hydrangea* 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 (*H.* subsect. *Asperae*, *H.* subsect. *Americanae* en *H.* 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 werd 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 op 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 *Hydrangea* 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: general supplementary material

Table S1.1: Abbreviations for genomic regions used throughout the thesis.

Abbreviation	Full name
accD	Acetyl-CoA carboxylase carboxyl transferase subunit beta
ITS	Internal transcribed spacer
matK	Maturase K
ndhA	NADH dehydrogenase ND1
ndhC	NADH dehydrogenase D3 subunit of the chloroplast NAD(P)H
ndhF	Chloroplast encoded NADH dehydrogenase unit
psaI	Photosystem I subunit I
psbA	Photosystem II reaction center protein A
rbcL	Ribulose bisphosphate carboxylase large chain
rpl32	Ribosomal protein L32
rps16	Ribosomal protein S16
SMC1	Structural maintenance of chromosomes 1
TIF3H1	Translation initiation factor 3 subunit H1
trnH	tRNA-His
trnK	tRNA-Lys
trnL	tRNA-Leu
trnV	tRNA-Val

Appendix 2: 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 validly published 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*
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*
Hydrangea	*obtusifolia*	(Hu) Y. De Smet & Granados	*Decumaria*

Hydrangea	*oerstedii*	Briquet	*Cornidia*
Hydrangea	*ofeliae*	Sodusta & Lumawag	*Dichroa*
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*

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	*tiliifolia*	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.

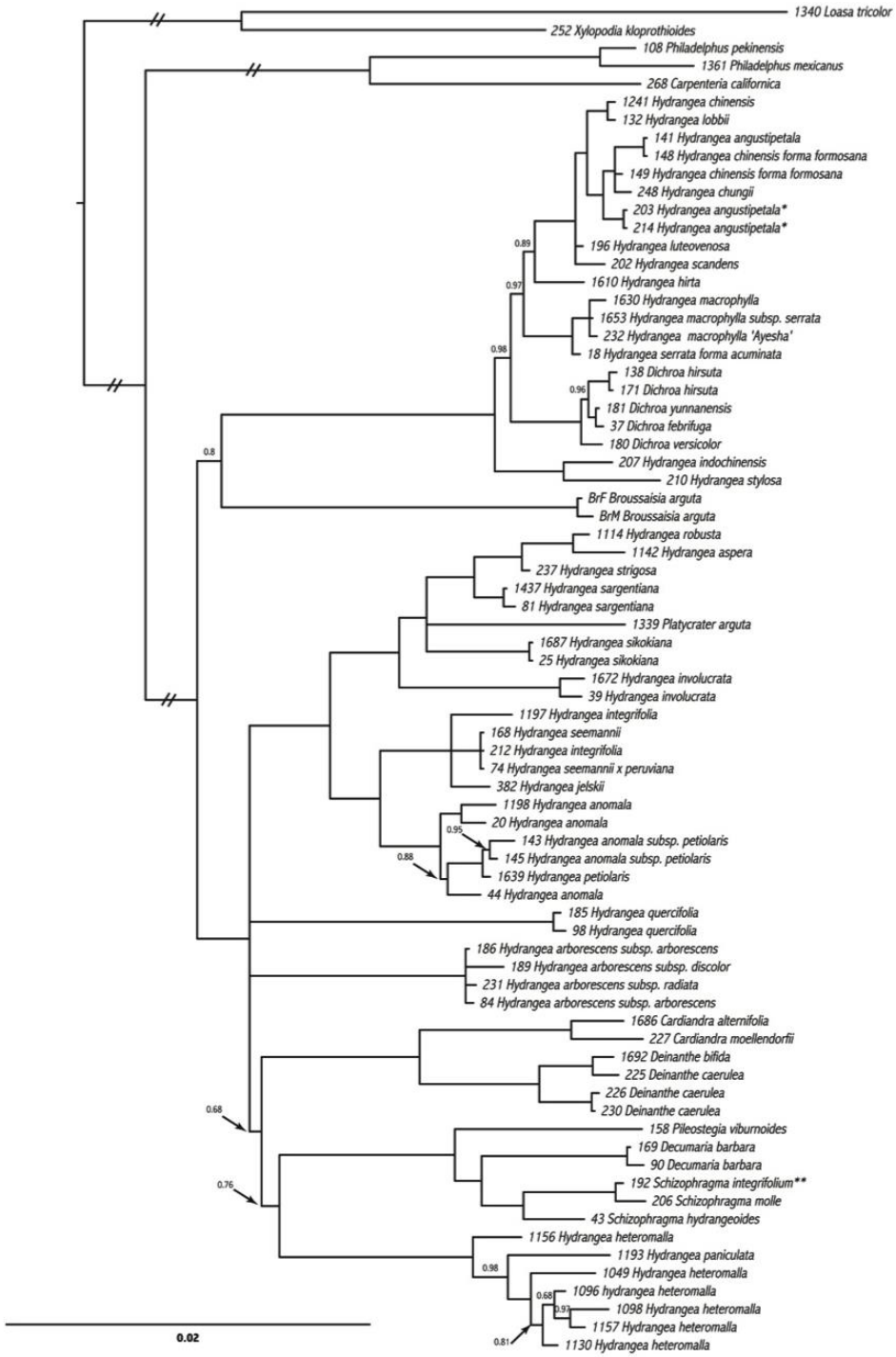


Figure S2.2: Single gene trees based on the analysis containing coded indels.

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.

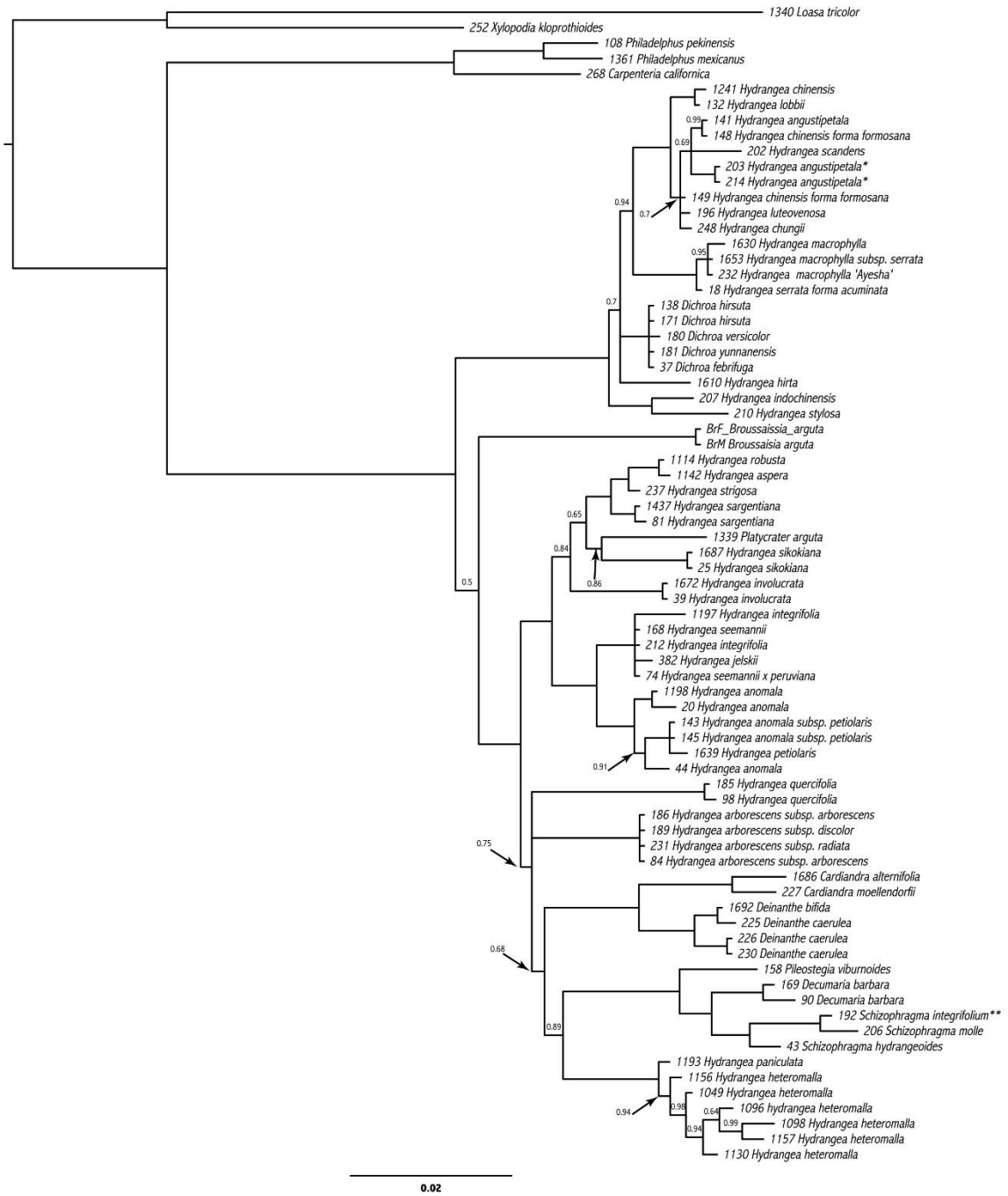


Figure S2.2B: The 50% majority rule consensus tree based on the *trnV-ndhC* IGS with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. No supported topological differences with the phylogenetic tree based on only nucleotide data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

