

**Molecular phylogenetic studies of the genera of tribe Polygonateae (Asparagaceae:
Nolinoideae): *Disporopsis*, *Heteropolygonatum*, and *Polygonatum*.**

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“Common experience teacheth, that in the world there is not to be found another herbe comparable to it.” John Gerard, 1597.

Gerard's Herball - Or, Generall Historie of Plantes, London: John Norton 1975.

Abstract

The Polygonateae (Asparagaceae) are a subtribe of the Nolinoideae that is redefined here to include three genera which are investigated here: *Disporopsis*; *Heteropolygonatum*, and *Polygonatum*. This group of genera, characterized by their axillary-flowered habit, are closely related but differ greatly in their morphology, cytology, and diversity. A molecular phylogeny is presented to show their relationships to one another and to closely related outgroups based on data from whole chloroplast genomes with low taxonomic sampling. The results show that the genera of the Polygonateae are each monophyletic, and also show that a fourth genus, *Maianthemum*, that was traditionally included in Polygonateae is not accurately placed there and should be excluded. A second set of analyses was based on an expanded dataset with high taxonomic sampling using a few selected loci from the chloroplast, mitochondrial, and nuclear genomes. The results confirmed the revised delimitation of Polygonateae that excludes *Maianthemum* and the respective monophyly of *Disporopsis*, *Heteropolygonatum*, and *Polygonatum*. Species-level relationships within each individual genus were analyzed, in part to allow assessment of the placement of many novel or obscure species that have been described (or resurrected) recently. Finally, the phylogenetic results from the expanded dataset were used to test various hypotheses regarding cytological evolution in the subtribe, and the results showed a pattern of descending (or in a few cases ascending) dysploidy that underlies the observed variation in chromosome numbers.

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Introduction

The tribe Polygonateae (Asparagaceae: Nolinoideae) includes three axillary flowered genera, *Disporopsis* Hance, *Heteropolygonatum* Tamura and Ogisu, and *Polygonatum* Mill., and the terminal-flowered *Maianthemum* Wigg.. These genera differ in their base chromosome numbers, their growth habit, rhizome shape, and their perigone morphology.

Polygonatum is circumboreal in distribution with the majority of the species-level diversity restricted to Asia, specifically China. The genus ranges from the Arctic Circle in the north and south to the Dalat Plateau in southern Vietnam (Floden 2014a). The breadth of climatic conditions inhabited by the genus also varies from tropical cloud-forest through temperate forest or scrub habitat to alpine scree. Those species from cloud-forest habitats are generally epiphytes whereas most other species occur as terrestrial herbs. *Polygonatum* offers distinctive opportunities to study diversification because of its classic Asian-North American disjunct distribution and the disparity of diversity between those areas, and the breadth of morphological and cytological infrageneric variation. The perigone is valvate though the shape varies across sections and within series and the variation in staminal filaments is often diagnostic for species recognition.

Heteropolygonatum was only recently recognized as a distinct genus delimited by its imbricate perigone (vs. valvate) and its uniform and distinct base chromosome number of $x = 16$. Of the ten species currently recognized (Floden 2014b), over half were first described in *Polygonatum*. All species are known from China with one species crossing the borders of Yunnan into Myanmar to the west and into Vietnam in the south (Floden 2014b). Most species are known from fewer than five localities and are allopatric. They grow as epiphytes or as lithophytes, but unlike epiphytic *Polygonatum*, all *Heteropolygonatum* species are deciduous (Floden 2014a, 2014b).

Disporopsis species are native to southeastern Asia with most species endemic to China (Chen and Tamura 2000). They are evergreen with fleshy green to yellow rhizomes, and have an imbricate perigone with the staminal filaments dilated and connate which form a corona-like structure (Hua 1892). The genus also has a uniform and distinct chromosome number of $2n = 2x = 40$. Recently several new species have been recognized based on morphology (Floden 2015a), but molecular data for the genus is restricted to a few samples used as outgroups in molecular studies.

The generic relationships of these genera (and *Maianthemum* not investigated fully here) are not well understood. Early studies with limited sampling of outgroups placed the four genera traditionally comprising the Polygonateae in a clade (Wu et al. 2000, Tamura et al. 2000) though more recent studies with broad taxon sampling have been equivocal (Kim et al. 2010). Meng et al. (2014) presented a molecular phylogeny based on four chloroplast loci in which *Disporopsis* and *Heteropolygonatum* are placed as sister genera and these sister to *Polygonatum*. Bayesian posterior probabilities provided high support but maximum likelihood bootstrap support was low (58). Their study only included maternally inherited chloroplast data and it also had low taxonomic sampling of all genera of the Polygonateae. Understanding the diversity of the tribe, especially within the highly diverse *Polygonatum*, is a primary goal of the current study though complete resolution is not the objective. Synthetic studies are hindered, however, by the poor

state of taxonomic knowledge of this tribe. Through studies of morphology, cytology, and basic molecular phylogenetics I aim to produce a comprehensive taxonomy of the core genera of the tribe Polygonateae to facilitate future systematic revisionary studies.

Tribe Nolinoideae encompasses a broad array of taxa including the rosette-forming desert genera *Calibanus* and *Nolina*, the perennial herbs in the Polygonateae, the tropical tree-like *Dracaena*, and numerous other genera (eMonocot Team 2015). *Polygonatum* is the most diverse genus in the tribe with as many as 83 species, including at least two species in North America and the remainder in Europe and Asia; 30 or more occur in China alone (Chen and Tamura 2000). Recent estimates recognize from 57–83 species (WCSP 2015, Tamura et al. 2014, Floden 2015b, 2015c), and my field and herbarium studies suggest that many species remain undescribed either because they have not been recognized or because field work in key areas has not been done.

The taxonomy of *Polygonatum* has not been comprehensively investigated on a worldwide basis since Baker (1875) who treated 23 species. Since then there has been a proliferation of names (approximately 220; IPNI 2014). Despite the taxonomic inquiry by numerous authors there have been substantial disagreements on specific boundaries and distributions (Komarov 1935, Abramova 1975, Tang 1978, Jeffrey 1980, 1982, Chen and Tamura 2000). This problem has been further aggravated by regional floristic treatments without systematic evaluations (e.g. no consultation of types), such that the resultant floras are usually in disagreement with neighboring regions regarding their alpha level taxonomy and conclusions in regional studies (Komarov 1935, Tang 1978, Chen and Tamura 2000). For instance, *Polygonatum* in North America has received intensive investigation but there is no agreement on the number of native species with varied numbers including five (Farwell, 1915), ten (Gates 1917), twelve (Bush 1927), four (Ownbey 1944), and two (Utech 2002). On par with the systematics in North America, *Polygonatum* has received scarce systematic attention on a world-wide level since Baker's treatment though recent interest has resulted in the description of three novel species (Cai et al. 2015, Floden 2014a, 2015b, 2015c, Tamura et al. 2014, Zhao and He 2014) and the discovery of several others (Floden, see Chapter 2). Comprehensive systematic work will likely reveal additional novel species and clarification of the application of names in synonymy.

Species-level diversity of *Polygonatum* is not only higher in eastern Asia, but so are cytological and morphological diversity. Previous morphology based systematics studies of *Polygonatum* have come to different conclusions regarding species number (Abramova 1975, Tamura 1993, Tamura et al. 1997), which is probably due to the difficult determination of some specimens in their preserved state. Classification of species-groups was first based on leaf arrangement, but this is variable and some leaf arrangements have evolved more than once in the genus (Meng et al. 2014). Specimens collected in flower are often difficult to determine due to the succulent nature of the perigone and the importance of the filaments in species delimitation. Without abrupt desiccation the perigone molds, obscuring the filament shape and surface texture. This molecular based study, which also includes data from morphology and cytology, is the first to be based largely on field collected samples cultivated to document the variation in growth habit, seed germination, and most importantly the morphology of the filaments. The ex-situ collection has provided material to build on previous cytological studies that will investigate cytology and polyploidy within the genera, assignment of generic status of anomalous taxa, placement of novel taxa, assessment of infrageneric relationships in *Polygonatum*, and potential discovery of

cryptic diversity due to polyploidy. Molecular data have already revealed novel species and aided in delimitation of others. Moreover, additional data will aid in the resolution of widespread species groups. Taxonomic revision will also aid in floristic projects covering Nepal (Floden ined.) and the Pan-Himalaya project in progress. Upon completion I estimate that there will be nearly 100 species recognized in *Polygonatum* supported by molecular data with the highest diversity in southwestern China and the adjacent Himalaya.

Not only are *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* poorly known at the species level but also their relationships to one another and to *Maianthemum*, included within the Polygonateae (Tamura 1997, Kim et al. 2010), are unresolved. My preliminary phylogenetic inferences using nrDNA ETS and 35 coding genes from the plastome resolved *Maianthemum* as sister to *Ophiopogon* and *Liriope*, which supports the classification of Baker (1875), but current classifications place it sister to *Polygonatum* due to their shared annual stems from sympodial rhizomes, elongate stems, axillary or terminal flowers, and baccate fruit (Conran and Tamura 1998). Despite their morphological similarities the genera differ by their chromosome number and karyotype (*Disporopsis* $x = 20$, *Heteropolygonatum* $x = 16$ distinctly bimodal, *Maianthemum* $x = 18$ asymmetric, and *Polygonatum* with a range from $x = 9$ –15. Species of *Polygonatum* with $x = 9$ –11 are typically asymmetric and $x = 12$ –15 are usually distinctly bimodal. Moreover, the placement of *Disporopsis* as sister to *Theropogon* and *Comospermum* and not to *Polygonatum* in a broad survey of the Asparagaceae (Kim et al. 2010) is in contrast to the results of more recent analyses (Meng et al. 2014). The placement of *Heteropolygonatum* in early molecular works relied on sampling only a single species of the genus (Wu et al. 2000, Tamura et al. 1997), and the most recent included only four of 10 (Meng et al. 2014).

Phylogenetic resolution is often hampered by low sampling of taxa and loci. Wu et al. (2000) and Tamura et al. (1997) both sampled fewer than 20 species including outgroups and focused on the use of the maternally inherited chloroplast *matK* gene. Meng et al. (2014) sampled only 30 species of *Polygonatum* and four *Heteropolygonatum* using four mostly invariant chloroplast markers with the resulting tree poorly resolved. Next generation sequencing (NGS) approaches produce large amounts of data at relatively low cost in comparison to traditional Sanger sequencing (Shaw et al. 2014). NGS data have the capability to resolve generic relationships, infrageneric relationships, paraphyletic lineages, and disjunct species that have not been resolved by traditional methods involving a few loci. These data will also have the potential to provide marker development with efficacy across the Nolinoideae to aid in generic and species delimitation in a targeted approach. Phylogenetic inferences will allow various hypotheses of biogeography, cytological evolution, divergence times, and rates of evolution to be tested.

Cytology of the Polygonateae is highly diverse in base number and in karyotype. Cytology and karyology studies in *Polygonatum* have a long history due to the large size of the chromosomes (3.5–11 μm), prevalence of polyploidy, variable base number ($x = 9$ –15), and correspondingly variable karyotypes between and within species (Zhao et al. 2014, Deng et al. 2009, Tamura, 1997, 1993, 1991a, b, Abramova 1975, Therman 1950, 1953a, 1953b, Suomalainen 1947). Base chromosome numbers in *Polygonatum* are associated with morphologically cohesive groups of species. For instance, species with $x = 9$ –11 have alternate leaves, and those with $x = 12$ –15 are either opposite, verticillate, although sometimes alternate (but then these evergreen), or a mixture of leaf arrangements (Tamura 1997, Meng et al. 2014). Despite the many publications that

document the cytology and karyology in *Polygonatum*, there still remains a great amount unknown, e.g. the phylogenetic distribution of chromosome number changes, the diversification of base number, karyotype, and prevalence of polyploidy. Knowledge of newly reported chromosome numbers from species not yet surveyed and additional counts of species already known has provided the largest dataset thus far of counts for the genus and allowed for modeling of chromosome evolution across these genera which is presented here.

Increased karyotype diversity was assumed to be the trend in *Polygonatum* based on the data from the long history of cytological study (see Zhao et al. 2014). The evolutionary trend assumed in *Polygonatum* has been that the simple alternate-leaved species are positioned at the base of the phylogeny and diversification has proceeded towards morphological and cytological complexity through ascending dysploidy (see Zhao et al. 2014, Deng et al. 2009). Recent phylogenetic inference (Meng et al. 2014) shows that the genus has had several possible reductions in karyotype diversity. Moreover, the trend within the Polygonateae has been reduced karyotype diversity with successive reductions starting from $n = 20$. Nonetheless, within some clades of *Polygonatum* there have been occurrences of ascending dysploidy within clades, but it is unclear what mechanisms and how many changes have resulted in the variability of chromosome number and karyotype in *Polygonatum*. It is also worthy to note that polyploidy is largely absent in section *Polygonatum* in Asia where the greatest diversity of species exists (see Zhao et al. 2014), but in North America both native species exhibit a range of cytotypes (Ownbey 1944). In contrast, polyploidy in section *Verticillata* is extensive in SW China.

Heteropolygonatum has a distinctly bimodal karyotype and most species are diploid (Bao et al. 1998). Two species, *H. ogisui* and *H. marmoratum*, have $2n = 4x = 64$ (Floden 2014b, Yamashita and Tamura 2001). Nonetheless, *H. marmoratum* is the most widespread and morphologically variable species in the genus and some samples of *H. marmoratum* have proven to be diploid (see Chapter 3). The diploid *H. marmoratum* from the border of Myanmar also differ in their floral morphology and several molecular changes which suggests that diploid and tetraploid populations might be better treated as independent species (Floden 2014b). Chromosome numbers are unknown for *H. ginfushanicum*, *H. parcefolium*, and *H. anomalum* (Floden 2014b). My own counts of both genera from my living collection will supplement missing data from several species not yet reported (*H. ginfushanicum*, *Polygonatum brevistylum*, *P. griffithii*, *P. nervulosum*, *P. wardii*, and several novel species). Study of these species will provide cytological and molecular data to resolve their phylogenetic placement as well as to explore the evolution of cytology in the Polygonateae.

Recent modeling programs that use molecular data in combination with cytological data have enabled testing hypotheses of karyotype evolution across families and genera (Glick and Mayrose 2014, Mayrose et al. 2010). Hypotheses on the mode of evolution of chromosome and karyotype in the Polygonateae can be tested utilizing ChromEvol which infers the chromosomal evolution (Glick and Mayrose 2014, Mayrose et al. 2010). Polyploidy in *Polygonatum* is largely restricted to *P.* section *Verticillata* (see Zhao et al. 2014). Inferences from the model-tested dataset will test several hypotheses regarding cytological evolution in these genera: 1) has diversification followed the presumed evolutionary trend towards increased cytological complexity resulting in higher number and greater asymmetry?; 2) has the bimodal karyotype in *P.* section *Verticillata* and *Heteropolygonatum* led to greater diversification relative to section

Polygonatum?; and 3) are cytological differences associated with certain groups of species based on their habitat (alpine vs. subtropical or epiphytic vs. terrestrial), or their latitudinal distribution, and are these associated with potential bursts of diversification?

A taxonomic revision based on a synthesis of multiple data sources has wide-reaching implications. Resolution of taxonomy in *Polygonatum* and *Heteropolygonatum* based on the living collection and data acquired from it will undoubtedly reveal much higher species diversity than currently accepted. Resolving the taxonomy of several species groups (*P. cyrtoneura*, *P. cirrhifolium*, and *P. verticillatum*) will give new insights and help prioritize efforts regarding their conservation. Each of the species groups comprise several morphological and often geographically disjunct forms that are supported by limited molecular sampling (Meng et al. 2014). Moreover, each of these *Polygonatum* are used as a wild food resource and as medicine and many of these are over utilized (Lohani et al. 2013, Kala and Ratajc 2012). Recognition of multiple species within each of these broadly circumscribed taxa would require reassessments of their rarity and where applicable, conservation strategies.

Polygonatum in Asia has a long tradition as both a food and as an ethnobotanical (Lohani et al. 2013, Lohani et al. 2012, Motohashi et al. 2003). Though sampling of species has been limited, numerous novel compounds have been reported (see Khan et al. 2012). The presence of compounds varies across lineages and thus far those sampled are not shared between different species groups. The highly resolved phylogeny with high species sampling will provide a resource to those who are assessing pharmacological potential of species in clades with known compounds.