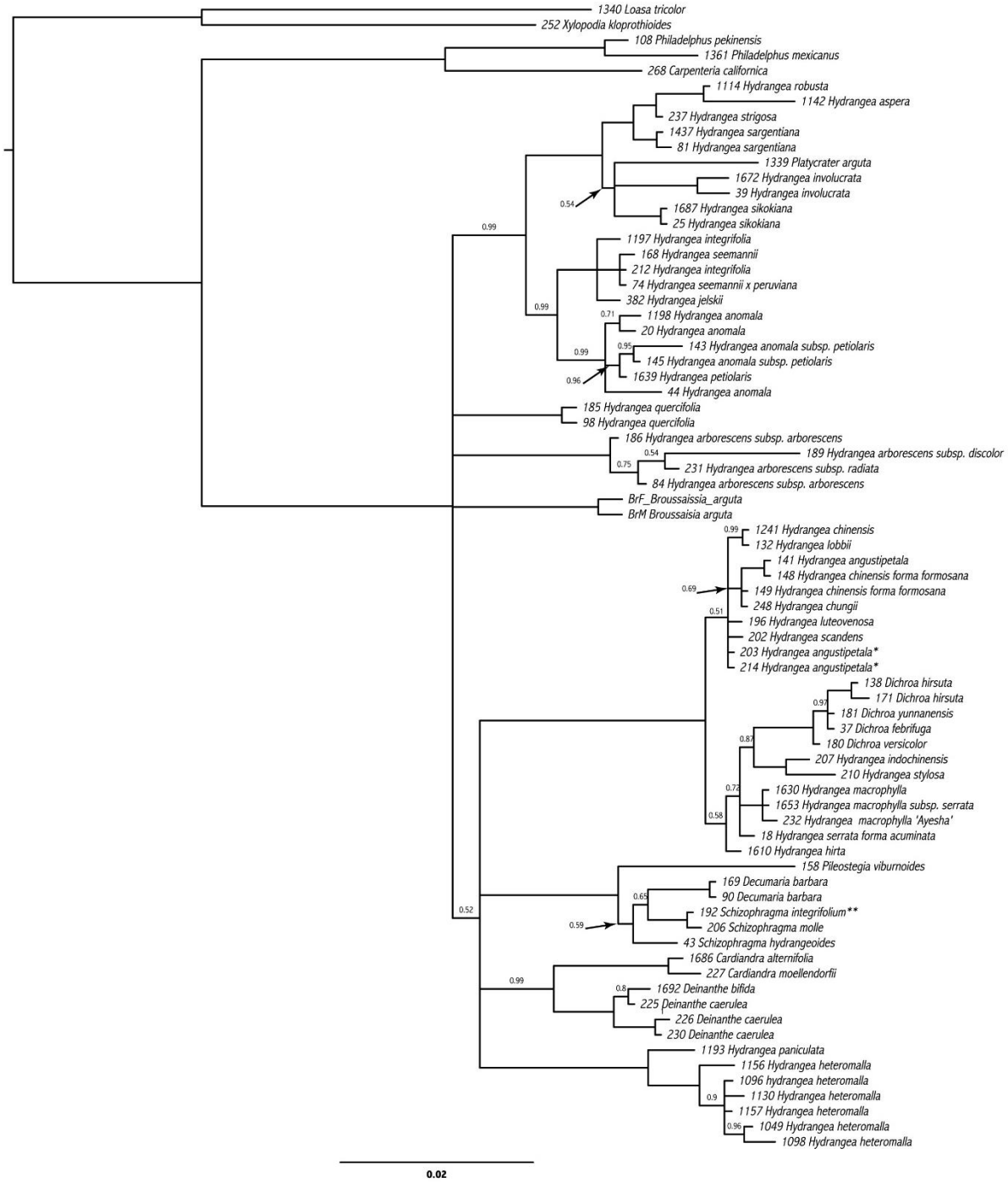


Figure S2.2C: The 50% majority rule consensus tree based on the *trnL-rpl32* IGS with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. No supported topological differences with the phylogenetic tree based on only nucleotide data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

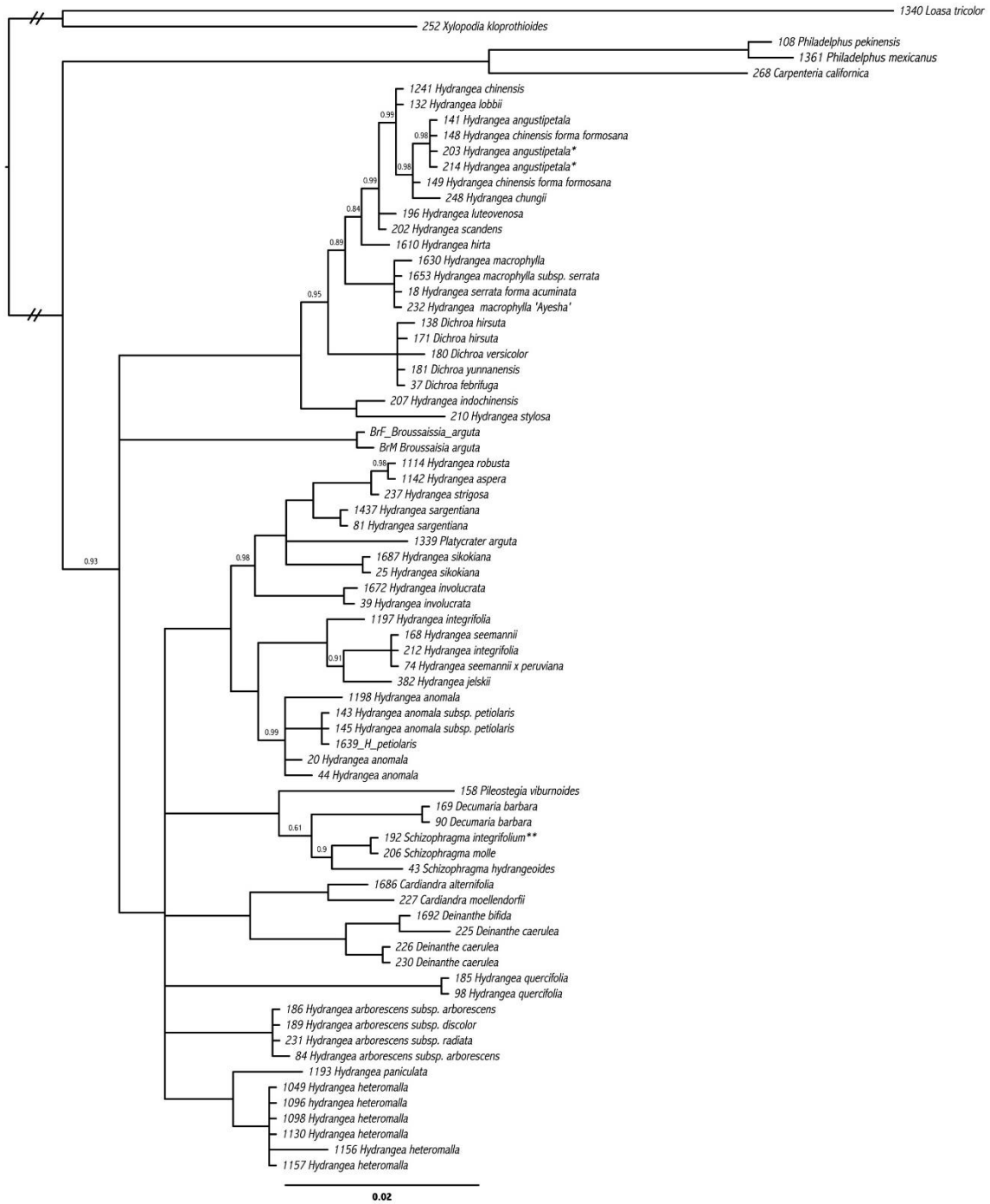


Figure S2.2D: The 50% majority rule consensus tree based on the *ndhA* intron without indel information, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. No supported topological differences with the phylogenetic tree based on only nucleotide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