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Chapter 1

The chloroplast genomes of *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* (Asparagaceae) and comparative analysis of two widely disjunct *Polygonatum* species.

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My primary contributions to this paper included study design, molecular work, library preparation of five samples, bioinformatics data analyses, and most of the writing.

Abstract

Polygonatum is widespread temperate genus with its greatest species diversity in the Eastern Himalaya and Indo-Burma biodiversity hotspots. A full assessment of the remarkable diversity of *Polygonatum* in these areas will require resolution of its circumscription, and generic and infrageneric relationships, which have been problematic. To obtain a comprehensive data set to resolve the taxonomic issues, the complete plastid genomes of 19 species of *Polygonatum* one of *Disporopsis*, and four species of *Heteropolygonatum* were sequenced with either Illumina or Ion Torrent NGS technology. The plastid genomes range from 153,821 to 155,580 bp in length with no major structural differences. Molecular phylogenetic analyses of the chloroplast coding regions gave evidence that tribe Polygonateae should be narrowed by exclusion of *Maianthemum* to include only *Polygonatum*, *Heteropolygonatum*, and *Disporopsis*. *Polygonatum* and *Heteropolygonatum* were shown to be reciprocally monophyletic, providing support for their recognition as distinct and for recent adjustments of their species composition. An expanded analysis using the *petA-psbJ* plastid gene region combined with nuclear ribosomal ITS data provided support for recognition of three distinct sections within *Polygonatum* that correlate with base chromosome numbers: *P. sect. Sibirica* ($x = 12$); *P. sect. Polygonatum* ($x = 9-11$); and *P. sect. Verticillata* ($x = 13-15$). Two species of *Polygonatum* that had previously been considered to have broad geographic distributions were shown to include multiple species. For *P. multiflorum* the Himalayan species is *P. govanianum*, but for *P. verticillatum* the data suggested that there are multiple Asiatic species for which the proper application of available names still needs to be determined.

Introduction

The Eastern Himalaya and the Indo-Burma ecoregions are two of the biodiversity hotspots that harbor numerous endemic plant species and where the remaining primary vegetation is under threat (Behera et al., 2002; Myers et al. 2000; Paul et al., 2005). Not only are these areas rich in endemic plant species, but recent studies have revealed previously unrecognized diversity in other groups, including geckos (Agarwal et al. 2014), avian fauna (Olsson et al., 2005), fish diversity in northeast India (Goswami et al., 2012), and in fungi (Van de Putte et al., 2012). Despite this, the diversity of some groups in the Himalaya remains understudied partly due to inaccessibility and logistical difficulties in conducting fieldwork there and also because of unrecognized cryptic speciation. As densely sampled molecular studies test the results of traditional taxonomic methods we are likely to see other studies that show that diversity has been underestimated.

The chloroplast genome is generally inherited from the maternal parent and portions of it are conserved across angiosperms so it is easily amplified and sequenced with universal primers to

resolve phylogeny, study population level genetic diversity, possible hybridization, and phylogeography (Birky, 1995; Provan et al., 2001). The genome structure is quadripartite with a large single copy (LSC), a small single copy (SSC), and two inverted repeats with much of the variation in the single copy regions (Provan et al., 2001; Sugiura, 1992). The low level of variability of the chloroplast often requires multiple genes or spacer regions to resolve species relationships (Dong et al., 2012, Shaw et al., 2007, 2014). Comparisons of individual regions across many genera first provided comparisons of potentially variable regions for phylogenetic estimation (Shaw et al., 2007, 2014). More recently the increase in the number of available plastid genomes has increased due to next-generation sequencing (NGS) technologies and have enabled higher taxonomic comparisons to target the potentially more variable regions for use in further phylogenetic studies (Scarcelli et al., 2011; Shaw et al., 2014). Although there has been broad sampling across angiosperms involved in the selection of molecular markers, they are frequently not variable within genera, nor do these regions always resolve close species-pair relationships (Dong et al., 2012). Dong et al. (2012) provided universal primers for 21 highly variable chloroplast loci developed from available plastid genomes of species-pairs from across angiosperms to aid in resolving closely related species. Scarcelli et al. (2011) provided an extensive list of possible monocot specific primers to aid in broad and population level phylogenetic studies. Within the Asparagales many genes have been sampled across families and these combined with data from whole plastid genomes provide sufficient coverage for comparative phylogenetic purposes to resolve familial and generic relationships (Kim et al., 2010; Seberg et al., 2012; Steele et al., 2012; Wilson, 2014), but generic phylogenetic studies have used traditional and limited set of markers that have often proven inadequate (Meng et al., 2014; Wang et al., 2014). These phylogenetically broad comparisons do not always reflect the same amount of diversity when comparing plastid genomes within a genus. It is often necessary to compare multiple plastid genomes within a genus to assess sufficiently variable regions to allow for phylogenetic resolution in larger species sampling efforts.

Within the Asparagales there are few plastid genomes available for comparative purposes (Wilson, 2014). Those available are predominantly from members of Orchidaceae (Kim et al., 2015). Presence of pseudogenes or losses of genes and variable spacer lengths have been reported in Orchidaceae (Chang et al., 2006; Pan et al., 2012; Yang et al., 2013), but other Asparagales available, including two species of *Iris* (Lee et al., 2015; Wilson, 2014), and the monospecific *Eustrephus* (Kim et al., 2016), reveal similar structure compared to other angiosperms. Another potential resource is found in studies that are not phylogenetic in design, but generate high coverage data which are often extensive and can be used for phylogenetic studies. The Sequence Read Archives (SRA) in Genbank is a repository for raw sequence reads from Rad-Seq studies and genome skimming from various NGS platforms. The potential of the SRA may provide extensive amounts of data from which primers for nuclear loci for many groups can be developed or plastid genomes of numerous lineages can be produced for comparative purposes. Here, these data are used to provide sampling of additional outgroups to increase the data in the analyses.

Polygonatum Mill. (Asparagaceae) is an example of a genus in which the greatest diversity is in the eastern Himalaya, including southwest China, and the Indo-Burma region. The genus is relatively large with between 70–80 species and the alpha taxonomic level sampling is still incomplete (Floden, 2015c, 2015e; WCSP, 2015). It is circumboreal in distribution with its

greatest diversity in Asia and particularly the eastern Himalaya and SW China (Meng et al., 2014; Tamura, 1991, 1993). The results reported here suggest that the actual species-level diversity of the genus in the Eastern Himalaya and Indo-Burma regions may be even higher than recognized, because of unrecognized cryptic speciation. The most obvious examples of this may involve individual species that are currently considered to have broad distributions spanning multiple regions or those disjunct within these regions as seen in *Gaultheria* (Lu et al., 2010; Zhang et al., 2017). Under current taxonomic treatments of *Polygonatum* there are several widespread disjunct species with broad, but incompletely understood morphological variation (Chen and Tamura, 2000; Jeffrey, 1980). Two species investigated here, *P. multiflorum* (L.) All. and *P. verticillatum* (L.) All., were described from Europe where they occur contiguously eastward into the Caucasus, but then are also considered to occur disjunct to the northwest Himalaya of India and Pakistan (Ali and Qaiser, 2005; Baker, 1875; Chen and Tamura, 2000; Hooker, 1875; Jeffrey, 1980). The morphological breadth in *P. verticillatum* across this distribution has led to the description of numerous entities, but these are not currently recognized. Similarly, *P. odoratum* (Mill.) Druce occurs from Europe to Japan and molecular data showed that it is paraphyletic (Park et al., 2011) and previous morphological studies have interpreted the variation as clinal with locally distinct geographic entities (Jeffrey, 1980; Tamura, 2009). Previous molecular phylogenetic studies in *Polygonatum* have been limited to employment of chloroplast markers that have proven to be mostly invariant combined with low taxonomic sampling that has not provided the types of data that are need to allow evaluation of whether an entity is a single widespread species or an aggregation of multiple species.

Heteropolygonatum Tamura and Ogisu is a sister genus to *Polygonatum* that differs by its imbricate perigone segments and its base chromosome number of $x = 16$ (Floden, 2014; Tamura et al., 1997). It is distributed in China and adjacent Vietnam though it should also be expected to occur in Myanmar (Floden, 2014). Nearly all species currently recognized in the genus were first described as *Polygonatum* species (Chao et al., 2014; Floden, 2014a, 2014b; Tamura et al., 1997), or were included under a broad circumscription of *P. punctatum* (Floden, 2014b). The monophyly of *Heteropolygonatum* was weakly supported in molecular studies by Tamura et al. (1997) and Wu et al. (2000), but the relationships of the genera of the Polygonateae were not clearly resolved. Meng et al. (2014) in analyses of chloroplast data recovered a *Heteropolygonatum* + *Disporopsis* clade sister to *Polygonatum*, but with relatively low support. Despite this the morphology of *Heteropolygonatum* suggests a closer relationship to *Polygonatum* than it does to *Disporopsis* and we seek to clarify the relationship here with an expanded set of molecular data.

Disporopsis Hance is a genus of ca. 10 species distributed across southeastern Asia with most species endemic to China (Conran and Tamura, 1998; Floden 2015d). The genus is delimited by its rhizome morphology, its evergreen stems, imbricate tepals, the staminal filaments dilated into a corona, and its base chromosome number of $x = 20$ (Conran and Tamura, 1998). Together with *Heteropolygonatum*, *Maianthemum*, and *Polygonatum* these genera form the Polygonateae (Conran and Tamura, 1998).

Next-generation sequencing technologies provide a cost-effective means with which to access extensive amounts of data that can provide insights into the systematic relationships of *Polygonatum* and *Heteropolygonatum*. The technology can be used to recover whole plastid

genomes, mitochondrial data, numerous nuclear markers, and the ribosomal cistron which can aid in resolving species relationships (Straub et al., 2012). Recent phylogenetic analyses of plastid sequence data for *Polygonatum* have provided some resolution with regards to infrageneric classification (Meng et al., 2014), but not species relationships. Chloroplast markers (*matK-trnK*, *trnC-petN*, *trnH-psbA*, and *rbcL*) show limited utility in resolving relationships within major clades (Meng et al., 2014), but resolved a clade containing *Heteropolygonatum* and *Disporopsis* as sister to *Polygonatum*. Broad sampling of *Polygonatum* species and using data from NGS may overcome difficulties in resolving relationships in the genus and also aid in marker development for future work.

Polygonatum is a genus that has multiple economic uses, and an accurate resolution of infrageneric and species relationships in *Polygonatum* using molecular data is needed to understand which species are employed, and monitor potential overexploitation and aid in conservation strategies. *Polygonatum* has value as a food resource (Wujisguleng et al., 2012; Zou et al., 2010), multiple ethnopharmacological uses and pharmacological potential (Bhatt et al., 2014; Khan et al., 2012; Khan and Khatoon, 2008; Liu et al., 2016), and multiple species are also cultivated as ornamentals. Several species are of conservation concern due to exploitation of their medicinal and food resource value. Particularly, *P. govanianum* and *P. verticillatum* are highly vulnerable in the Himalaya with their restricted distributions and their understudied taxonomy (Bhatt et al., 2014; Butola and Bodola, 2008; Chawla et al., 2012; Chung et al., 2014; Hamayum et al., 2006; Kala, 2005; Kala and Ratajc, 2012; Lohani et al. 2012, 2013; Man and Samant, 2011; Poonam et al., 2011; Rafiq, 1995). Several other recently described species are known only from single populations (Cai et al., 2015; Floden, 2015c; Tamura et al., 2014; Zhao and He, 2014), others are narrow endemics (An et al., 2016; Floden 2012, 2014a, 2014b, 2015a, 2015e), and some are known only from their type gatherings (e.g. *P. griffithii* Baker and *P. longistylum* Y.Wan and C.Z.Gao). In contrast some species are broadly circumscribed, with expansive noncontiguous distributions and are also morphologically highly variable. For instance, *P. cyrtoneura* Hua is highly variable in leaf, rhizome, and flower shape and the staminal filaments vary in the position of insertion and their surface texture (Floden, pers. obs.). *Polygonatum cyrtoneura* has also been shown to be polyphyletic (Meng et al., 2014). Moreover, *P. verticillatum* shares similar variation in features while also exhibiting cytological variation (Chen and Tamura, 2000; Jeffrey, 1980; Mehra and Pathania, 1960; Mehra and Sachdeva, 1976) and flavonoid variation associated with the cytotypes (Sachdeva et al., 1986) that hints at unexplored diversity. Molecular phylogenetic studies will aid in uncovering and understanding diversity, biogeography, and cytological evolution in *Polygonatum*.

Here we compare the plastid genomes of 21 *Polygonatum*, one species of *Disporopsis*, and four species of *Heteropolygonatum*. In this sampling we include: two widespread and potentially improperly circumscribed species, *P. multiflorum* and *P. verticillatum*, to compare chloroplast sequences of the European and Himalayan plants to one another; species from seven of Tang's (1978) series divisions; and all three sectional divisions (Meng et al., 2014) with the inclusion of plastid genomes downloaded from Genbank for two species. Analyses of these data seek to address several questions; 1) are *Polygonatum* and *Heteropolygonatum* reciprocally monophyletic when analyzed with higher species and molecular sampling, and are they sister genera or is *Disporopsis* the sister to *Polygonatum*; 2) are the disjunct species of *Polygonatum* composed of independent evolutionary lineages that should be treated as distinct species; and 3)

are there regions of the plastid genome with suitable levels of variability that will aid in future molecular-based studies of *Polygonatum* and the Polygonateae.

Materials and Methods

Sampling

A total of 26 samples of ingroup taxa were sampled here with an additional two species included from Genbank (Table 1.1). One species of *Disporopsis*, four species of *Heteropolygonatum* (including one placed in that genus at its inception and three recently transferred to that genus Chen et al., 2014; Floden, 2014b), one species of *Polygonatum* first described under *Heteropolygonatum* were included (Floden, 2014a). Further sampling in *Polygonatum* included representatives of each of the three sections, as well as from series recognized in earlier taxonomic treatments (Tamura et al., 1997; Tang, 1978) totaling 21 samples. A goal in sampling was to identify variable markers for future molecular work. Other goals in sampling were to assess molecular variation in taxa that are widespread, morphologically variable, or have been placed in synonymy. Two samples of *P. kingianum* Collett and Hemsl. (here treated as *P. huanum* and *P. uncinatum*) were included because of their highly incongruent morphology compared to one another, and two perigone color forms of *P. mengtzense* that grow at different elevations (see Floden, 2014a). Two samples each of *P. multiflorum* (the Himalayan one treated here as *P. govanianum*), and of *P. verticillatum* were included to allow comparison of these widespread species. Additionally, samples of the eastern North American species, *P. biflorum* and *P. pubescens* were included.

Molecular protocols

Total genomic DNA was extracted from live leaf tissue or from silica preserved samples using Qiagen DNeasy Plant Minikits (Carlsbad, CA, USA) following manufacturers protocols with minor modifications. The duration of the heat treatment was increased to 1 hour, and the subsequent cold treatment was 20 minutes which has improved success in PCR and sequencing in *Polygonatum* and related genera. Libraries were prepared from total genomic DNA following manufacturer protocols for their respective sequencing platforms. Five of the samples were prepared and sequenced at the University of Tennessee Genomics Core (<http://mbrf.utk.edu/>) by AF on an Ion Torrent PGMTM (Thermofisher Scientific), and the remaining samples were prepared and sequenced at Global Biologics LLC (Columbia, Missouri, USA) on an Illumina HiSeq with 100 bp reads.

Plastid Genome Assembly

Reference guided assemblies using the nearest reference plastid genome in the Asparagales available, *Asparagus officinalis* (provided by C. Pires, Univ. MO, Columbia), was performed using Geneious 7.2.8 (Kearse et al., 2012). Additionally, the sample of *Polygonatum biflorum* was also assembled de novo using the proprietary algorithm implemented in Geneious for comparative purposes. Of the 10 000 (default setting) contigs assembled, the largest contigs (>5000 bp) were regions of the chloroplast. These were then assembled together manually using a template assembly of *P. biflorum* as a guide. Comparison of the reference guided assembly and the de novo assemblies revealed numerous incongruent regions that necessitated additional checks for accuracy.