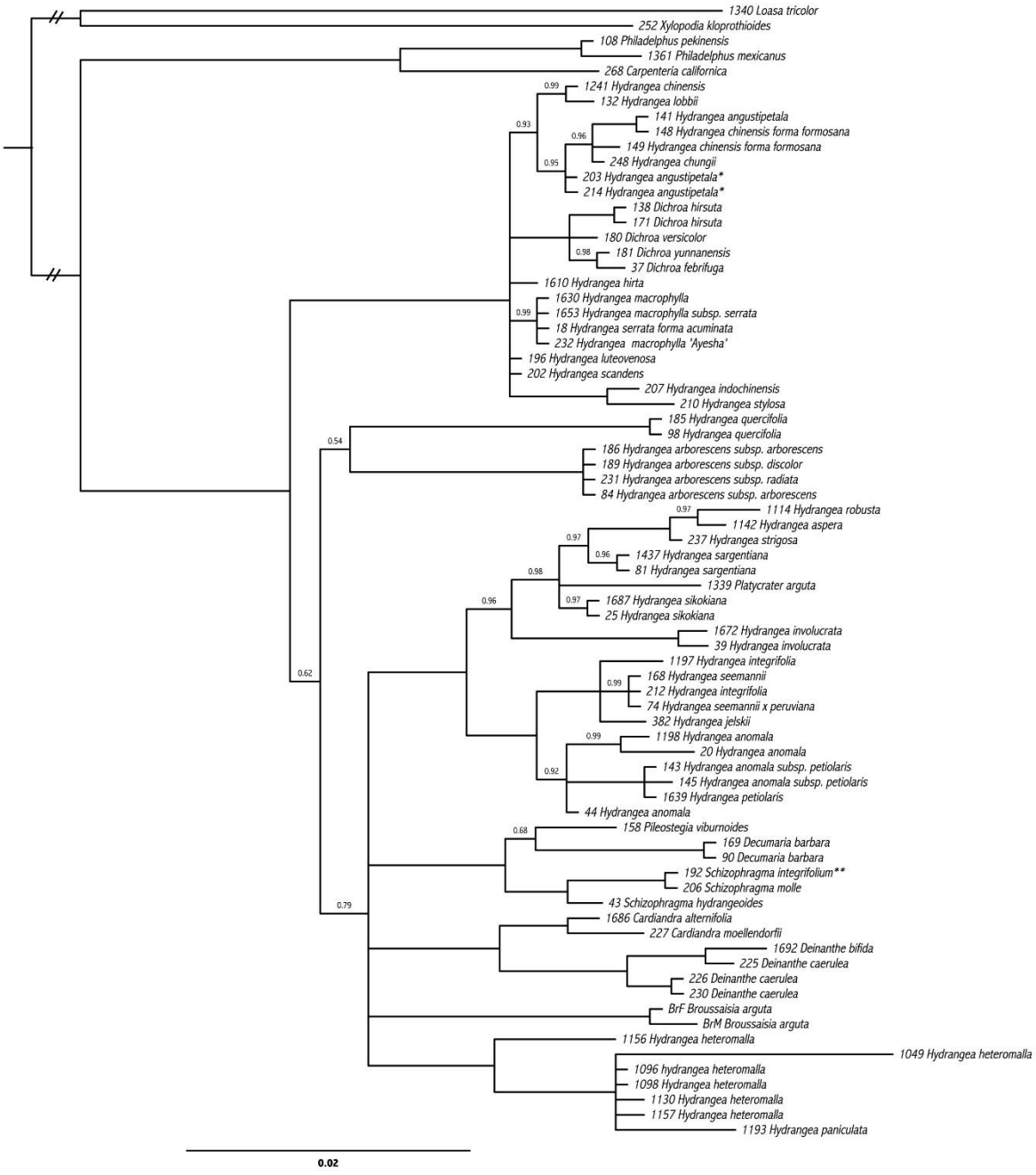
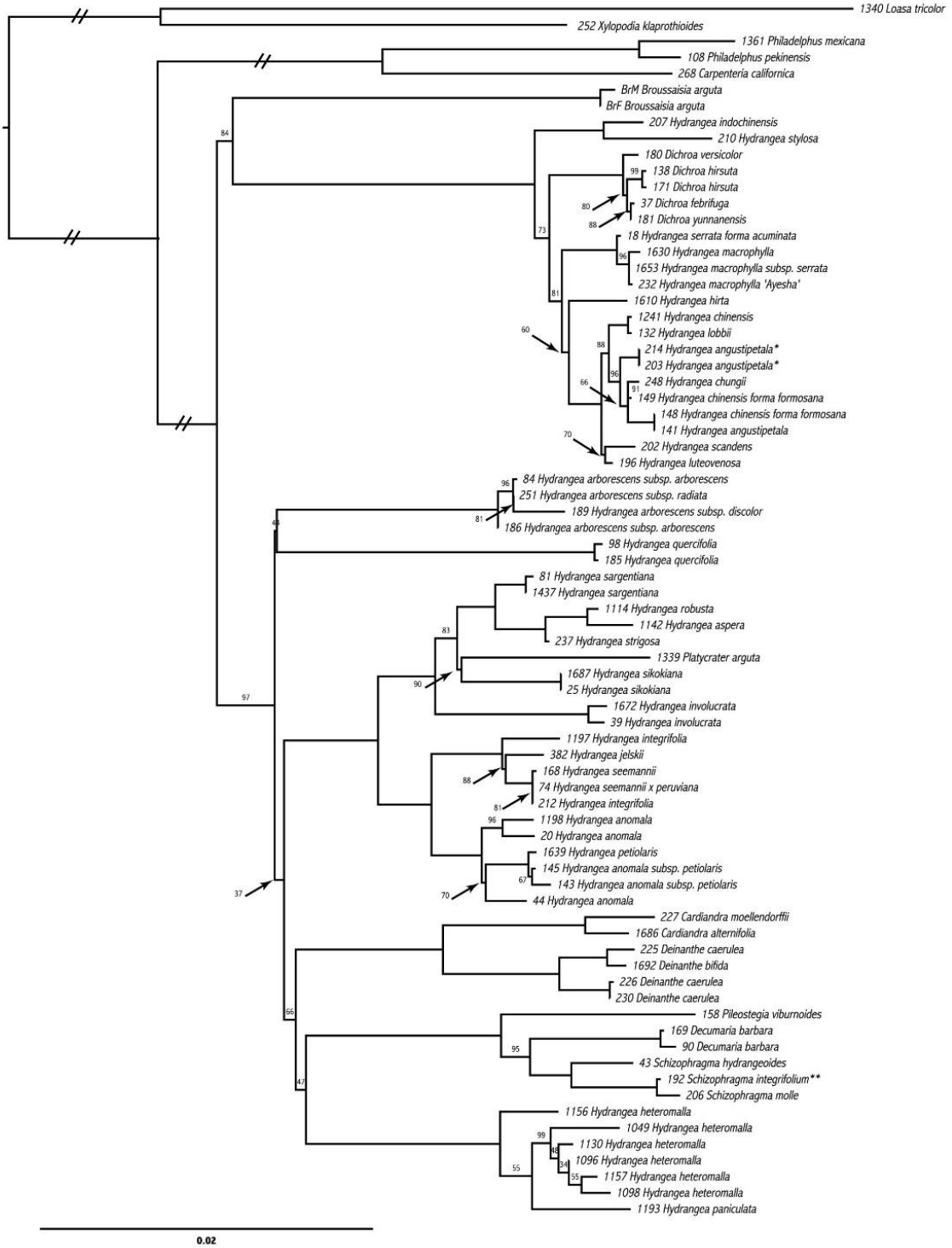


Figure S2.2E: The 50% majority rule consensus tree based on ITS with indels coded, posterior probabilities obtained from Bayesian Inference indicated on the respective branches when below 1. No supported topological differences with the phylogenetic tree based on only nucleotide data (not shown).

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*** = *Schizophragma integrifolium* var. *fauriei*.



Appendix 3: 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. Cires Rodríguez, 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	1921 m	1	LT838907	LT838936	LT838965	LT838995	LT854706	LT854735	LT854762	LT854792
H. strigosa	YDS1032	China, Sichuan (29.57, 103.44)	W	541 m	1	LT838911	LT838937	LT838966	LT838996			LT854763	LT854793
H. strigosa	YDS1035	China, Sichuan (29.57, 103.44)	W	548 m	1	LT838912	LT838938	LT838967	LT838997	LT854707	LT854736	LT854764	LT854794
H. robusta	YDS1086	China, Sichuan (29.67, 102.94)	W	1906 m	1	LT838913	LT838939	LT838968	LT838998	LT854708	LT854737	LT854765	LT854795
H. aspera	YDS1101	China, Sichuan (29.83, 102.7)	W	919 m	1	LT838914	LT838940	LT838969	LT838999	LT854709	LT854738	LT854766	LT854796
H. robusta	YDS1114	China, Sichuan (29.69, 102.61)	W	1862 m	1		LT838941	LT838970	LT839000	LT854710	LT854739	LT854767	LT854797
H. aspera	YDS1137	China, Sichuan (29.6, 102.06)	W	2194 m	1	LT838915	LT838942	LT838971	LT839001	LT854711		LT854768	LT854798
H. kawakamii	YDS1176	Taiwan, Yilan (24.39, 121.36)	W	1974 m	1	LT838929	LT838943	LT838972	LT839004	LT854712	LT854756	LT854769	LT854799
H. longifolia	YDS1183	Taiwan, Yilan (24.54, 121.51)	W	803 m	1	LT838922	LT838944	LT838973	LT839002	LT854713	LT854740	LT854770	LT854800
H. longifolia	YDS1203	Taiwan, Taichung (24.2, 121.48)	W	952 m	1	LT838923	LT838945	LT838974	LT839003	LT854714	LT854741	LT854771	LT854801
H. kawakamii	YDS1227	Taiwan, Taichung (24.22, 121.27)	W	2030 m	1	LT838930	LT838946	LT838975	LT839005	LT854715	LT854757	LT854772	LT854802
H. aspera	YDS1349	Nepal, Khinti Khola	H	1898 m	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	1331 m	2	LT838931	LT838949	LT838979	LT839012	LT854719	LT854758	LT854788	LT854806
H. sargentiana	YDS1437	China, Hubei (31.33, 110.48)	W	1315 m	2	LT838918	LT838950	LT838980	LT839011	LT854720	LT854744	LT854776	LT854807
H. strigosa	YDS1459	China, Hubei (31.31, 110.48)	W	1357 m	2	LT838932	LT838951	LT838981	LT839014	LT854721	LT854759	LT854789	LT854808
H. strigosa	YDS1462	China, Hubei (31.31, 110.48)	W	1403 m	2	LT838933	LT838952	LT838982	LT839007	LT854722	LT854760	LT854790	LT854809
H. sargentiana	YDS1468	China, Hubei (31.31, 110.48)	W	1443 m	2	LT838919	LT838953	LT838983	LT839015	LT854723	LT854745	LT854777	LT854810
H. strigosa	YDS1485	China, Hubei (31.34, 110.51)	W	740 m	2	LT838934	LT838954	LT838984	LT839013	LT854724	LT854761	LT854787	LT854811
H. longipes	YDS1489	China, Hubei (31.53, 110.34)	W	1725 m	2	LT838920	LT838955	LT838985	LT839016	LT854725	LT854747	LT854778	

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

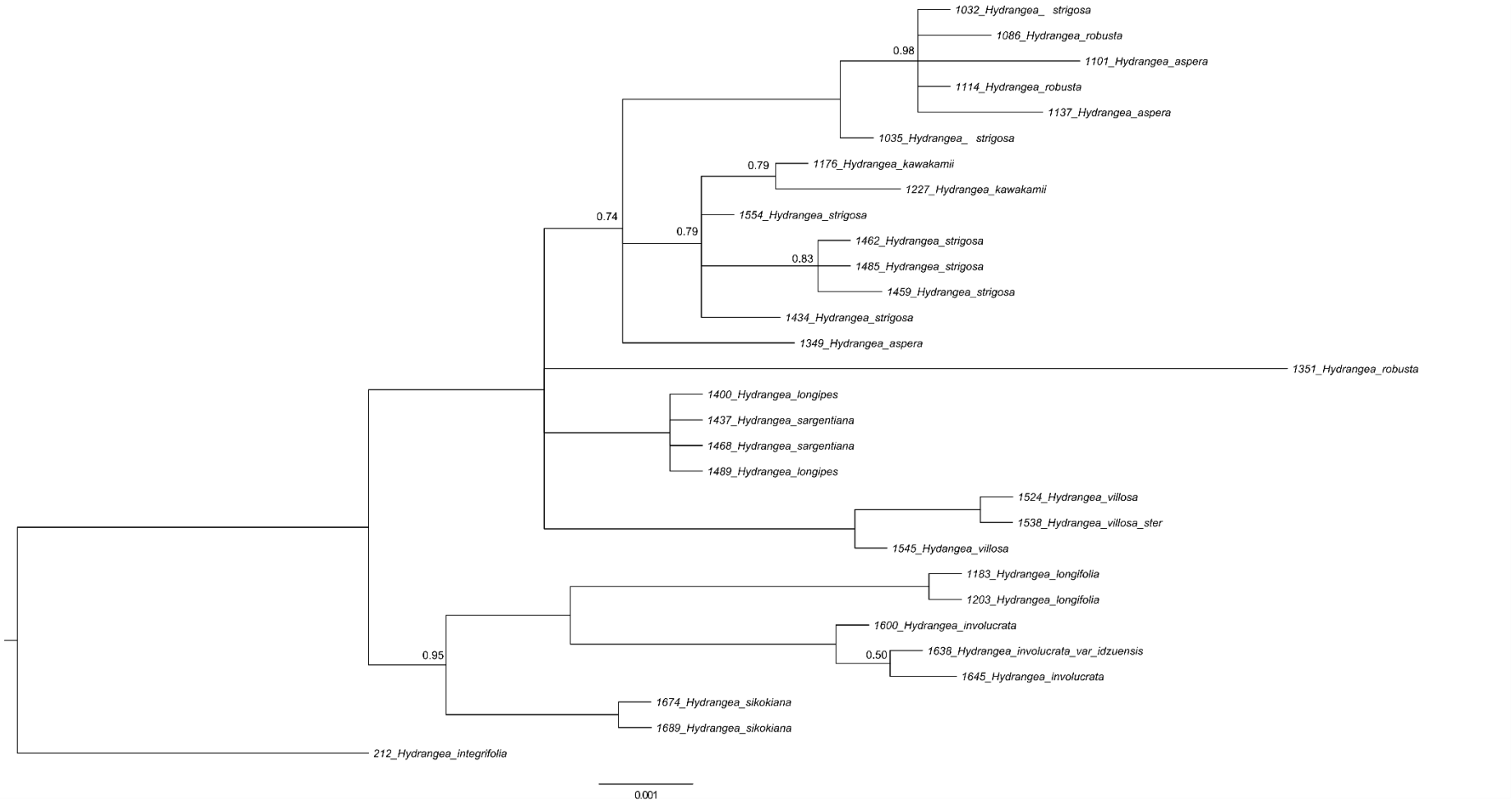


Figure S3.2: The 50% majority-rule consensus tree based on the *rpl32-ndhF* IGS. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