Regions of low coverage were checked for accuracy by two methods that achieved improved the accuracy of the assembly. First, an iterative approach to recovering these regions was utilized which was essentially walking the assembly across the genome. Regions between 200–500 bp long without ambiguities that were adjacent to areas of low coverage, or those with greater than 10 ambiguities per 100 bp, were extracted and used as templates. For each series level taxonomic division a single template with the highest coverage was selected, e.g. *P. urceolatum* for the Punctatum-group. The raw reads for each sample were then re-assembled to the templates, using 5–10 iterations at default Geneious assembly parameters. Second, to test for accuracy of walking the assembly across smaller template regions the following regions in the reference guided assemblies that had ambiguous regions were checked by Sanger sequencing for one species within each series division: *accD-psaI*, *atpB-rbcL*, *atpH-atpI*, *clpP-psbB*, *ccsA-ndhD*, *ndhC-trnV*, *petA-psbJ*, *petD-rpoA*, *psaI-ycf4*, *psbD-trnT*, *rpl16* intron, *rpl36*, *rpoB-trnC*, *rpoC1* intron/exon 1, *trnG-atpA*, *trnK-rps16*, *rpl32-trnL*, *trnH-psbA*, *trnS-trnG*, and *ycf1*, and *ycf2* using primers and protocols in Shaw et al. (2007) and Scarcelli (2011).

SRA Plastid Genome Assemblies

Sequence read archives (<https://www.ncbi.nlm.nih.gov/sra>) of several Asparagales genera were downloaded and used to assemble whole or partial plastid genomes for use in phylogenetic analyses (Appendix A). Individual reads were assembled to the *Polygonatum oppositifolium* plastid genome using default Geneious parameters. A consensus sequence with gaps retained of each was made. Then the SRA was reassembled back to the initial template assembly. These assemblies were not fully walked across the genome to provide complete intergenic spacers because only some of the genes were used in the analyses here.

Plastid Genome Annotation

Annotation and visualization of plastid genomes was performed both in Geneious and using CpGAVAS (Liu et al., 2012). For *Disporopsis*, *Heteropolygonatum* and *Polygonatum* plastid genome sequences for one sample of each were fully annotated and all start and stop codons were checked. This fully annotated sample was then used to annotate all other ingroup samples. These were compared to the annotations for the two Genbank samples included here. For the SRA plastid genomes a 75% similarity threshold was used in Geneious to annotate the genes.

Nuclear genome comparison

The availability of some ITS sequences in Genbank for *Polygonatum*, particularly samples of *P. verticillatum* and *P. multiflorum* from India (KM887356.1, KM887393.1, respectively), allowed the comparison here of a common marker from the nuclear genome. Raw reads of the *Heteropolygonatum* and *Polygonatum* samples were assembled to de novo segments of the ribosomal repeat containing 26S, ITS1, 5.8S, ITS2 18S, and ETS, and 5S using default parameters in Geneious. For purposes here only the combined internal transcribed spacer region (ITS1, 5.8S rDNA, ITS2), were used in the analyses. Additional ITS sequences from the Asparagaceae and from *Polygonatum* were downloaded from Genbank (Appendix B). Several additional ITS sequences were obtained for this study for *P. cirrhifolium*, *P. sibiricum*, and *P. verticillatum* from Asia. Protocols for amplification and sequencing followed (Vargas et al., 1998) to complete sampling of taxa within the Polygonateae (Appendix B). Despite the concerns with using ITS (Alvarez and Wendel, 2003; Keller et al., 2010) it has been recommended as a

possible barcode for plants (Chase et al., 2007; Hollingsworth et al., 2011; Kress et al., 2005). Difficulties in sequencing ITS from *Polygonatum* are well known and the China Plant BOL Group (2011) reported a high failure rate in sequencing ITS in *Polygonatum*. Meng et al. (2008) designed *Maianthemum* specific primers for their study, but these failed to amplify samples of *Polygonatum*.

Phylogenetic analyses of the plastid genome based on complete plastid genome gene data

Comparison of regions of the plastid genome were performed using DnaSP v5 (Librado and Rozas, 2009). An alignment of the samples with complete plastomes was assessed for variable regions using the “sliding window” option with a “window length” of 1500 and a “step size” of 500. From the output of DnaSP regions with greater variability were extracted using Geneious. Some of the regions were expanded to incorporate regions of variation adjacent to window. These regions were assessed for the number of variable sites, parsimony informative and uninformative characters, pairwise differences, and indels in each region.

Plastid genome sequences from Genbank (Asparagaceae SRA data, Iridaceae, two species of *Polygonatum* (Appendix A)), and the plastid genomes produced for this study of *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* were aligned using MAUVE whole genome aligner (Darling et al., 2010) in Geneious. Adjustments were made manually to reduce or better align indels. Pairwise comparisons of ingroup taxa (*Disporopsis*, *Heteropolygonatum* and *Polygonatum*) were made with the entire plastid genome. For phylogenetic inference the total alignment was edited to include only gene regions where coverage was present for most taxa limited by the Asparagaceae dataset from Steele et al. (2012). Intergenic spacers were excluded because assemblies of the SRA data were mostly incomplete and the data from Steele et al. (2012). The total aligned matrix of gene regions was 44,066 bp in length.

ITS and ITS + chloroplast analyses

Additional analyses included independent inferences of ITS and the *petA-psbJ* region of the plastid genome. Analyses of the nuclear ribosomal ITS were performed to compare its topology to that of the plastome results and the analyses of *petA-psbJ*. A single locus from the plastome, *petA-psbJ*, was also selected to assess the use of a single marker as in phylogenetic reconstruction with higher sampling and as a possible barcode. The *petA-psbJ* intergenic spacer sampled here was approximately 1700 bp in length and was selected from several candidate regions showing similar amounts of interspecific variation (Supp. material). This region was selected from several variable regions because we encountered difficulties in sequencing the other regions partially due to homopolymers. Amplification primers were designed using Primer3 (<http://bioinfo.ut.ee/primer3/>, Rozen and Skaletsky, 2000) (F- 5'-GGCTTTGAATGTAGGGGCTG-3', R-5'-CGATGGTTAGCTGTTACGG-3'). PCR protocols followed Shaw et al. (2007) with an increase in the elongation step given the length of the selected region. Sequencing used the forward primer designed for this study and the *petA* primer from Shaw et al. (2007) which provided approximately 1700 bp of readable sequence. The two *Polygonatum* samples from Genbank (KT695605.1, KT722981.1) were included even though their *petA-psbJ* intergenic spacers were incomplete based on comparisons to Sanger sequences of the same species. This spacer was excluded for many of the outgroup taxa because it was absent from their source datasets. ITS was used for additional samples of tribe Nolinoideae produced here (Appendix B) and for outgroup taxa from the chloroplast studies

when available. The nuclear ribosomal ITS and chloroplast *petA-psbJ* were concatenated after comparing their resulting phylogenies which had similar topology, but with dissimilar support values.

Phylogenetic methods

Alignments for reduced data analyses were performed with MAFFT v7.017 (Katoh et al., 2002). Indels were viewed as informative characters following the recommendation of Dessimoz and Gil (2010) and coded using FASTGAP 1.2 (Borchsenius, 2009), which uses the simple gap coding methodology of Simmons and Ochotorena (2000). For the plastid genome matrix a single model of nucleotide substitution was selected based on the Akaike Information Criterion (AIC) using MrModelTest v2 (Nylander, 2004). For the combined analyses of ITS + *petA-psbJ* the two loci were tested individually for the best model of nucleotide substitution. For all molecular datasets Maximum Likelihood (ML) analyses were performed using the RAXML 7.2.8 (Stamatakis, 2006) GUI version (Silvestro, 2012). 1000 bootstrap replications with a GTR- Γ -I model of evolution and a search for the best scoring tree were performed. The plastid genome gene analyses were also compared with Bayesian inference which used the MrBayes plugin (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) in Geneious with 10 000 000 generations on four incrementally heated chains sampled every 1000 generations. The burn-in and convergence were assessed using Geneious with the EES for the runs reaching 200, and the burn-in stage trees were excluded. The remaining trees were considered to represent the posterior probability (PP) and internodes with PP > 0.95 were considered to be statistically significant.

Results

Plastid Genome

The number of reads obtained on the Illumina Hi-Seq for the samples of *Heteropolygonatum* and *Polygonatum* ranged from 1.9–19 million 100 bp reads. The number of reads obtained on the Ion Torrent for the five samples ranged from 430k–839k and their read length from 80–600 bp. Whole plastid genomes were recovered for all samples except *P. multiflorum*, run on Ion Torrent, for which only a partial sequence totaling 137,883 bp was recovered. Depth of coverage for the chloroplast ranged between 10–50 $\times$ for the chloroplast for the Illumina reads. Reads from the Ion Torrent resulted in 3–20 $\times$ coverage for four of the five samples. The sample of *P. multiflorum* was incomplete with numerous gaps in the sequence.

Walking the assembly across the genome generally added several hundred bases to the flanking ends of template contigs. This resulted in increased coverage for areas with low coverage, resolved ambiguous characters, and added continuity between contigs. This was often necessary to complete small gaps in the sequence or resolve intergenic spacers. In addition, it was found that the middle of large intergenic spacers that had exhibited numerous ambiguous base calls after assembly to templates were fully recovered with the genome walking approach. These intergenic spacers were frequently several hundred base pairs in length (50–360 bp) that were missed in whole-chloroplast template assemblies. These intergenic spacers were nearly fully recovered in de novo assembly of *P. biflorum* (when using default settings in Geneious). Conversely, this method frequently led to reductions in spacer length and duplications that had been recovered from when assembled to a template. Sanger sequencing provided confirmation

that genome walking was an accurate method for filling in gaps and resolving ambiguities in the template assemblies.

Whole plastid genomes were assembled for nearly all samples. Complete plastid genomes in *Heteropolygonatum* and *Polygonatum* ranged in length from 153,821 bp (*P. orientale*) to 154,995 bp (*P. verticillatum* BSWJ2417). Only partial plastid genomes were assembled for samples of *Disporopsis jinfushanensis*, *Polygonatum multiflorum*, and *P. pubescens*, but the similarity of the nearly complete *Disporopsis* to *P. biflorum* was 97%. The incomplete *Disporopsis* plastid genome was 155,580 bp in length which is larger than all other samples assessed for this study. Across all samples of *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* pairwise plastid genome comparisons showed a range of 95–99% similarity. *Disporopsis* was 95–97 % similar to samples of *Heteropolygonatum* and *Polygonatum*. *Heteropolygonatum* and *Polygonatum* samples ranged from 95–97 % similar to one another. Evaluations of the comparable regions present in both the incomplete plastid genome of *P. multiflorum* and in *P. govanianum* showed that they were 99.31% similar to one another. Comparisons of the two samples of *P. verticillatum* originating from Europe (3862) and from the Himalaya (3860), respectively, showed that they were only 98.5% similar.

Pairwise comparison of only the chloroplast genes of *P. sibiricum* (KT695605.1) and *P. verticillatum* (KT722981.1) from Genbank showed them to be 99.2% similar whereas comparisons of this sample of *P. verticillatum* (KT722981.1) to the two samples of *P. verticillatum* analyzed here showed 98.8% (3860) and 98.9% (3862) similarities. The identity of KT722981.1 is unlikely to be *P. verticillatum*, but rather it is more closely related to, if not conspecific with *P. sibiricum* given the origin of the sample as a Chinese medicinal material. Here it is treated as *P. sibiricum* because of the high similarity to KT695605.1 and the results of the phylogenies (Figs. 1.1–1.2).

The overall structure of the plastid genomes of *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* are typical of most other angiosperms and similar to other Asparagales plastid genomes available for comparison. There are 128 genes in the Polygonateae plastid genome with 75 unique protein-coding genes, 4 rRNA genes, and 31 tRNA genes. The *infA*, *ycf15*, and *ycf68* genes had several internal stop codons each. This suggests that they have become pseudogenes. The large single copy (LSC) and the small single copy (SSC) are ca. 87000 and 19500 bp in length, respectively, and the inverted repeats (IR_A and IR_B) are ca. 25000 bp in length. No structural rearrangements in the boundaries of the inverted repeats were observed among the genera of the Polygonateae. Boundaries of the inverted repeats were identical across all samples, and the positions were similar to those reported for *Iris gatesii* (Wilson, 2014). Variation in sizes of the inverted repeats and LSC and SSC were due to retractions or expansions in genes or spacers, most of which formed characters associated with clades recovered in the phylogeny. The larger, although incomplete plastid genome of *Disporopsis* is largely due to expansions of the intergenic regions. All *Heteropolygonatum* samples exhibited a notable 42 bp loss in *accD* relative to *Disporopsis* and *Polygonatum*. *Heteropolygonatum* had an insertion in the *accD-psaI* spacer that was 109 bp in *H. pendulum* and *H. alternicirrhosum*, but was expanded to 143 bp in *H. altelobatum* and *H. marmoratum*. In the *petA-psbJ* spacer *Heteropolygonatum* samples had a 39 bp loss relative to *Disporopsis* and most *Polygonatum* samples. The Punctatum-group exhibited 25 bp loss starting 1bp upstream from the loss in *Heteropolygonatum*. In contrast to

these and other numerous indels, *Heteropolygonatum* and *P.* section *Polygonatum* exhibited numerous variable length indels and some of these were also seen in *P.* series *Grandiflora* sensu Abramova (1975). *Polygonatum* samples exhibited numerous large differences in indel lengths and positions that correlate with traditionally defined clades (Jeffrey, 1982; Tang, 1978).

Plastid genome phylogenetic analyses and marker analyses

Results of the phylogenetic analyses based on numerous plastid genes did not recover a monophyletic Polygonateae (Fig. 1.1). Instead, *Maianthemum* was placed sister to *Calibanus* and *Dasyilirion* (formerly *Nolinaceae*) (63/0.79, bootstrap proportion/Bayesian posterior probabilities). The succeeding two clades were the Ophiopogoneae (100/1) and the Aspidistreae (97/1). The remaining Polygonateae formed a well-supported clade (100/1). These two clades of the traditionally defined Polygonateae differ in their terminal vs. axillary inflorescences.

Within the axillary flowered Polygonateae *Disporopsis* was recovered as sister to *Heteropolygonatum* and *Polygonatum* (100/1) (Fig. 1.1). All genera of the Polygonateae were monophyletic with full support, although only a single species was sampled for *Disporopsis*. Within *Polygonatum*, *P.* section *Polygonatum* was recovered as monophyletic and sister *P.* section *Sibirica* (100/1). As previously noted a sample (KT722981) identified as *P. verticillatum* but likely misidentified was placed with *P. sibiricum* (100/1). Within section *Polygonatum*, *P. multiflorum* was the earliest diverging lineage (60/0.88) followed by *P. govanianum*. The next two successive clades were the two North American species sister to one another (100/1) followed by *P. yunnanense* sister to *P. acuminatifolium* (85/1) and *P. orientale* + *P. arisanense* (45/0.61). *Polygonatum* section *Verticillata* was monophyletic (100/1). The earliest diverging lineage contained *P. huanum* and *P. uncinatum* (100/1) which was sister to the remainder of section *Verticillata*. The next clade recovered the opposite leaved species (100/1) with the deciduous *P. cathcartii* sister (100/1) to the evergreen species *P. oppositifolium* and *P. tessellatum*. In the next succeeding clades *P. verticillatum* from the Himalaya was the earliest diverging lineage with verticillate leaves (55/0.83) to the two remaining clades that contained *P. stewartianum* and the sample of *P. verticillatum* from Europe (100/1). The last clade within *P.* sect. *Verticillata* included the five samples of the Punctatum-group (100/1) though *P. mengtzensense* was paraphyletic with respect to *P. urceolatum* in the ML analyses, but not in the Bayesian analyses.

Analyses of plastid genome variation

Variable regions of the plastome assessed here can be seen in Table 2. Several regions that were tested for phylogenetic work proved difficult to sequence due to polynucleotide repeats (*accD-ycf4* and *trnV-ndhC*). The *rps4-trnL* region had a high number of variable characters (193) and sequence divergence (37.9%) but this was largely due to a large insertion in samples of *Heteropolygonatum*. A portion of *ycf1* had the highest number of variable characters that were parsimony informative.