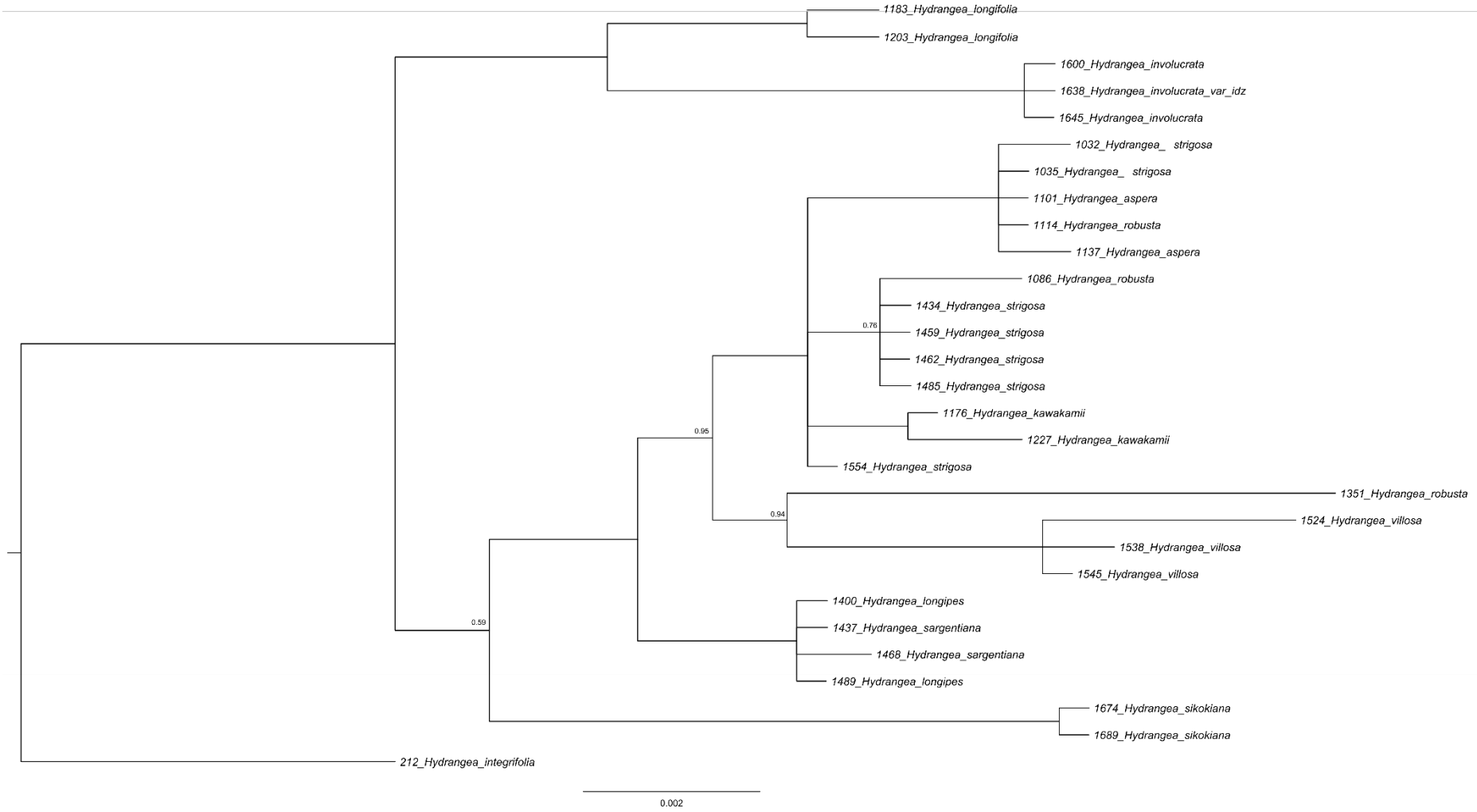


Figure S3.3: The 50% majority-rule consensus tree based on the *ndhA* intron. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

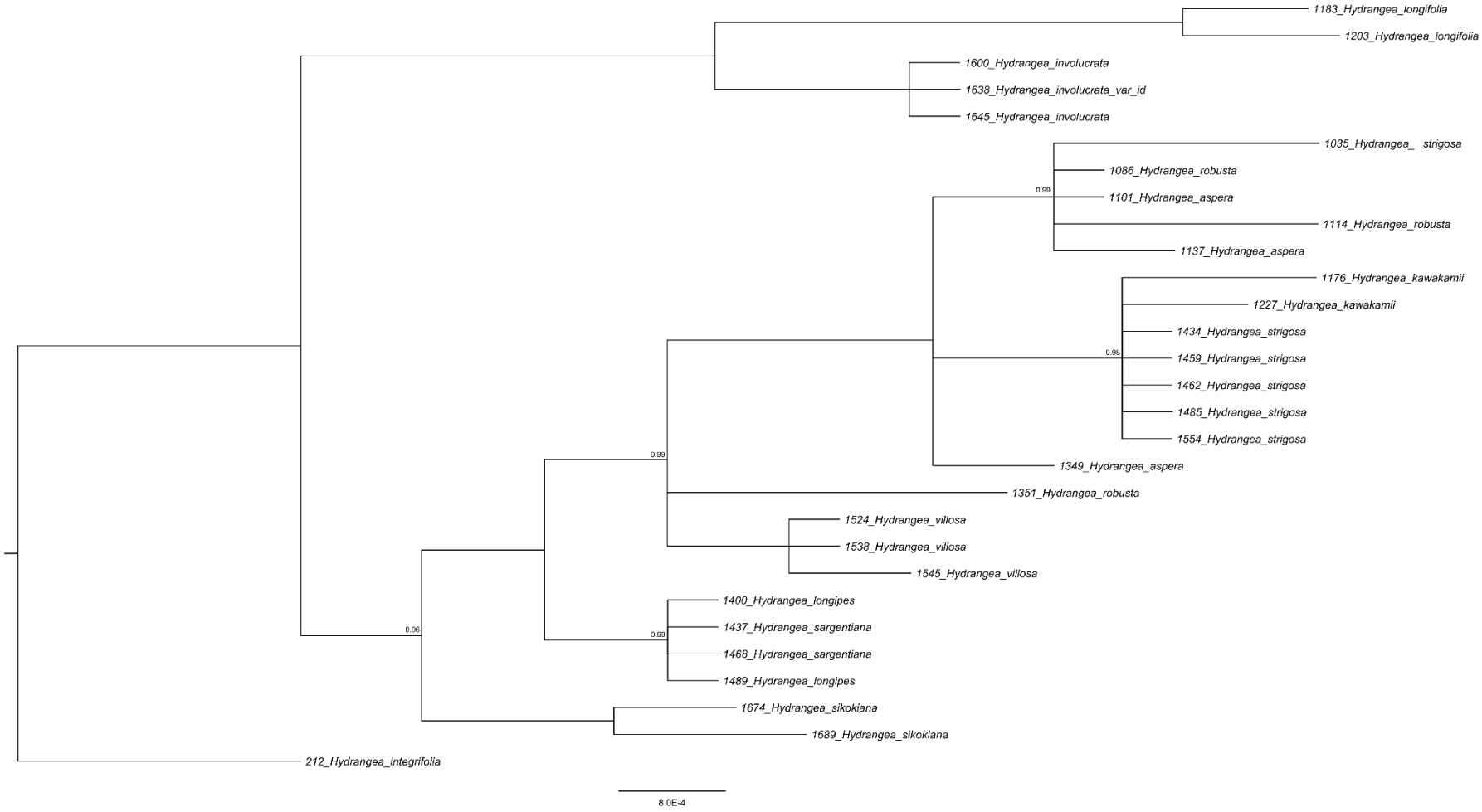


Figure S3.4: The 50% majority-rule consensus tree based on the *trnL-rpl32* IGS. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