Analysis of the *petA-psbJ* region combined the plastome samples with ones obtained by Sanger sequencing (Appendix B). The total *petA* region alignment was 1750 bp in length. The results of the phylogenetic analyses revealed a topology that was similar to that of the analyses of ITS (supp. material) though resolution and support values were low. Indels in the alignment were shared across series divisions (based on morphology and cytology) of *Polygonatum* sensu Tang

(1978) and between genera. Coding indels provided another 239 characters. The axillary-flowered Polygonateae were recovered as monophyletic (99) with high support for *Disporopsis* (100), *Heteropolygonatum* (100), and *Polygonatum* (95). Topology within *Polygonatum* supported the recognition of three sections; *Polygonatum* (86), *Sibirica* (90), and *Verticillata* (74). Within *P.* sect. *Verticillata* the samples of *P. verticillatum* were highly paraphyletic and recovered in several clades. Two clades in *P.* sect. *Verticillata* were recovered with high support; the Punctatum-group (99) and the evergreen series *Oppositifolia* sensu Abramova (1975) that excluded the deciduous *P. cathcartii*. In the Punctatum-group *P. annamense* and *P. punctatum* were sister (100) to a clade with *P. urceolatum* nested within the two samples of *P. mengtzensense*.

Nuclear ribosomal ITS

Nearly complete ribosomal repeats were recovered from the NGS reads and from the SRA data. Here only the internal transcribed spacer region (ITS) was used for analyses. The ITS region was approximately 630 bp in length. The total alignment including outgroups was 692 bp in length with an additional 64 characters after coding for indels. Pairwise comparisons of *Polygonatum govanianum* to *P. multiflorum* revealed only a 96% similarity, whereas comparisons of *P. govanianum* to *P. orientale* or *P. yunnanense* were 99% similar. Samples of *P. verticillatum* were 98% similar to one another.

Two samples of *Polygonatum* from Genbank were excluded from analyses based on comparisons with samples produced here: ITS sequences from *Polygonatum odoratum* (JF421534.1) and *P. verticillatum* (KM887356.1) were not able to be aligned with other *Polygonatum* samples. Pairwise differences were 26% and 79% similar, respectively, to the closest *Polygonatum* samples. BLAST results for JF421534.1 gave 99% similarity to *Veratrum nigrum*. An alignment that included KM887356.1 shows that the sequence was comparable to other *Polygonatum* species upstream of a large poly-G and poly-C region which complicates sequencing efforts in many *Polygonatum* samples, but divergent downstream from it. The China Plant BOL Group (2011) reported a high failure rate in sequencing ITS in *Polygonatum* and Meng et al. (2008) found difficulties in sequencing the sister genus *Maianthemum* probably because of this region. Few additional ITS sequences were produced for this study and others were included from Genbank; several samples of *P. verticillatum*, one of *P. sibiricum*, and several of *P. cirrhifolium* (Appendix B).

Analyses using the ITS data alone failed to recover a monophyletic Polygonateae (supp. material). *Maianthemum*, with low support, was sister to the Ophiopogoneae + Aspidistreae + the remaining genera of the Polygonateae (32). The axillary-flowered Polygonateae were recovered as monophyletic (100). *Heteropolygonatum* was the earliest diverging lineage followed by *Disporopsis*, though support for *Disporopsis* + *Polygonatum* was low (36). Within the monophyletic *Polygonatum* (82) sectional support for *P.* sect. *Verticillata* was high (99), but *P.* sect. *Sibirica* sister to *P.* sect. *Polygonatum* were both low (27, 27). *Polygonatum multiflorum* and *P. govanianum* were not recovered as sister species. The latter was sister to *P. yunnanense* (48), and *P. multiflorum* was sister to *P. cyrtoneura* (30). In *P.* sect. *Verticillata* support values were low, but samples of *P. verticillatum* were paraphyletic. The Punctatum-group was recovered with high support (100).

Plastid + ITS

Phylogenetic analyses using ITS + *petA-psbJ* plastid genome sequence data also revealed Polygonateae not to be monophyletic (Fig. 1.2) with similar topology to that of the plastid genome analyses although major nodes were not well supported (Fig. 1.2). *Maianthemum* (100) was recovered with low support (65) as sister to a monophyletic Ophiopogoneae comprised of *Liriope*, *Ophiopogon*, and *Peliosanthes* (100). The Aspidistreae were paraphyletic with *Aspidistra* sister to *Convallaria* and *Speirantha* (= *Convallarieae*) (81) and *Reineckea* and *Tupistra* (incl. *Rohdea*) sister to these (100). This clade was placed between *Maianthemum* + Ophiopogoneae clade and the axillary-flowered Polygonateae (48). The axillary-flowered genera of the Polygonateae were monophyletic; *Disporopsis* + *Heteropolygonatum* + *Polygonatum* (99). The sister relationship of *Heteropolygonatum* to *Polygonatum* received moderate support (84). *Polygonatum* was monophyletic, but support for this was not high (76). Within *Polygonatum* the three sections of Meng et al. (2014) were recovered with a topology of *P. sect. Sibirica* sister to *P. sect. Polygonatum* (36), and *P. sect. Verticillata* sister to these (65). Clades within *P. sect. Verticillata* that were recovered with high support were the evergreen opposite-leaved species (100) which excluded the deciduous *P. cathcartii*, the Punctatum-group (100), *P. stewartianum* + *P. verticillatum* BO s.n., and *P. huanum* + *P. uncinatum* (97). The relationships in the Punctatum-group were *P. punctatum* sister to *P. annamense* (100) and those were sister to a clade containing *P. urceolatum* sister to the two *P. mengtzensense* samples (99). The multiple samples of *P. verticillatum* were paraphyletic. *Polygonatum* “Himalayan Giant”, sold as *P. verticillatum* in the nursery trade was recovered as the earliest lineage in *P. sect. Verticillata*. Other samples of *P. verticillatum* also from the Himalaya formed a clade nested within *P. sect. Verticillata* and one from China was placed in with several samples of *P. cirrhifolium*.

Discussion

Results of molecular phylogenetic analyses of the gene regions of the chloroplast provided strong support for the reciprocal monophyly of *Polygonatum* and *Heteropolygonatum*, an issue that has not been clearly resolved (Fig. 1.1). The placement of *Disporopsis* with respect to *Heteropolygonatum* and *Polygonatum* also remains to be fully resolved. In the plastid analyses *Heteropolygonatum* was sister to *Polygonatum*, but ITS resolved *Disporopsis* as the sister genus. *Heteropolygonatum* has been significantly altered by the recent transfers of several species formerly included in *Polygonatum* (Chen et al., 2014; Floden, 2014b) as well as by the transfer of one species, *P. urceolatum*, to *Polygonatum* from *Heteropolygonatum* (Floden, 2014a). Molecular support for the monophyly of both *Heteropolygonatum* and *Polygonatum* correlates with a change in base chromosome number that suggests that cytological evolution may be a key aspect in their divergence.

Morphological synapomorphies and the exact species composition of *Heteropolygonatum* have not been evident until recently. The monophyly of *Heteropolygonatum* is well-supported (Figs. 1.1–1.2) both in the whole plastid genome comparisons and also in the combined ITS and *petA-psbJ* analyses which resolve it as sister to *Polygonatum*. Earlier molecular work with a single sample of *Heteropolygonatum* in each study did not strongly support the monophyly of the two genera (Tamura et al., 1997; Wu et al., 2000). Recent analyses with four species of *Heteropolygonatum* using several chloroplast markers place it sister to *Disporopsis* (Meng et al., 2014), but the results here a much larger sampling of the plastid genome placed *Heteropolygonatum* as sister to *Polygonatum*. Results of plastid and ITS + *petA-psbJ* sequence

data the monophyly and relationships of *Disporopsis* as sister to *Heteropolygonatum* and *Polygonatum* are recovered, but only well-supported in the former (Fig. 1.2). These molecular data support the recent circumscription of *Heteropolygonatum* characterized by its base chromosome number of $x = 16$ and the morphological synapomorphy of imbricate tepals (Floden, 2014b; Tamura et al., 1997).

The analyses of the combined chloroplast *petA-psbJ* genes and spacer region and ITS sequence data provide clarification of the circumscription and placement of Polygonateae, and also provide insights into the relationships of several outgroup taxa. These data support the monophyly of a Polygonateae comprised exclusively of *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* but excluding *Maianthemum*. The exclusion of *Maianthemum* from this clade is in contrast to recent systematic assessments, including some based on molecular phylogenetic data (Conran, 1989; Conran and Tamura, 1998; Dahlgren et al., 1985; Meng et al. 2008, 2014; Seberg et al., 2012). Nonetheless, it can be noted that Baker (1875) recognized that *Maianthemum* was not closely allied to Polygonateae when he placed *Maianthemum* in his tribe Tovariae which included *Speirantha* among the genera sampled here, rather than in the Convallarieae in which *Polygonatum* was placed. The results of our analyses placed *Maianthemum*, with a base of $x = 18$, as sister the Ophiopogoneae with a base of $x = 18$ (rarely 17) rather than sister to the variable Polygonateae which have $x = 9–20$ (Conran and Tamura, 1998; Wang et al., 2013). *Maianthemum* has a strictly terminal inflorescence whereas the other Polygonateae have axillary inflorescences (*Heteropolygonatum* and some *Polygonatum* species can have a pseudoterminal inflorescence at the terminal leaf (Floden, 2014a)). In Chen et al. (2013) and Meng et al. (2014) *Maianthemum* was included in the Polygonateae based on four plastid markers, but in Seberg et al. (2012) where multiple loci from the plastid genome and from the mitochondrion were sampled, *Polygonatum* was resolved sister to *Ophiopogon* whereas *Maianthemum* was placed sister to the remaining Nolinoideae. In Kim et al. (2010) ML analyses recovered a paraphyletic Polygonateae where *Disporopsis* was placed sister to the Dracaenaceae and *Polygonatum* and *Maianthemum* were sister genera. In the same study Bayesian analyses recovered a monophyletic Polygonateae with *Disporopsis* sister to a clade of *Polygonatum* and *Maianthemum*. Kim et al. (2012) showed a weakly supported monophyletic Polygonateae sister to the remaining unresolved Nolinoideae, but *Heteropolygonatum* was not sampled. Wang et al. (2014) recovered a weakly supported Polygonateae (51/66) with *Heteropolygonatum* sister to *Polygonatum*. The results here recover similar conflicting topologies of the axillary-flowered Polygonateae and exclude *Maianthemum* in all analyses.

Molecular phylogenetic results (Figs. 1.1–1.2) provided further evidence for the division of *Polygonatum* into three major clades corresponding to sections *Polygonatum*, *Sibirica*, and *Verticillata* (Meng et al., 2014). In addition to morphology, these sections are largely characterized by a constant or a distinct range of chromosome numbers that have been well documented (Tamura, 1990, 1993, 1997; see Zhao et al., 2014 for an overview). Deng et al. (2009) and Zhao et al. (2014) determined that three distinct groups could be recognized that correspond to the molecular resolution of sectional divisions in Meng et al. (2014) and are also supported by the results here; these being *P. sect. Polygonatum* ($x = 9–11$), *P. sect. Sibirica* ($x = 12$), and *P. sect. Verticillata* ($x = 13–15$). Further divisions within sections have been shown to be paraphyletic (Meng et al., 2014) though several series sensu Abramova (1975) and Tang (1978) within *P. sect. Verticillata* were well supported (Figs. 1.1–1.2). *Polygonatum* series

Grandiflora, represented here by *P. huanum* and *P. uncinatum*, was placed sister to the remaining clades comprising *P.* section *Verticillata* in the plastid analyses, but within *P.* sect. *Verticillata* in the combined analyses. Series *Oppositifolia* and series *Punctata* were also well supported within the broadly delimited *P.* sect. *Verticillata*. Though taxonomic sampling here is still relatively low, with only 19 species of *Polygonatum* out of an approximate 80 or more, preliminary data suggests (not shown) that with higher sampling these series level divisions are still well supported.

The low taxonomic sampling in the analyses of the plastid genome and of ITS+*petA-psbJ* which included six species of *Polygonatum* sect. *Polygonatum* did not recover previously proposed divisions based on filament shape and chromosome numbers (Figs. 1.1–1.2). Similar results were reported by Tamura et al. (1997). Traditional morphological series in *P.* sect. *Polygonatum* were based on the presence of foliaceous bracts on the inflorescence, perigone size, stem shape, and the staminal filament shape (Abramova, 1975; Komarov, 1935; Tamura, 1991, 1993, 1997; Tang 1978). Later classification also incorporated chromosome number; series *Bracteata* with $x = 9$, series *Inflata* with $x = 11$, and series *Polygonatum* with $x = 10$ (Tamura, 1993, 1997). Tamura et al. (1997) and Meng et al. (2014) showed that there are clades with multiple base numbers suggesting there is labile dysploidy in this section. Subclassification schemes of *P.* sect. *Polygonatum* should incorporate additional species and more molecular loci to enable resolution of relationships. A proper series level classification will aid in the understanding the relationships of these species and their biogeography is of interest because *P.* sect. *Polygonatum* is the only section in the genus that occurs in the New World.

The species of *Polygonatum* series *Punctata* were shown to be clearly monophyletic and nested within *Polygonatum* sect. *Verticillata* (Figs. 1.1–1.2). *Polygonatum annamense* and *P. punctatum* were recovered sister to one another, but *P. mengtzense* was paraphyletic with respect to *P. urceolatum* in the plastid genome based ML phylogeny (Fig. 1.1), but not in the Bayesian analyses. Inclusion of ITS data recovered both *P. mengtzense* samples as sister to *P. urceolatum* (Fig. 1.2). *Polygonatum mengtzense* is interesting in that it occurs as two color forms that grow at different elevations in Vietnam, but apparently co-occur in adjacent China (Floden 2014a). Not only are the perigone colors different, but the higher elevation red form can be distinguished by its papillose stem, papillose leaf surface at magnification, and more prominently zig-zag stem (Floden, 2014a). The lower elevation form appears more similar to *P. urceolatum*, e.g. white perigone and epapillose leaves, to which it was placed sister in the analyses of the gene regions of the plastid genome (Fig. 1.1). Nonetheless the two samples here, FMWJ 13292 (high elevation) and FMWJ13066 (low elevation), are recovered as sister in the combined analyses (Fig. 1.2). These analyses do not include all the species in this group which may change the topology and relationships recovered here. Additional analyses will need to include multiple samples of *P. mengtzense* and *P. urceolatum*, and also the recently described *P. costatum* (Floden, 2015c) to better understand the relationships within *Polygonatum* series *Punctata*.

Detailed analyses of plastid genome sequence data showed that two different species of *Polygonatum* are polyphyletic under their current circumscriptions (Figs. 1.1–1.2). The two species are *P. multiflorum* (previously including *P. govanianum*) and *P. verticillatum*, both of which are considered to have widespread geographical distributions extending non-contiguously from Europe to Asia. Based on plastid genome sequence data for each species the European and

Asian populations of each species should be recognized as distinct species (Figs. 1.1–1.2). For *P. multiflorum* and *P. govanianum*, additional data from analyses of ITS with an additional sample of *P. govanianum* supported their separation as distinct and placed *P. govanianum* as sister to *P. yunnanense*. Data from ITS analyses that included multiple samples of *P. verticillatum* provided additional evidence of its polyphyly where the Asiatic samples did not form a single entity but were shown to be highly polyphyletic (Fig. 1.2).

The current circumscription of *Polygonatum multiflorum* is here narrowed to include only material from Europe and westernmost Asia in the Caucasus. The material previously included in *P. multiflorum* from the Himalaya should be recognized as a distinct species, for which the name *P. govanianum* applies (see below). The two species are supported as distinct lineages by the molecular data and also can be differentiated by data from morphology and cytology. Despite some morphological similarities, there are distinct differences between *P. govanianum* and *P. multiflorum*. In *P. govanianum* the primary abaxial veins of the leaves are scabrellous and the minutely papillose filaments are inserted proximal of the middle of the tube, which is in contrast to the glabrous leaves and pubescent filaments inserted distal of the middle in *P. multiflorum*. Secondly, the perigone is generally larger in *P. govanianum* than in *P. multiflorum* (14–20 vs. 10–12 mm). Moreover their base chromosome numbers differ: *P. multiflorum* has a base of $x = 9$ (Baltisberger et al., 2002; Suomalainen, 1947) and *P. govanianum* has a base of $x = 11$ (Mehra and Pathania, 1960; Mehra and Sachdeva, 1976; Pandita and Mehra, 1982). Mehra and Pathania (1960) reported counts of $2n = 2x = 22$ from Dalhousie and Simla (Dan Kund and Kala Top, and Narkanda and Huttoo, respectively), India, and Mehra and Sachdeva (1976) reported the same number from the Nainital Hills. Pandita and Mehra (1982) reported the same number from Kashmir, but two collections from Ahrabal and Zojila were reported by them with $2n = 2x = 28$. It is unclear what these plants represent, but the vouchers are sterile, and the number corresponds to a diploid with $x = 14$, so the identity of this sample is uncertain. Also, the geographic distributions of *P. govanianum* and *P. multiflorum* are wholly allopatric and they occupy different habitat types. *Polygonatum multiflorum* occurs in Europe and adjacent Asia where it is distributed across Turkey and parts of the Caucasus. It is a ubiquitous component of mesic woodland throughout Europe (Hermy et al., 1999). *Polygonatum govanianum* is endemic to the NW Himalaya in northeast Pakistan, Kashmir, and NW India and occurs in mixed coniferous forest (Chawla et al., 2012; Hooker, 1875; Ilyas et al., 2013; Kala and Ratajc, 2012). Of the species of *P. sect. Polygonatum*, only *P. orientale* occurs in the intervening region between these two areas, and it is distinguished from both by its pubescent abaxial leaf surfaces and smaller perigone.

A second case involving the need to revise the classification of a species previously interpreted to be widespread involves *Polygonatum verticillatum*, which probably includes multiple distinct species as currently circumscribed. Understanding the amplitude of variation in morphology among the verticillate species of *Polygonatum* in Asia is known to present difficulties (Chen and Tamura, 2000; Jeffrey, 1980, 1982; Noltie, 1994). Molecular data (Figs. 1.1–1.2) revealed that *P. verticillatum* is polyphyletic. In the plastid genome data *P. verticillatum* from Europe was placed as sister to *P. stewartianum*, a species morphologically distinct from *P. verticillatum* (Tamura, 1993), from China whereas the sample of *P. verticillatum* from the Himalaya was placed as the sister lineage to two clades; one containing *P. verticillatum* and *P. stewartianum*, and the other formed by the Punctatum-group. In the tree based on the ITS + *petA-psbJ* similar topologies

were recovered, but inclusion of additional samples showed further dimensions of the paraphyly of the current circumscription of *P. verticillatum* (Fig. 1.2). The level of variability in morphology and molecular sequence data suggests that there is not one, but rather multiple species currently treated under the name *P. verticillatum* in Asia. There are multiple names available for those from India and the Sino-Himalaya and plants from this region vary in their flower color, leaf shape, leaves per whorl, the presence or absence of cirrhose leaf apices, habitat, altitudinal distribution, and chromosome number. Under current circumscription of a broad *P. verticillatum* between the European and the Indian sample examined here there are documented counts of $x = 14$, the only count for *P. verticillatum* documented in Europe, and $x = 14$ – 15 in Asia where polyploids have been documented for both base numbers (Mehra and Pathania, 1960; Mehra and Sachdeva, 1976; Pandita and Mehra, 1982; Sachdeva et al., 1986; Sachdeva and Malik, 1977). Some cytotypes correlated with flavonoid profiles (Sachdeva et al., 1986), suggesting that they may be distinct entities. It is unclear which of the numerous potential names apply to the various samples of Himalayan *P. verticillatum* included in the analysis. The Himalayan sample from Sikkim (BSWJ2417) used for the plastid genome data is morphologically distinct from the European plant and from *P. stewartianum*. This sample of *P. verticillatum* is sister to another sample of *P. verticillatum* from the same region and these were in turn sister to *P. leptophyllum* also formerly placed in synonymy with *P. verticillatum* (Fig. 1.2). Moreover, the Sikkim sample has a different chromosome number ($2n = 4x = 60$, Floden, unpublished) and *P. stewartianum* and *P. verticillatum* both have $x = 14$. Other samples of *P. verticillatum* are also morphologically distinct from the two used for plastid genome samples. Additional extensive sampling from Asia is required to determine the diversity in this region currently placed under the name *P. verticillatum*.

The results of the analyses of ITS and *petA-psbJ* data showed that several other taxa appear to be non-monophyletic. One example is the verticillate leaved *Polygonatum cirrhifolium* from the Sino-Himalaya. Two samples, JF977841 and JF977842, obtained from Genbank were not placed with other samples of the species (Fig. 1.2). The other samples were placed in two weakly supported clades sister to one another. Under its current circumscription *P. cirrhifolium* is highly variable in perigone color, shape, and the insertion position of the staminal filaments. There is also cytological variation across a broad area of distribution from Nepal eastward to central China (see Zhao et al., 2014). These results show that the presence of a cirrhose leaf apex is not a unifying character defining a single species or species group. It is present in the *P. kingianum*-group, *Polygonatum* sect. *Sibirica*, numerous other verticillate-leaved species, and also even in *Heteropolygonatum alternicirrhosum*.

The monospecific *Polygonatum* sect. *Sibirica* was only recently elevated to sectional rank, and the results here corroborate its recognition as a distinct section (Meng et al., 2014). However, there appears to be some confusion regarding identification of samples of *P. sibiricum*. The species is morphologically distinct based on a combination of characters including verticillate leaves with cirrhose apices and a style that is two to three times as long as the ovary (Chen and Tamura, 2000). Collections out of anthesis are hard to differentiate from other verticillate-leaved species in areas of sympatry. Our ITS + *petA-psbJ* data for a sample of this species (CPC 01553B) from northeast Sichuan are congruent with results from samples in Genbank (KT695605.1, KT722981.1, the latter is reported as *P. verticillatum*). Based on its placement in the tree in a clade with *P. cirrhifolium* (Fig. 1.2), a third sample of *P. sibiricum* from Genbank

EU850003 appears to be misidentified. No samples from the Himalaya received under that name share molecular substitutions with *P. sibiricum* from north-central and northeast China (Floden, unpublished data) nor do they have the long style characteristic of this species. This raises doubts about whether *P. sibiricum* actually occurs in the Himalaya, despite the fact that this name has long been used for Himalayan plants (Baker, 1875; Clarke, 1876; Deng et al., 2009; Mehra and Pathania, 1960; Mehra and Sachdeva, 1976; Noltie, 1994; Sen, 1973).

The North American species of *Polygonatum* are well-differentiated in their morphology. *Polygonatum biflorum* is glabrous and *P. pubescens* has pubescent abaxial leaf surfaces. They also differ in their filament shapes, but share the same chromosome number. Comparison of their plastome, partial in the latter species, shows similar pairwise differences to other closely related species, differing by 0.5–1%. The biogeographic origin of the NA species is presumed to be of eastern Asian origin across the Bering land bridge (Wang et al., 2016). Nonetheless, in Meng et al. (2014) *P. biflorum* and *P. pubescens* were sister to *P. filipes* and *P. desoulavyi* from eastern and northeastern China. In Wang et al. (2016) *P. multiflorum* was sister to the *P. filipes* + *P. desoulavyi* clade and these sister to the two NA species. These two topologies are conflicting and further taxonomic sampling is required to resolve the North American species origin and assess the variation in *P. biflorum*.

Phyllotaxy in *Polygonatum* has traditionally been used as the major character to separate sections in the genus (Abramova, 1975; Baker, 1875; Komarov, 1935; Meng et al., 2014; Tamura, 1997; Tang, 1978), but the results presented here suggest that the understanding of this trait is still incomplete. Noltie (1994) noted the artificiality of subdividing the genus on the basis of phyllotaxy due to the observed variation for the trait within a single species. Meng et al. (2014) assessed the evolution of phyllotaxy within *Polygonatum* and found that it is labile, with whorled leaves having arisen twice. Within *P. sect. Verticillata* the Punctatum-group has reverted mostly to alternate phyllotaxy, but two Punctatum-group species included in the plastid genome data occasionally have opposite leaf pairs or a terminal whorl; *P. punctatum* and *P. annamense* (Fig. 1.1). *Polygonatum oppositifolium*, *P. cathcartii*, and *P. tessellatum* did not form a clade in the combined ITS and *petA-psbJ* despite the results of the plastid genome analyses in which they formed a clade. These species are mostly strictly opposite though *P. cathcartii* can occasionally have a terminal whorl of three leaves (Chen and Tamura, 2000; Noltie, 1994) and *P. tessellatum* has whorled leaves throughout. The placement here of *P. tessellatum* sister to *P. oppositifolium* suggests a reversion to whorled leaves after a loss of that character in this clade. The labile nature of phyllotaxy in the genus is still not fully understood with only limited sampling of taxa thus far in molecular analyses and future work may reveal more complexity and variation in this trait within clades.

The results of analyses based on complete plastid genome data were successful in resolving the broad relationships in *Polygonatum* and also in resolving the three accepted sectional divisions. Assessment of variable regions of the plastid genome (Table 2) provide some regions to test for future phylogenetic work. Two regions here based on primers from Scarcelli et al (2011) did not provide clean sequence for *accD-ycf4*. Primers designed to test *ndhC-trnV* and *rps4-trnL* also did not provide clean sequence even though amplification was successful. Utility of a single marker, *petA-psbJ*, tested here provides resolution of sections and some series within *P. sect. Verticillata* and recovers a topology that is almost totally congruent with the one based on four

markers (Meng et al., 2014). As a single marker this serves to distinguish sectional placement and series placement of most species tested (Floden, unpublished). In some cases *petA-psbJ* has sufficient characters to delimit some species or species pairs within series, e.g. *P. mengtzense* and *P. urceolatum* from *P. punctatum* and *P. annamense*. Of the variable regions the portion of *ycf1* assessed here may provide the best single marker from the plastid genome for phylogenetic work or barcoding utility. The low level of variability of regions of the plastid genome make it necessary to use multiple markers to accumulate enough characters which should be combined with mitochondrial and nuclear genomic data to resolve relationships of sections and series and also delimit species.

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Appendix A

Chloroplast genomes from Genbank and from the Genbank Sequence Read Archive (SRA)*.

Asparagus racemosus SRX460513*, *Aspidistra saxicola* SRX313502*, *Iris gatesii* Foster KM014691.1, *I. sanguinea* Hornem. KT626943.1, *Polygonatum sibiricum* Redouté KT695605.1, *P. verticillatum* KT722981.1, *Sansevieria trifasciata* Prain SRX268224*.

Appendix B

Nuclear ribosomal ITS and *petA-psbJ* sequences from Genbank* and produced for this study; species, Genbank accession ITS, *petA-psbJ*, collector, and herbarium.

Asparagus officinalis L., HM357931.1*, *Aspidistra hainanensis* Chun and How, _____, *Floden s.n.* (TENN), *A. sp.*, _____, *Floden s.n.* (TENN), *Convallaria majalis* L., _____, TCM (TENN), *Disporopsis bodinieri* (H. Lév.) Floden, _____, *Floden et al. 1924* (TENN), *Iris laevigata* Fisch. ex Fisch. and C.A. Mey, DQ277643.1*, *Liriope sp.*, _____, CPC _____ (TENN), *Maianthemum racemosum* (L.) Link, _____, (TENN), *Maianthemum racemosum* var. *amplexicaule* (Nuttall) LaFrankie, _____, *Floden & Schilling* __, *Maianthemum stellatum* (L.) Link, EU850032.1*, *Maianthemum stenolobum* (Franchet) S. C. Chen and Kawano, _____, *Floden* ____ (TENN), *Ophiopogon bockianus* Diels, _____, CPC (TENN), *O. clavatus* C. H. Wright ex Oliver, _____, *Floden s.n.* (TENN), *O. clavatus*, _____, MD10-173 (TENN), *O. sp.*, _____ FMWJ13427 (TENN), *Peliosanthes macrostegia* Hance, _____, *Floden s.n.* (TENN), *Polygonatum cirrhifolium* (Wall.) Royle, EU850005.1*, *P. cirrhifolium*, JF977841.1*, *P. cirrhifolium*, JF977842.1, *P. cirrhifolium* JF977843.1*, *P. cirrhifolium*, JF977844.1*, *P. cirrhifolium*, JF 977845.1*, *P. cyrtoneura* Hua EU850001.1*, *P. govanianum* KM887393.1*, *Polygonatum leptophyllum* (D. Don) Royle, _____, Bajhang (RBGE), *Polygonatum sibiricum* Redouté, _____, CPC 01553B (TENN), *Polygonatum sp.* “Himalayan Giant,” _____, *Floden* ---- (TENN), *P. verticillatum* (L.) All., _____, Manaslu (RBGE), *P. verticillatum*, JF977847.1*, *Reineckea carnea* Andrews, _____, *Floden s.n.* (TENN), *Rohdea japonica* (Thunb.) Roth, _____, *Floden s.n.* (TENN), *Speirantha gardenii* (Hook.) Baill., _____, *Floden s.n.* (TENN), *Tupistra sp.*, _____, *Floden s.n.* (TENN), *Tupistra sp.*, _____, *Floden s.n.* (TENN), *T. tonkinensis* Baill., _____, FMWJ (TENN).

Appendix C

Table 1.1. Plastid genome sequences or partial plastid genomes used here. Taxon, voucher, Genbank numbers (to be submitted). “–” indicates only partial recovery for sample. All vouchers at TENN.

Taxon	voucher	Genbank #
<i>Disporopsis jinfushanensis</i> Z. Y. Liu	MD10-117	–
<i>Heteropolygonatum altelobatum</i> (Hayata)	BSWJ8961	####
Y.H.Tseng, H.Y.Tzeng & C.T.Chao		
<i>H. alternicirrhosum</i> (Hand.-Mazz.) Floden	Floden s.n.	####
<i>H. marmoratum</i> (H. Lév.) Floden	Floden 2458	####
<i>H. pendulum</i> (Z.G.Liu & X.H.Hu) M.N.Tamura & Ogisu	Wang s.n.	####
<i>Polygonatum acuminatifolium</i> Komarov	Floden s.n.	####
<i>P. annamense</i> Floden	BSWJ9752	####
<i>P. arisanense</i> Hayata	BSWJ1369	####
<i>P. biflorum</i> Ell.	Floden s.n.	####
<i>P. cathcartii</i> Baker	BSWJ2429	####
<i>P. govanianum</i> Royle	CC4752	####
<i>P. huanum</i> H. Lév.	Floden 2461	####
<i>P. mengtzense</i> F.T. Wang & Tang	FMWJ 13292	####
<i>P. mengtzense</i> F.T. Wang & Tang	FMWJ 13066	####
<i>P. multiflorum</i> (L.) All.	TCM10-164	–
<i>P. oppositifolium</i> Royle	BSWJ2375	####
<i>P. orientale</i> Desf.	TCM s.n.	####
<i>P. pubescens</i> (Willd.) Pursh	Floden 2376	–
<i>P. punctatum</i> Royle	BSWJ2395	####
<i>P. stewartianum</i> Diels	CLD325	####
<i>P. tessellatum</i> F.T. Wang & Tang	MW275	####
<i>P. uncinatum</i> Diels	BO33	####
<i>P. urceolatum</i> (J.M.H. Shaw) Floden	Floden 2416	####
<i>P. verticillatum</i> (L.) All.	BO s.n.	####
<i>P. verticillatum</i>	BSWJ2417	####
<i>P. yunnanense</i> H. Lév.	BO-2011	####

Table 1.2. Comparison of character differences in chloroplast markers with high variability.