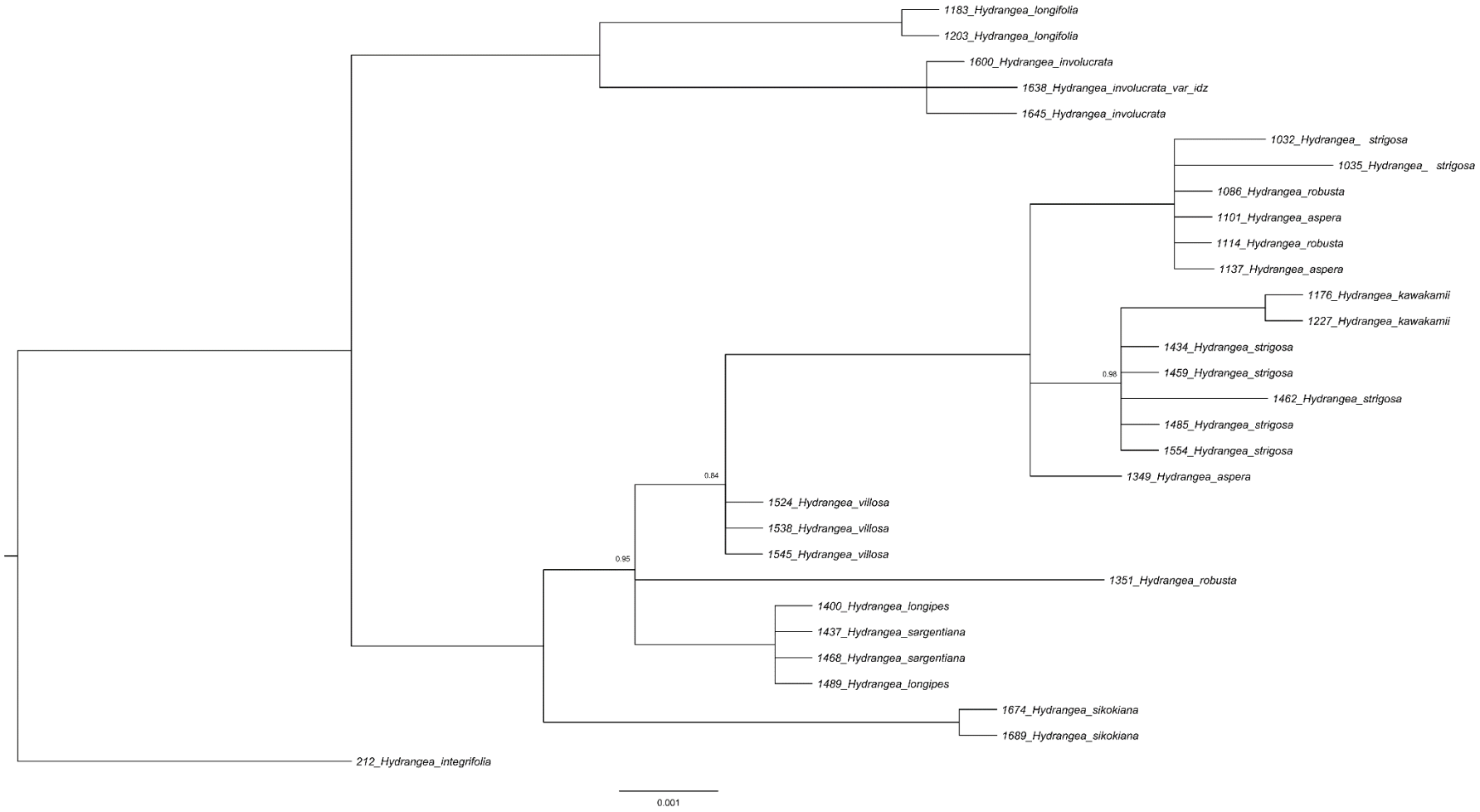


Figure S3.5: The 50% majority-rule consensus tree based on the ITS region. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

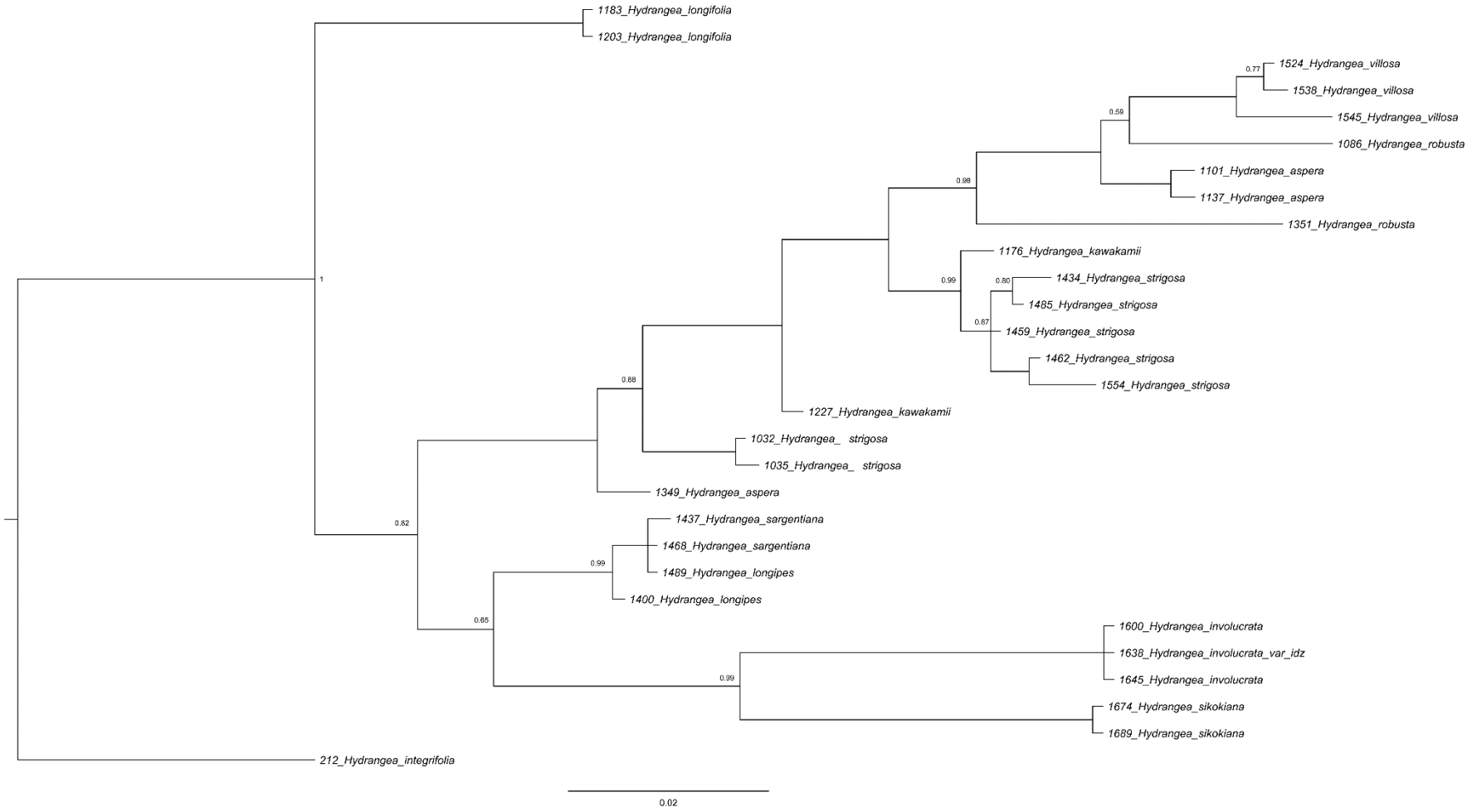


Figure S3.6: The 50% majority-rule consensus tree based on the *SMC1-22* region. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

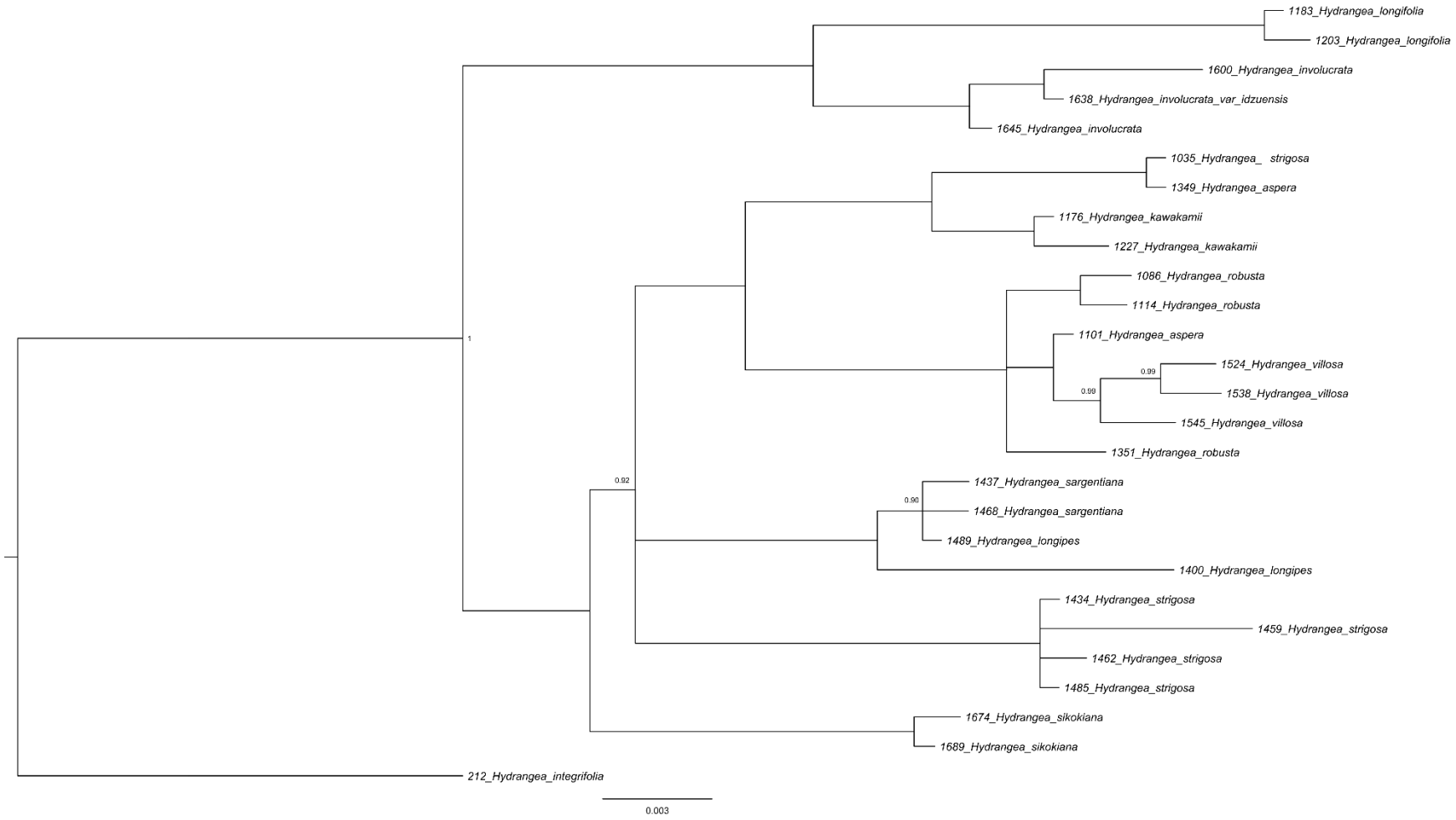


Figure S3.7: The 50% majority-rule consensus tree based on the *SMC1-44* region. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.

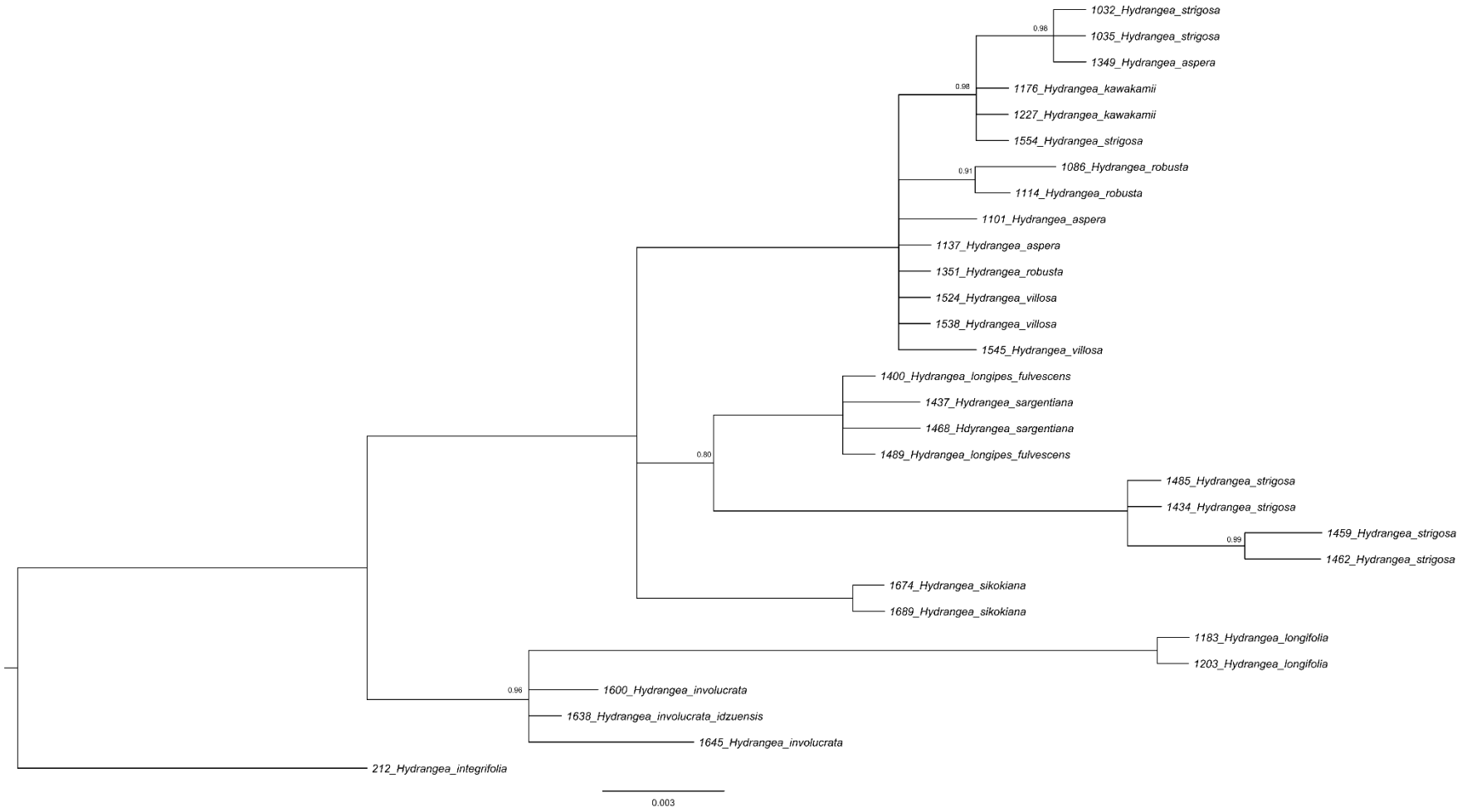
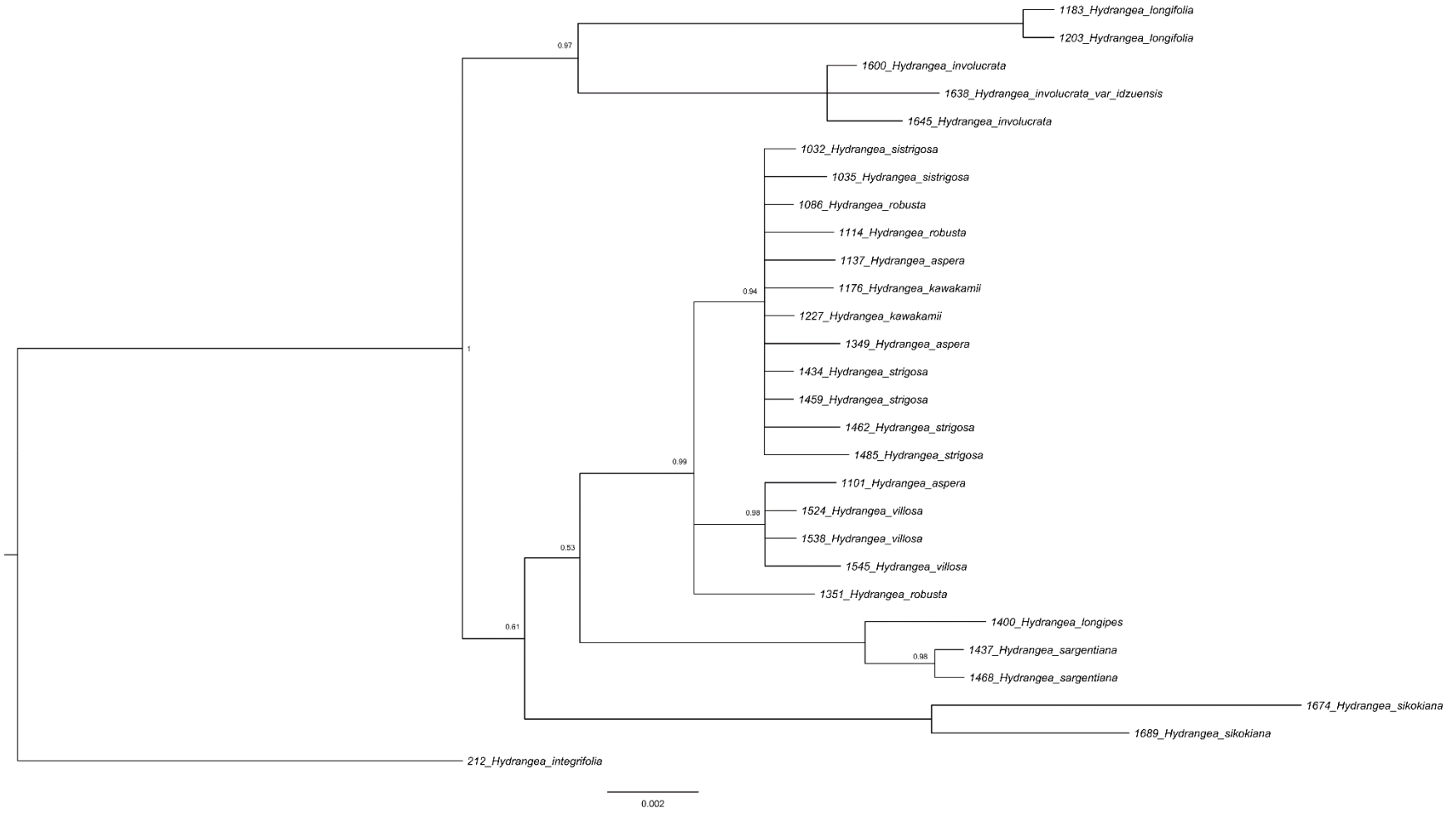