Locus	<i>accD-ycf4</i>	<i>clpP-psbB</i>	<i>ndhC-trnV</i>	<i>ndhF-rpl32</i>	<i>petA-psbJ</i>	<i>rps4-trnL</i>	<i>trnL-ndhJ</i>	<i>trnS-rps4</i>	<i>trnQ-rps16</i>	<i>ycf1-partial</i>
Locus Length (bp)	1649	1521	1896	1630	1442	1803	2476	2270	1616	1677
Constant positions	1528	1448	1823	1512	1387	1610	2408	2221	1482	1504
Uninformative positions	103	40	42	62	20	162	26	17	63	107
Parsimony informative positions	18	33	31	56	35	31	42	32	71	66
Variable positions	121	73	73	118	55	193	68	49	134	173
Sequence divergence (%)	0.2–11.38	0–4.7	0.43–14.04	0.69–9.35	0.18–7.08	0–37.9	0.38–16.02	0.14–16.02	0.03–11.16	0.21–12.25
Total indels	5	7	13	13	11	8	29	17	13	7

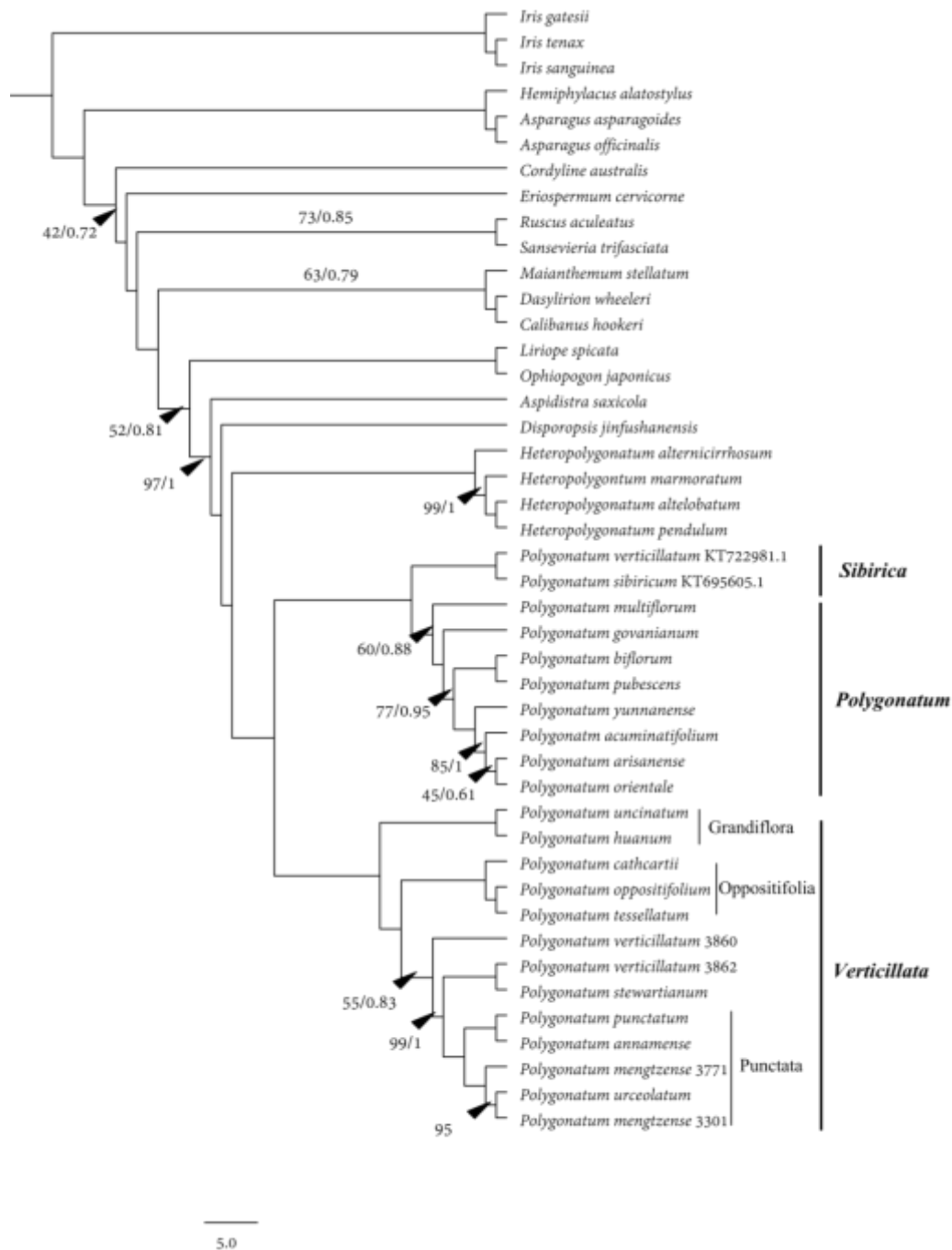


Figure 1.1. Phylogeny of *Heteropolygonatum* and *Polygonatum* using 44 066 bp of the coding regions of the chloroplast on a proportional branch length best-scoring Maximum Likelihood tree. Branch supports < 99/1 are shown and are Maximum Likelihood bootstrap proportion values from 1000 replications and Bayesian posterior probabilities from 10 000 000 iterations (ML/PP). The ML results did not resolve *P. mengtzense* and *P. urceolatum*, but Bayesian analyses recovered *P. mengtzense* as monophyletic, thus the PP value is not shown here. Sectional divisions within *Polygonatum* are in bold with monophyletic series divisions in section *Verticillata* labeled.

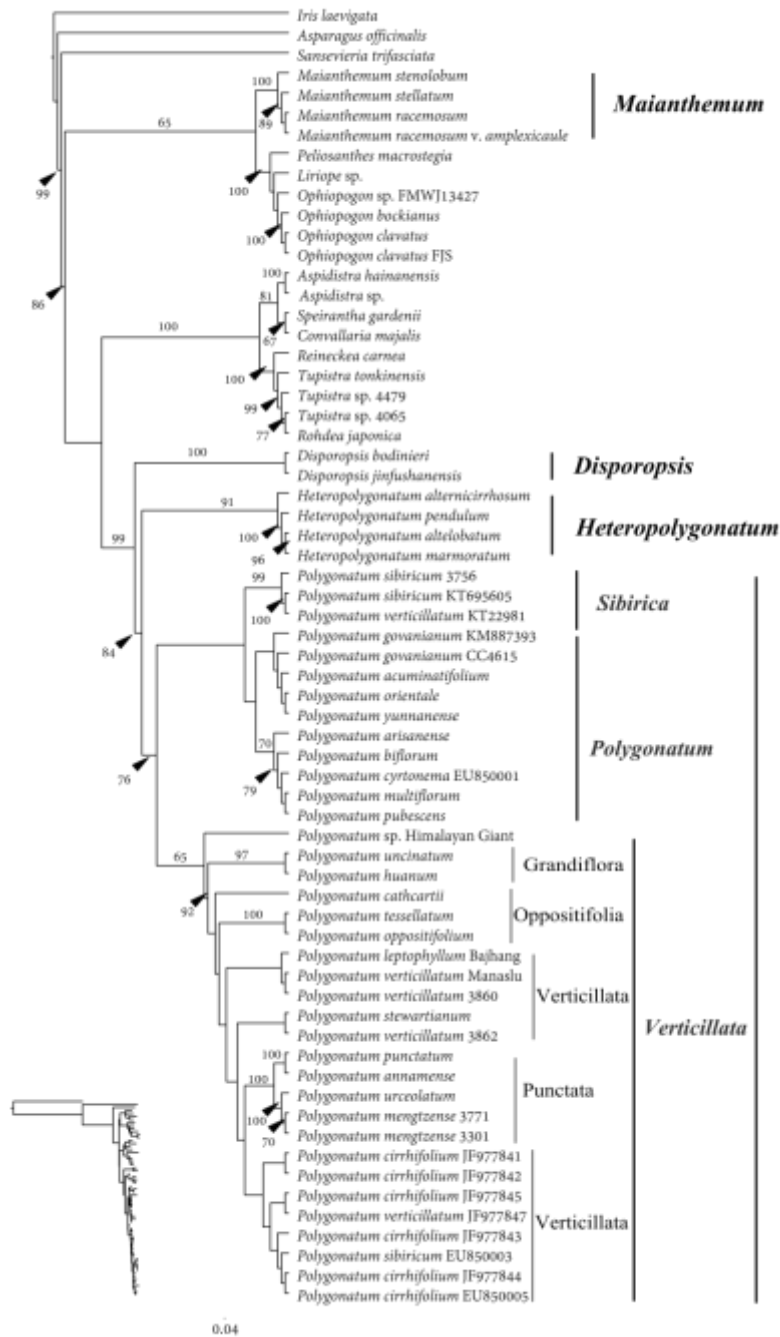


Figure 1.2. Maximum likelihood phylogeny with proportional branch lengths inferred from the combined plastid *petA-psbJ* and ITS datasets showing relationships of *Polygonatum* and related genera. ML bootstrap support values ≥ 65 are shown from 1000 bootstrap replications. The inset shows branch lengths of the phylogram. Genera of the *Polygonateae* s.l. are labeled and the sections of *Polygonatum* are in bold italic with the series of *P.* sect. *Verticillata* labeled.

Chapter 2

Resolving *Polygonatum* (Asparagaceae); a phylogeny based on molecular data from the nuclear and plastid genomes.

This chapter has been written for submission to *The Botanical Journal of the Linnaean Society*.

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My primary contributions to this paper included study design, molecular work, data analyses, and most of the writing.

Abstract

The Polygonateae (Asparagaceae) have been shown to include three with a moderate level of species diversity. *Disporopsis* and *Heteropolygonatum* with 10 species each, and *Polygonatum* with 80+ species. Previous molecular phylogenetic estimations of *Polygonatum* have been limited in scope and not resolved species relationships, but revealed sectional. Here we provide a first comprehensive phylogeny of the Polygonateae using four molecular markers, chloroplast DNA (*petA-psbJ*), nuclear ribosomal ETS and ITS, and mitochondrial *rps10*, and 285 accessions including most 9 *Disporopsis*, 6 *Heteropolygonatum*, and 66 species of *Polygonatum*. We performed maximum likelihood and Bayesian analyses on the individual and concatenated datasets. These data support the mutual monophyly of the genera of the Polygonateae. The results support previous conclusions of three sectional divisions; *P.* sect. *Polygonatum*, *P.* sect. *Sibirica*, and *P.* sect. *Verticillata*, with some series classification schemes supported within the latter section. These data also reveal novel placements for several species; *P. wardii* as sister to *P.* sect. *Verticillata*, and *P. brevistylum* and *P. nervulosum* embedded in *P.* series *Oppositifolia*, and show that the diversity within *Polygonatum* has been greatly underestimated.

Introduction

For botanists in North America and Europe, *Polygonatum* (Asparagaceae: Nolinoideae) is a ubiquitous and pedestrian genus with a few woodland species that are well-differentiated from one another. It is only in Asia where the genus exhibits striking diversity in habit, leaf arrangement, perigone color and structure, and occupies diverse habitats from subtropical low elevations to high alpine scree (Tamura et al. 1997, Jeffrey 1980). There it has also been appreciated as a crude drug of the traditional medical systems across Asia and is also widely consumed as a vegetable (Khan et al. 2012, Khan and Khatoon 2008). Despite some recent studies, the full diversity of the genus in Asia is still not well appreciated or understood. The current study makes a much broader sampling than has been previously attempted.

Polygonatum is the largest genus in tribe Nolinoideae which contains such arid-growing genera as *Nolina*, *Beaucarnea*, and *Dasyllirion* (Conran and Tamura 1998). In contrast, *Polygonatum* and the other three genera in the subtribe Polygonateae, *Disporopsis*, *Heteropolygonatum*, and *Maianthmeum* (incl. *Smilacina*) are generally forest dwelling herbs (Conran and Tamura 1998). *Disporopsis* and *Heteropolygonatum* are both endemic to Southeast Asia and have fewer than a dozen species in each (Floden 2014, 2015) whereas, *Polygonatum* has over 80 species which range from sea level to over 4500 m elevation and occur variously in mesic forest understories in temperate climates (Jeffrey 1980), as epiphytes in cloud forests (Floden 2014, 2015), and frequently in *Kobresia*-alpine habitats in the Himalaya (Noltie 1994). *Polygonatum* is circumboreal in distribution, but its center of diversity is in southwestern China and the eastern Himalaya where approximately 40 of the 80 or more species occur (Chen and Tamura 2000,

Meng et al. 2014, WCPS 2015). The genus ranges from the Arctic Circle in the north and to as far south as the Dalat Plateau in southern Vietnam.

Morphologically *Heteropolygonatum* and *Polygonatum* are well-defined (Tamura et al. 1997a, Floden 2014) and recent molecular data from the maternally inherited chloroplast support their generic distinction (Meng et al. 2014). *Heteropolygonatum* has a base chromosome number of $n = 16$ and has imbricate tepals whereas *Polygonatum* species have a range of base numbers from $n = 9–15$ and they have valvate tepals (Tamura et al. 1997, Floden 2014). Examination of morphological characters has also shown that the diversity in *Heteropolygonatum* is greater than had been estimated with several species added nearly doubling the size of the genus (Floden 2014).

Polygonatum has a long taxonomic history, but it remains comparatively understudied despite its significant ethnobotanical history. The last comprehensive systematic revision of *Polygonatum* dates to Baker (1875) in which only 23 species were included. Since Baker's (1875) treatment the number of species included in the genus has risen to approximately 83 (Floden 2015, WCSP 2015) with over 200 proposed names (IPNI 2016). More recently, cytotaxonomic and morphological studies, and infrageneric classification schemes have focused on a limited number of species of the genus and encompassed only some of the diversity (Tamura 1991, 1993, 1997). Revisionary studies (Tamura 2009, Jeffrey 1980, 1982) and floristic studies have been constrained by their regional perspective (Komarov 1935, Abramova 1975, Tang 1978) and these are often incongruent with one another on the circumscription of species recognized when the regions covered overlap. These studies have frequently resulted in misapplication of synonyms in several taxa and resulted in undescribed diversity in the genus (An et al. 2016, Cai et al. 2015, Floden 2012, 2013, 2014a, 2014b, 2015, Tamura et al. 2014, Zhao and He 2014).

Infrageneric classifications of *Polygonatum* vary in the morphological characters or cytological data that they utilize and in their ultimate arrangements (Fig. 2.1). Many follow Kunth (1850) and Baker (1875) in first breaking the genus into three groups based on phyllotaxy; the arrangement of the leaves which are alternate, opposite, or verticillate. Abramova (1975) classifies the groups also using stem, floral, and cytological data. Tang (1978) further subdivides the genus, but only considering the Chinese species, into numerous series using a similar suite of characters, some of which are unique to Eastern Asian species, e.g. the foliaceous bracts on the inflorescences of some species (except for some teratological forms). Jeffrey (1980, 1982), following Tang's classification, provides an account of the Eastern Asian species (including Japan and Korea). Tamura (1993, 1997) offers an alternative scheme using chromosome number and characters of the staminal filaments resulting in an alternate leaved *P.* section *Polygonatum* with base chromosome numbers of $x = 9–11$, and all other species placed in section *Verticillata* with $x = 12–15$. Molecular data contradicts this and supports two larger sections, *P.* sect. *Polygonatum* and *P.* sect. *Verticillata*, and a monospecific section, *P.* sect. *Sibirica* (Meng et al. 2014). Nonetheless, the molecular analyses of Meng et al. (2014) sample only 30 of the 83 species. A useable, natural classification based on molecular data, or molecular and other characters, for *Polygonatum* are desirable to understand their relationships, aid floristic studies, benefit conservation strategies, and facilitate description of additional novelties.

Series level classifications within sections of *Polygonatum* have been proposed by numerous authors (Komarov 1935, Abramova 1975, Tang 1976 (see Jeffrey 1982), Tamura 1991, 1993, 1997). To delimit these series the authors have used numerous characters to define them such as phyllotaxy, stem characters, cytology, perigone shape, and the shape and surface of the filaments (Komarov 1935, Abramova 1975, Tang 1976, Tamura 1991, 1993, 1997, Tamura et al. 1997). Early phylogenetic work does not support series divisions in *P. sect. Polygonatum*, but several series within *P. sect. Verticillata* are well-supported (Floden and Schilling unpubl.). In addition to shared base chromosome numbers within proposed series there are karyotypic differences, growth habit, seed shape and testa characters, and fruit colors that have not been previously used to delimit series. Moreover, limited pharmacological studies have shown that some species might have polysaccharides and steroidal compounds that are possibly restricted to series (Khan et al. 2012). Dense taxonomic sampling and multiple loci will provide better understanding of *Polygonatum* species relationships at the subsectional level that will aid in understanding evolution of morphology, cytology, chemical compounds with pharmacological potential, and biogeography.

Though numerous classification schemes have been promulgated based on morphological and cytological characters, many species still have never been examined in detail and placed within an infrageneric classification, and for many the placement has not been tested by molecular data. *Polygonatum brevistylum* and *P. nervulosum*, both Sino-Himalayan species were placed in *P. section Polygonatum* based on their alternate leaves (Baker 1875), but no cytological or life history data are known for these two species nor have more recent classification systems included them. The latter was described from Sikkim, but is also known from Bhutan (Noltie 1994), whereas *P. brevistylum* is now known from the Indo-Burma region of the eastern Himalaya (Floden unpubl.). Another species, *P. wardii* is known from only a few collections other than the type and was not observed by Chen and Tamura (2000) for the Flora of China treatment though it was included. It has also not been placed into a section or series which may present a challenge because its phyllotaxy is variably alternate, opposite, or whorled on a stem. At least one other species from Central Asia, *P. sewerzowii*, is placed as a monotypic series (Abramova 1975), though it appears morphologically similar to *P. sibiricum*. It differs from *P. sibiricum* in its tubular perigones and the presence of single leaves occasionally between whorls. Higher taxonomic sampling will undoubtedly better resolve infrageneric relationships in *Polygonatum* and provide a better background to reveal cryptic species.

The recent descriptions of numerous novel species has revealed previously undocumented diversity in *Disporopsis* (Floden 2014, 2015), *Heteropolygonatum* (Floden 2014a, Chao et al. 2013), and *Polygonatum* (An et al. 2016, Cai et al. 2015, Floden 2014a, b, 2015, Tamura et al. 2014, Zhao and He 2014). The need for systematic work in this group is made evident by these recent discoveries eastern Himalaya, Indochina, and the Indo-Burma ecoregions. Most of the novel species have limited areas of distribution (Floden 2014a, b, 2015) or are known from single localities (Tamura et al. 2014, Zhao and He 2014). Not only are these species rare, but several of them have characters or combinations of characters not previously reported in *Polygonatum*, e.g. *P. luteoverrucosum* and *P. angelicum* have opposite leaves and verrucose perigone surfaces (Floden 2015), *P. gongshanense* has opposite leaves, an elongated 3-lobed ovary apex, and spurred staminal filaments (Zhao and He 2014), and *P. campanulatum* G.W. Hu has alternate leaves, a broadly campanulate yellow perigone, and spurred staminal filaments (Cai

et al. 2015). These discoveries highlight the need for continued exploration, but they also emphasize the need for molecular phylogenetic studies to place these in the larger context of the classification scheme of *Polygonatum*.

The results of molecular phylogenetic data from the whole chloroplast genome data show high paraphyly and polyphyly in several species groups in *Polygonatum* that suggest there is a need for extensive systematic work in the genus (Floden and Schilling unpubl., Meng et al. 2014). Limited molecular and taxon sampling in Meng et al. (2014) (30/83+ spp.) and the absence of numerous potentially evolutionarily significant taxa, e.g. those with synapomorphies or apomorphies, presents an opportunity to resolve the infrageneric relationships in *Polygonatum*. In Meng et al. (2014) *P. cyrtoneura* was recovered in three clades, and *P. cirrhifolium* was unresolved. Floden and Schilling (unpubl.) resolved the taxonomy of *P. govanianum* and *P. multiflorum* and uncovered polyphyly in the concept of *P. verticillatum* in phylogenetic comparisons of the coding regions of their plastomes. Additionally, Park et al. (2011) uncovered paraphyly in the widespread *P. odoratum*. Broader taxonomic and molecular sampling will help to resolve these and other taxonomic problems in *Polygonatum*.

Despite a long history of botanical exploration in the Himalaya and southwest China, many of the plant groups are still poorly understood on a systematic basis. This is due to multiple factors that affect field work necessary for study which include high elevation, rugged topography and poor accessibility, and unpredictable weather that make planning in advance difficult. Not only have the field conditions hampered progress in many groups and in *Polygonatum*, there are also other factors one must take into account. The flowering season of *Polygonatum* species can vary from the early spring at lower elevations and early to mid-summer for the higher elevation species which makes for multiple field excursions to cover a single area. Not only are there elevation gradients within regions, but the great latitudinal range of the genus, from southern Vietnam to Siberia, make many successive field seasons requisite. Due to this great range of flowering periods field exploration is often limited to a particular group or elevation range to collect specimens in anthesis. Study of specimens is vital to resolving their systematics. Unfortunately, many specimens are collected while still in bud or in early fruiting stages, and those collected in anthesis are not always properly prepared. Frequently specimens in flower do not have any flowers dissected so that the staminal filaments are visible. If the perigone is not cut lengthwise before preservation the tissues of the filaments and the perigone wall often join into a single mass so that determination can be difficult. When comparing closely related species the most important morphological features are the shape of the perigone and the internal position of the staminal filaments and their shape. Species of *P.* section *Polygonatum* often occur in monospecific populations, but the higher elevation members of *P.* sect. *Verticillata* in the Himalaya and Hengduan Shan of SW China can co-occur with three or more species at a site (P. Bruggeman pers. comm, Floden unpublished data).

The goals of this study are to infer a highly sampled species level phylogeny across all former series level classification schemes (see Abramova 1975, Tang 1978, Jeffrey 1980, 1982) to provide a better understanding of the infrageneric relationships within *Polygonatum*, and the generic relationships of *Heteropolygonatum* and *Disporopsis* with respect to *Polygonatum*. Dense species sampling, and the first sampling of many species, permits the opportunity to assess infrageneric relationships in *Polygonatum*. In some cases species boundaries are unclear,

e.g. *P. verticillatum* (Floden and Schilling unpubl.), and sampling in these species complexes seeks only to assess the variation and not to resolve species boundaries. In other cases only a single exemplar could be obtained. With these data we assess the relationships of the three axillary-flowered genera of the Polygonateae and the infrageneric relationships within *Polygonatum*. This phylogeny presents the most comprehensive species sampling of the genus using multiple loci to provide an up to date phylogeny for *Polygonatum* and the Polygonateae.

Materials and Methods

Taxonomic Sampling

Nomenclature generally follows Chen and Tamura (2000) for most Chinese species though not necessarily their taxonomic concepts, Jeffrey's (1980) taxonomy for many Chinese species, Tamura (2009) for Japanese species, Noltie (1994), and Floden (2012, 2013, 2014a, b, 2015) for species in eastern Asia where the greatest diversity exists and the taxonomy, though well-studied, remains unresolved. In addition some names proffered here are from older floras and were used to accommodate samples that do not fit a currently accepted taxon in the above works, but do exhibit molecular and morphological differences that warrant recognition. Sectional divisions follow Tamura (1997) and Meng et al. (2014). Series level divisions follow a summation of Tang (1978) and Abramova (1975) in Jeffrey (1980), excluding the concept of series *Sibirica* which is now recognized as a distinct monospecific section (Meng et al. 2014).

A total of 285 samples were included in the total matrix which included 66 species, at least four undescribed species, and 70+ taxa of *Polygonatum* (Table 1). All European (5/5), North American (3/3), and Japanese (9/9, but only 1 nothospecies) species were included. Species from all three sections and from the series level classification *sensu* Tang (1978) were included. Multiple samples were made of widely distributed species from disparate parts across their ranges, and of morphologically variable species, e.g. *P. cirrhifolium* and *P. verticillatum* s.l. to include the range of molecular, morphological, and also cytological variation. *Polygonatum govanianum* (as *P. multiflorum* KM887393, Malik and Babbar, unpubl.) was included here only in the ITS analyses. In addition partial ITS sequences of *P. cobrense*, from the United States southwestern mountains, and *P. sewerzowii* from Central Asia, are included only in the ITS analyses. Sampling of closely related genera included most of the described species of *Disporopsis* (9/10) with high sampling in the morphologically variable *D. arisanensis* and *D. undulata*, and *Heteropolygonatum* (6/10). Outgroup taxa included at least one species of each genus of the former Convallariaceae (in which *Polygonatum* was also placed) which are now included in a broadly delimited Asparagaceae.

Extraction

Total genomic DNA was extracted using Qiagen DNeasy Plant Mini Kits (Qiagen, Valencia CA) from fresh cultivated material for the majority of samples, with a small number of samples taken from herbarium vouchers (Table 1). Adjustments were made to the standard protocols including increasing the duration of the heat treatment to 1 hour at 60 C, and the icing step to 15 minutes.

Both nuclear and chloroplast loci were analyzed. Amplifications were performed in 20 µl reactions using 10–20 ng of genomic DNA, 10× PCR buffer (Promega), 1.8–2.25 mM MgCl₂, 0.2 mM each dNTP, 1.25 units of Taq polymerase, and 0.2 µM each primer. A portion of the nuclear external transcribed spacer (ETS) region was amplified and sequenced using 18sETS

(3'TGCTACCTGGTTGATCCTGC) and a forward primer (5'GGTTTTGGATMTGCATGTCCC) designed using Primer3 (Untergasser et al. 2012) based on results from next generation sequence (NGS) data. A portion of the mitochondrial *rps10* gene was identified from de novo assemblies of NGS data (Adams et al. 2000, 2002). Primers for the region were designed using Primer3 (Untergasser et al. 2012). This segment was amplified using the primers (F-CCACCTTCTTGCTCAATCCAC, R- TATCGAGAGGTTGGTGTAGTACA) and sequenced with the forward primer which was located just upstream of a small segment where the majority of the sequence variation was observed across the tribe Nolinoideae outgroups and within the Polygonateae. This region was selected due to nucleotide changes shared among species in sections and series. The chloroplast locus included a portion of the *petA* gene and *petA-psbJ* intergenic spacer. This was amplified with primers developed from NGS plastome data for Polygonateae samples (F- GGCTTTGAATGTAGGGGCTG, R- CGATGGTTAGCTGTTCACGG). Sequencing used the forward primer designed for this study and the *petA* primer from Shaw *et al.* (2007). This provided a larger segment of the *petA* gene and most of the *petA-psbJ* intergenic spacer than the primers in Shaw et al. (2007). This region incorporated fixed differences and indels associated with informal taxonomic groups in our plastome studies (Floden and Schilling unpubl.).

Nuclear ribosomal ITS region

Previous analyses of *Polygonatum* and some related genera have not included the nuclear ribosomal internal transcribed spacer (ITS), one of the proposed standard barcodes (Li et al. 2011, Michel et al. 2016), either due to difficulty in PCR or in obtaining clean sequencing data. To obtain sequence data for *Maianthemum*, Meng et al. (2008) reported using specifically designed primers which have not proven successful for other genera. These primers (MF and MR), a suite of other universal ITS primers (ITS 1–5, ITS-Leu), and four primers designed for this study were tested in various combinations for *Polygonatum* and *Maianthemum* with nearly 100% success in amplification, but only about 20% success rate in obtaining clean sequence data. Internal primers anchored in the 5.8s region were used in various combinations with external primers and gave good amplification, but often performed no better in obtaining clean sequence. Li et al. (2011) had similar difficulties and reported a 70% failure rate in sequencing ITS for *Polygonatum*. Genera of the Aspidistreae (including Convallarieae), *Peliosanthes* in the Ophiopogoneae, and *Disporopsis* were exceptions and sequence was obtained under standard protocols without the need to employ to special protocols (see below). After attempting multiple published and newly designed primers the nuclear ITS region of *Heteropolygonatum* and *Polygonatum* was ultimately amplified and sequenced with ITS-Leu and ITS-4 (Vargas et al. 1998)) after PCR amplification was done using the a special protocol described below.

Amplification and Sequencing of nuclear ribosomal ITS

Cloning of some of the PCR products from ITS obtained using standard universal ITS primers (ITS4 and ITS-Leu or ITS4 and ITS5) was done to determine if multiple copies of ITS were present in a single sample. Three samples representing two sections of *Polygonatum* were used. Purified PCR products were ligated into pGEM-T (Promega, Madison, WI) following the manufacturer's instructions. Competent Top10 F9 (Invitrogen, San Diego, CA) cells were transformed via electroporation. At least six positive colonies per plate were sequenced. For only one sample, *P. orientale* SB sn, was there sequence variability among clones, with three different sequences of different lengths. Results of a BLAST search of the clone (clone1) most similar in

length and sequence showed 98–99% identity percentages to *Polygonatum* samples in Genbank, but sequences obtained for the other two clones (clone2 and clone 3) were only 89–91% similar to *Polygonatum* in Genbank. Comparisons of the three clones obtained from the above sample showed different nucleotide substitutions that accounted for the dissimilarity in addition to their sequence lengths, 500 for clone2 and 769 bp for clone3, compared to ca. 600 for the average length of the ITS region in *Polygonatum* and clone1. Cloned sequences that were highly similar in BLAST search results to *Polygonatum* ITS sequences showed 98–99% similarity to ITS sequence data assembled from Illumina NGS data (Floden and Schilling unpubl.). Despite cloning the ITS region from three samples, there was no evidence that more than a single copy of ITS was present. This was also consistent with assembly of the ITS region from 22 *Polygonatum* from NGS data.

Single Molecule PCR

A third approach to obtaining clean ITS sequence data for *Polygonatum* involved single molecule PCR (smPCR; Marcussen et al. 2012). Aliquots of DNA extracts that were diluted 1:10 or 1:30 (depending upon DNA concentration measured by a NanoDrop 2000 (ThermoScientific)) were run in PCRs of 4–8 reactions per sample using primers ITS-4 and ITS-Leu. For samples used here dilutions between 1:10 and 1:30 were found to be the weakest dilutions that would still amplify. All successful amplifications (usually fewer than 40% of reactions) were sequenced. Often amplification of some samples failed completely and these were run again with the same concentration of template DNA until a band was observed. All successfully amplified samples were sequenced using one or both amplification primers. The protocol resulted in as few as one to as many as seven successful PCR amplifications being obtained from single samples, and when multiple amplifications were obtained the resulting sequences were identical to one another. This method also recovered only a single copy from *P. orientale* SB sn which had given three copies when cloned. This suggests that cloned products and direct sequencing from undiluted DNA resulted in co-amplified products rather than multiple copies of ITS even though the secondary products were similar to ITS in BLAST results. For some DNA extracts from herbarium specimens of *Polygonatum* that did not amplify using the standard protocols, it was possible to obtain sequence using this method. For presumably degraded herbarium or silica preserved samples the internal primers, ITS2 and ITS3, were coupled with the external primers ITS4 and ITS-Leu, respectively, to obtain partial ITS sequence data; which lacked only a segment of the invariable 5.8s region. For nearly all *Heteropolygonatum* and *Polygonatum* samples smPCR was used to obtain ITS sequence data.

PCR products were purified and sequenced using ABI Prism BigDye Terminator cycling conditions at the Biology Molecular Resource Facility at the University of Tennessee on an ABI 3100 automated sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA). Chromatograms were edited in Geneious 7.1.9 (Biomatters Ltd., New Zealand). For some samples sequences were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

Phylogenetic analyses

Sequences were aligned using default settings in Mafft v7.023b (Katoh et al. 2002) and adjustments made manually in Geneious and/or MEGA6 (Tamura et al. 2013) to better align repeat regions and provide consistent alignment for putatively homologous indels. Indels were treated as characters because they carry important phylogenetic signal (Dessimoz and Gil 2010,

Warnow 2012) and there were observed indel events associated with former series level divisions *sensu* Tang (1978). Gaps were coded with Fastgap 2.1 (Borchsenius 2009) which uses the simple gap-coding methods of Simmons and Ochotorena (2000). Individual loci were analyzed independently to compare their topologies. Nuclear ribosomal ETS and ITS were treated as a single locus in the final analyses. MrModeltest v2 (Nylander 2004) was used to determine the best model of nucleotide substitution based on AIC. Tree were estimated using RaxML 7.2.8 (Stamatakis 2006) and Mr. Bayes 3.2.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003, Ronquist et al. 2012). RaxML used rapid tree search of 1000 replicates to assess bootstrap support values (Felsenstein 1978) under a model of GTR- Γ -I. Bayesian analyses were performed in Geneious using the MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) plugin. The Bayesian MCMC algorithm was run for 10 000 000 generations with four incrementally heated chains starting with random trees and sampling one per 1000 generations. These were run to convergence based on the average standard deviation of split frequencies falling below 0.01 and ESS values rising above 200. After the burn-in trees (first 25%) were excluded, the remaining trees were considered to represent the posterior probability (PP) of distribution.

Results

Sequence data for 282 taxa in the Nolinoideae and Polygonateae are newly reported here (Table 2.1). Although DNA extracts from live material were easily amplified most extracts from herbarium preserved samples proved recalcitrant to PCR amplification. Even extracts made from recent herbarium collections (collected on or after the year 2000) either failed to amplify or were only successful for very short sequence segments <300 bp in length, e.g. *Polygonatum* cf. *nervulosum* Miede 00-002-06. Silica dried samples also gave significantly reduced quality and quantity of DNA when compared to fresh DNA extractions as measured on a NanoDrop 2000. For example, a fresh extraction of *P. uncinatum* (B11) measured 68.1 ng/ μ l of DNA with 260/280 of 1.81 whereas a fresh silica preserved sample of *P. uncinatum* (B33) was 8.9 ng/ μ l of DNA with 260/280 of 2.62. Another older silica preserved sample of *P. verticillatum* (Gagnidze et al. 214) measured only 1.8 ng/ μ l of DNA with a 260/280 of 1.21.