Figure S3.8: The 50% majority-rule consensus tree based on the *TIF3H1* region. Posterior probabilities obtained from Bayesian inference indicated on the respective branches when below 1.



Appendix 4: 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, specimens collected at, or near type locations are indicated with an asterisk), 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)	548 m	1
Hydrangea robusta	1086	YDS1086	China, Sichuan (29.67, 102.94)	1906 m	1
Hydrangea aspera	1101	YDS1101	China, Sichuan (29.83, 102.7)	919 m	1
Hydrangea robusta	1114	YDS1114	China, Sichuan (29.69, 102.61)	1862 m	1
Hydrangea aspera	1137	YDS1137	China, Sichuan (29.6, 102.06)	2194 m	1
Hydrangea kawakamii	1176	YDS1176	Taiwan, Yilan (24.39, 121.36)	1974 m	1
Hydrangea longifolia	1183	YDS1183	Taiwan, Yilan (24.54, 121.51)	803 m	1
Hydrangea longifolia	1203	YDS1203	Taiwan, Taichung (24.2, 121.48)	952 m	1
Hydrangea kawakamii	1227	YDS1227	Taiwan, Taichung (24.22, 121.27)*	2030 m	1
Hydrangea longipes	1400	YDS1400	China, Hubei (31.05, 110.95)	~1300 m	2
Hydrangea strigosa	1434	YDS1434	China, Hubei (31.33, 110.48)*	1331 m	2
Hydrangea sargentiana	1437	YDS1437	China, Hubei (31.33, 110.48)*	1315 m	2
Hydrangea strigosa	1459	YDS1459	China, Hubei (31.31, 110.48)*	1357 m	2
Hydrangea sargentiana	1468	YDS1468	China, Hubei (31.31, 110.48)*	1443 m	2
Hydrangea strigosa	1485	YDS1485	China, Hubei (31.34, 110.51)*	740 m	2
Hydrangea longipes	1489	YDS1489	China, Hubei (31.53, 110.34)	1725 m	2
Hydrangea villosa	1524	YDS1524	China, Hubei (30.17, 110.97)*	882 m	2
Hydrangea villosa	1538	YDS1538	China, Hubei (30.16, 110.78)*	1055 m	2
Hydrangea involucrata	1600	YDS1600	Japan, Hinohara (35.72, 139.11)	263 m	3
Hydrangea involucrata	1638	YDS1638	Japan, Oshima island (34.71, 139.43)	367 m	3
Hydrangea involucrata	1645	YDS1645	Japan, Shiga (35.93, 137.13)	626 m	3
Hydrangea sikokiana	1674	YDS1674	Japan, Tokushima (33.95, 134.4)	925 m	3
Hydrangea sikokiana	1689	YDS1689	Japan, Tokushima (33.91, 134.29)	1143 m	3
Hydrangea strigosa	1074	YDS1074	China, Sichuan (29.63, 103.04)	1108 m	1
Hydrangea aspera	1164	YDS1164	China, Sichuan (30.41, 102.65)	1532 m	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 5: 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 subsp. *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.

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

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.

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

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.

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. Cires, 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

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

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

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

YDS1645	*H. involucrata*	Japan	Shiga prefecture	Gero	626	35	55	40,2	N	137	7	54,9	E	3	04/08/2012
YDS1646	*H. involucrata*	Japan	Shiga prefecture	Gero	628	35	55	4,3	N	137	7	58,6	E	3	04/08/2012
YDS1647	*H. involucrata*	Japan	Shiga prefecture	Gero	597	35	52	35,8	N	137	10	34,6	E	3	04/08/2012
YDS1649	*H. involucrata*	Japan	Shiga prefecture	Gero	610	35	52	4,1	N	137	10	43,4	E	3	04/08/2012
YDS1650	*H. involucrata*	Japan	Shiga prefecture	Gero	648	35	55	21,3	N	137	19	2,7	E	3	04/08/2012
YDS1651	*H. involucrata*	Japan	Shiga prefecture	Gero	645	35	55	12	N	137	19	19,1	E	3	04/08/2012
YDS1652	*H. involucrata*	Japan	Shiga prefecture	Gero	656	35	55	12,4	N	137	19	19,2	E	3	04/08/2012
YDS1655	*H. involucrata*	Japan	Shiga prefecture	Gero	665	35	55	9,9	N	137	19	23,4	E	3	04/08/2012
YDS1656	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	852	35	34	33,4	N	137	50	15	E	3	05/08/2012
YDS1659	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	845	35	34	26,8	N	137	50	11,1	E	3	05/08/2012
YDS1660	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	1038	35	35	13,7	N	137	49	53,5	E	3	05/08/2012
YDS1661	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	998	35	35	5,8	N	137	49	58,4	E	3	05/08/2012
YDS1668	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	982	35	34	58,8	N	137	49	59,9	E	3	05/08/2012
YDS1669	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	971	35	34	56,2	N	137	49	58,9	E	3	05/08/2012
YDS1671	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	953	35	34	52,6	N	137	50	1	E	3	05/08/2012
YDS1672	*H. involucrata*	Japan	Nagano prefecture	Takamori-cho	941	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

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

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

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	927	33	56	50,1	N	134	24	5,8	E	3	07/08/2012
YDS1674	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	925	33	56	50,6	N	134	24	5	E	3	07/08/2012
YDS1677	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	925	33	56	50,1	N	134	24	5,9	E	3	07/08/2012
YDS1678	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	928	33	56	50,7	N	134	24	5,9	E	3	07/08/2012
YDS1679	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	931	33	56	50,5	N	134	24	5,4	E	3	07/08/2012
YDS1687	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	1109	33	54	42,5	N	134	17	23,2	E	3	07/08/2012
YDS1688	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	1140	33	54	42,6	N	134	17	21,8	E	3	07/08/2012
YDS1689	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	1143	33	54	42	N	134	17	22,3	E	3	07/08/2012
YDS1690	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	1140	33	54	42,9	N	134	17	22,6	E	3	07/08/2012
YDS1691	*H. sikokiana*	Japan	Tokushima prefecture	Kamikatsu	1140	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

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

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

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

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

Box S5.1: 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 1.9). 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 *H.* 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 *H.* 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.

Curriculum Vitae

YANNICK DE SMET

Curriculum Vitae | 2020

PERSONALIA

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EDUCATION

OLVP Bornem – Techniek Wetenschappen
September 2001 – July 2005

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

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

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

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."

Professional experiences

Ghent University – Research group Spermatophytes – PhD student
October 2010 – 2014 | FWO-mandate |
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

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.

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)

LANGUAGES

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

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 –Ghent –2019 (5 days, certified)

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

De Smet, Y., Goetghebeur, P., Chatrou, L., Samain, M.-S. Taxonomic treatment of *Hydrangea* sect. *Asperae*.

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

VARIA

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).