The plastid *petA-psbJ*, mitochondrial *rps10*, and nuclear ETS regions were readily amplified by PCR when using fresh material of *Polygonatum*. The *petA* primer from Shaw et al (2007) failed as a sequencing primer in all samples of *Disporopsis* giving difficult to read sequence and their *psbJ* primer did not provide clean sequence reads for most taxa. Only partial *petA* sequences were included for outgroup genera of tribe Nolinoideae and *Disporopsis* due to difficulties in sequencing the complete region used for *Heteropolygonatum* and *Polygonatum* samples. For *rps10* amplification in some outgroup samples was unsuccessful in some samples of outgroup taxa, e.g. *Convallaria*, *Maianthemum*, *Ophiopogon*, *Peliosanthes*, and *Speirantha*. The use of *rps10* was thus restricted to ingroup taxa; *Disporopsis*, *Heteropolygonatum*, *Maianthemum*, and *Polygonatum*. It proved difficult to obtain clean sequence across the entire ETS region for some samples because of the presence of two variable length internal polynucleotide repeats. Higher template input in PCR of the ETS region often resulted in brighter and more defined bands in gel electrophoresis which often resulted in cleaner sequence reads. But, it was difficult to obtain clean sequence data for the ETS region from certain species groups, e.g. *P. cyrtoneura* and *P. series Grandiflora*, though the lengths of the polynucleotide repeats did not differ from closely

related species groups. When amplification and sequencing failed repeatedly for a locus in some taxa those samples were not included in individual data matrices.

Phylogenetic Analyses

The results of phylogenetic analyses of the individual loci show support for outgroup tribe relationships and section divisions in *Polygonatum* (Figs. 2.2–2.5). Intergeneric relationships of the outgroups were similar across loci. The individual data sets revealed short backbone branch lengths, but high support across the loci for many for clades within *Polygonatum* (Figs. 2.2–2.5). A total evidence approach of the four combined loci was used, but data matrix lengths and topologies for the individual loci are briefly described below. Support values given in individual analyses are bootstrap proportions from maximum likelihood results. Support value ranges of 85–100 are considered well-supported, 75–84 as moderate, and <75 as weakly supported. In the combined analyses support values are given as bootstrap/posterior probabilities where the topologies of the trees are congruent. Significant incongruent placement of branches or positions of clades that differ between the two phylogenetic methods are mentioned in the results.

ETS

The alignment for the ETS region was 449 bp in length and 509 bp in length with gaps coded. Sequences across samples ranged from 381–420 bp in length. Part of the disparity in length differences were due to the trimmed ends of some *P. cyrtonema* samples. The Aspidistreae, Convallarieae, and Ophiopogoneae were monophyletic (Figs. 2.2). Polygonateae was not recovered as monophyletic; *Maianthemum* was placed sister to the Ophiopogoneae (Fig. 2.2). Topologies within the remaining Polygonateae placed *Heteropolygonatum* + *Disporopsis* + *Polygonatum* as sister genera with high support.

ITS

The aligned ITS region, including portions of the adjacent 18SrDNA and 28SrDNA was 716 bp in length and 781 bp in length with indel coding. Sequences across samples ranged from 636–651 bp in length. The trees obtained from analysis of ITS data were topologically similar to those obtained from ETS with a few exceptions (Figs. 2.2–2.3). *Convallaria* was sister to *Speirantha*, and *Aspidistra* was recovered as a distinct clade (Fig. 2.2) (93) not sister to the other genera of the Aspidistreae which formed a clade (99). *Maianthemum* was placed sister Ophiopogoneae. Within the remaining Polygonateae the genera were resolved as *Disporopsis* + *Heteropolygonatum* + *Polygonatum*. The three sections in *Polygonatum* were resolved, but were not well-supported. In ITS, for which some taxa were only sampled for this locus, the results of their placement are significant (Figs. 2.2–2.3). *Polygonatum govanianum* SBB-1268 and the sample obtained for this study were sister to a clade containing *P. megaphyllum*, *P. nodosum*, and *P. yunnanense*. A partial sequence of *Polygonatum cobrense* with several heterozygous base calls was placed sister to *P. biflorum* 3664, in a paraphyletic clade including the North American species. A partial ITS sequence for *P. sewerzowii* resulted in a placement that was sister to the remainder of the genus (tree not shown), though pairwise comparison of ITS1 and ITS2 to *P. sibiricum* and *P. zamlanscianense* was ca. 98% which was similar to the pairwise difference between *P. sibiricum* and species of either *P.* section *Polygonatum* or *P.* section *Verticillata*.

ETS + ITS

The aligned matrix for the combined ETS and ITS was 1151 bp in length and 1298 bp in length with the indels coded as characters. Topology of the outgroups was similar to the ETS results (Figs. 2.2–2.3). *Maianthemum* was not placed as sister clade to the Ophiopogoneae, but instead placed sister the Ophiopogoneae + the remaining Polygonateae. Within the axillary flowered Polygonateae analyses of ETS + ITS placed *Disporopsis* as sister to *Polygonatum* (100), and *Heteropolygonatum* sister to these two genera (100) (Figs. 2.2–2.3). Infrageneric relationships within *Polygonatum* had higher support values over a similar topology of the individual analyses of ETS and ITS. All three sections of *Polygonatum* were recovered. Within *P.* section *Verticillata* there was weak support (21) for *P.* series *Oppositifolia* which included the alternate leaved species *P. brevistylum* and *P. nervulosum*. *Polygonatum* series *Grandiflora* was monophyletic (53) with *P. vietnamicum* sister to the remainder of the species (90), but their relationships were largely unresolved. The next successive clade was a group of unresolved Sino-Himalayan verticillate species followed by a well-supported series *Punctata* (98). The remainder of the clades of section *Verticillata* was a mixture of the European *P. verticillatum* forming a clade and numerous Sino-Himalayan species that were mostly not well-resolved. *Polygonatum* section *Polygonatum* was not well resolved in the ETS + ITS dataset with low support and short branch lengths (tree not shown).

Plastid *petA-psbJ*

The plastid *petA-psbJ* region and intergenic spacer was 1686 bp in length without the indels coded and 1800 bp in length with coded indels. Sequences across samples ranged from 1260–1546 bp in length. Sequencing difficulties in *Disporopsis* and other outgroups required that the sequences were trimmed preceding a large polynucleotide repeat which resulted in a region with a range of 1260–1270 bp in length. *Heteropolygonatum* samples ranged from 1404–1507 bp in length; several species had two large indels near the middle of the intergenic region. In *Polygonatum* the range was 1367–1546. *Polygonatum zamlanscianense* and DNA samples 4429 and 3772 shared large deletions in the *petA* region, 1596 bp in the former and 1679 bp in length for the latter two. The trees that were obtained based on *petA-psbJ* sequence data included a polytomy of the Ophiopogoneae + *Maianthemum* + Polygonateae (100) (Fig. 2.2). *Heteropolygonatum* was sister to *Polygonatum* (98) with *Disporopsis* sister to these (96) (Fig. 2.2). Relationships within *Polygonatum* were largely unresolved and sectional divisions were not recovered (Fig. 2.3). Within *Polygonatum* there was a large series of polytomies and very short branches with some well-defined clades. Most of *P.* section *Polygonatum* was recovered as a large polytomy, but some species and species groups were moderately to well-supported.

rps10

The mitochondrial *rps10* alignment was 419 bp in length before gap coding and 445 with gaps coded. Samples ranged from 373–382 bp in length. This marker had a generally low amount of variation within genera. Results of *rps10* did not resolve the relationships of the successfully amplified outgroup taxa to one another, nor were the samples of *Maianthemum* or *Ophiopogon* recovered as monophyletic (Fig. 2.3). Variability in *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* was restricted to differences between clades or species groups. Within *Polygonatum* the infrageneric relationships recovered in analyses of *rps10* were mostly unresolved (Fig. 2.3). Series *Punctata*, *Oppositifolia*, and *Grandiflora* formed clades on a large polytomy in which the relationships of the sections to one another were unresolved. This locus

had fixed differences that were associated with sectional and series divisions, some species groups within series, and also for some species (e.g., *P. autumnale*, and *P. sp. Macer sn*). Within *P. series Punctata* samples of *P. punctatum*, *P. annamense*, and *P. costatum* differed from samples of *P. mengtzense* and *P. urceolatum*.

Combined Analyses

The total DNA matrix of the concatenated loci with indels coded was 3512 bp in length. Based on the individual and combined analyses *Maianthemum* should be excluded from the *Polygonateae*, and for the purposes of the remainder of this paper *Polygonatae* will be considered to consist of only *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* (Figs. 2.4–2.5). The trees obtained from analysis of the combined data (Figs. 2.4–2.5) showed similar topology to ITS alone, but some nodes had lower support values. Outgroup relationships recovered were *Speirantha* + *Convallaria* (100/1), *Aspidistra* sister (97/1) to *Reineckea* + *Tupistra* (including *Rohdea*) (100/1) which comprise the Aspidistreae. *Maianthemum* was placed sister (64) to a monophyletic Ophiopogoneae, *Peliosanthes* + *Liriope* + *Ophiopogon* (100/1) in the ML analyses, but placed in a polytomy with the Ophiopogoneae and the remaining Polygonateae in the Bayesian analyses. Sister to these was the remaining Polygonateae (100/1). The remaining genera of the Polygonateae, i.e., those with axillary inflorescences, were *Disporopsis* (100/1) sister to *Heteropolygonatum* (100/1) + *Polygonatum* (100/1) (Figs. 2.4–2.5).

The results of the combined data analysis provided some resolution of species relationships within both *Disporopsis* and *Heteropolygonatum* (Figs. 2.4–2.5). *Disporopsis longifolia* was placed as sister to the remainder of the species in the genus (94/1). The next successive clades in *Disporopsis* were *D. aspersa* sister to *D. yui* (41) and this clade sister to the remaining species (84/1). The ML and Bayesian analyses were incongruent; in the former support values were low, but in the latter there was a large polytomy. In *Heteropolygonatum*, *H. alternicirrhosum* was the earliest diverging species in that genus (100/1) followed by *H. roseolum* (97/1) (Fig. 2.5). *Heteropolygonatum marmoratum* samples were polyphyletic (Fig. 2.4). Two samples were sister to the remaining species of *Heteropolygonatum* (86/1) which were split into two clades; one containing the other samples of *H. marmoratum* sister to *H. altelobatum* (75/1), and this clade sister (49/0.65) to *H. anomalum* that was placed sister to *H. pendulum* (91/1) placed sister (94/1) to the two samples of *H. ginfushanicum*.

Within *Polygonatum* several clades were recovered that are consistent with proposed sectional subdivisions and some traditional series divisions, but also provided some novel results (Fig. 2.5). *Polygonatum* section *Sibirica* (70/1) was sister to *P. section Polygonatum* (92/0.99) with high support (97/0.98). In section *Polygonatum* support values were low in the backbone of the clade. Species groups were generally well-supported. Sequence variation was observed in some widespread species, e.g. *P. odoratum* and *P. orientale*. Despite exhibiting sequence variability, the *P. orientale* samples were recovered in a single clade (80). Likewise, samples of *P. inflatum* (98), *P. latifolium* (93), and *P. filipes* (98) formed clades. The North American *P. pubescens* was sister to *P. biflorum* which was in turn sister to a large clade of Eurasian species. *Polygonatum amabile* from Japan was recovered as sister to several samples of *P. lasianthum* var. *coreanum* (88) (the nominal *P. lasianthum* was not sampled). *Polygonatum arisanense* exhibited differentiation in molecular and morphological data, but was resolved as monophyletic (99) in a weakly supported trichotomy with *P. macranthum* (98) and weakly monophyletic *P. odoratum*

(24), though European samples formed a clade (78) sister to all Asian samples (78). All samples of *P. falcatum* from Japan and South Korea formed a clade with no distinctive geographic topology in the results (97). The species in *P.* section *Polygonatum* with bracteate inflorescences were not recovered as a monophyletic clade and were dispersed in the early lineages of this clade. A clade comprised of *P. acuminatifolium*, the hybridogenic *P. desoulavyi*, *P. govanianum*, *P. inflatum*, *P. involucratum*, *P. megaphyllum*, *P. nodosum*, *P. orientale*, and *P. yunnanense* was weakly supported (24). The bracteate *P. cryptanthum* samples were placed in between a sample of *P. humile* from Japan and multiple samples of *P. humile* from China and South Korea (Fig. 2.5). *Polygonatum amabile* from Japan was recovered as sister to several samples of *P. lasianthum* var. *coreanum* (83) and this clade was sister to a *P. arisanense* (94) and *P. falcatum* clade (97). *Polygonatum odoratum* was weakly monophyletic (16) with geographic structuring in the topology. The ultimate clade of Eurasian species was largely unresolved though species mostly formed clades, e.g. *P. macranthum* (94) and *P. multiflorum* (85). Notable among section *Polygonatum* species, *P. cyrtoneura* was polyphyletic and samples were placed in three clades. Within *P.* section *Verticillata* (65/0.99) the first diverging clade contained the four samples of *P. wardii* (100/1; Fig. 2.5). The samples of the European *P. verticillatum* were placed in a clade (86/0.98) sister to *P. roseum* (42/1), which were separate from all Sino-Himalayan samples of *P. verticillatum* (Fig. 2.5). Within *P.* section *Verticillata* support values were very low (Fig. 2.5). The widespread *P. hookeri* was recovered in two distant clades as were samples of *P. graminifolium* and *P. kansuense*. Series *Punctata* and series *Grandiflora* in *P.* section *Verticillata* were well-supported (100/1, 93/1, respectively). Species relationships within series *Grandiflora* were not well-resolved, but *P. vietnamicum* was sister to the remainder of the clade, and *P. uncinatum* was sister to two samples of the paraphyletic *P. kingianum* (Fig. 2.5). Series *Punctata* was mostly well-resolved with the southernmost species, *P. annamense*, sister to the remaining species (100; Fig. 2.5). *Polygonatum punctatum* and *P. costatum* were sister species (29/0.97) and *P. mengtzensense* and *P. urceolatum* were sister to one another (98/1). Within the larger verticillate species clade samples of *P. prattii* were placed in a well-supported clade (95/1) sister to *P. trinerve* (75/0.65). Series *Oppositifolia* was not well-supported (56/0.93; Fig. 2.5). Several alternate leaved species were placed in series *Oppositifolia*; *P. brevistylum*, *P. nervulosum*, and *P. sp. nov* (DNA4652). Samples of *P. nervulosum* were placed in two different clades; one sister to *P. brevistylum* and *P. sp. nov* (DNA4652), and the other sister to *P. cathcartii*. The three samples of *P. brevistylum* were also polyphyletic with two sister to one another and the other sister to *P. sp. nov* (DNA4274 and DNA4275). The other samples of the broadly distributed *P. cathcartii* were also polyphyletic with samples recovered in several of the smaller clades within series *Oppositifolia*. *Polygonatum angelicum* and *P. luteoverrucosum* were paraphyletic in a clade sister to a possible novel taxon and *P. oppositifolium* and *P. tessellatum* which were unresolved.

Discussion

This study, based largely on a living collection representing most of the diversity in the Polygonateae, was essential to obtaining DNA sequence data because of the low quantity of DNA obtained from herbarium specimens, and this collection has enabled chromosome counts, morphological observations, and seed germination studies as an aid to comprehensive systematic studies of the Polygonateae, specifically *Polygonatum*. Molecular techniques to amplify and obtain clean sequence of the ITS region for *Polygonatum* and other obstinate genera of the tribe Nolinoideae provides the first extensive survey of this proposed barcoding region for the genus

which has allowed the most comprehensive phylogenetic analyses of *Polygonatum* to date. The phylogenetic results of this study have shown novel phylogenetic placement of several *Polygonatum* species, corroborated the three sectional divisions in the genus, shown extensive polyphyly in several species groups, and has also provided a foundation from which further systematic studies will be based.

The relationships of the Nolinoideae are well-supported based on several independent loci, but surprisingly *Maianthemum* is not placed with the other genera of the Polygonateae (Figs. 2.2–2.5). Results of the molecular phylogenetic analyses provide high support for tribal relationships of the genera of the former Convallariaceae: the Aspidistreae; the Convallarieae; and the Ophiopogoneae (Figs. 2.2–2.5). In contrast to previous studies, the monophyly of the Polygonateae – if *Maianthemum* is included – is not supported. Only in the chloroplast data is *Maianthemum* placed in the same clade (Fig. 2.3) as it also was based on four chloroplast markers in Meng et al. (2014). In analyses of *petA* the placement of *Maianthemum* is unresolved with regards to the Ophiopogoneae and the other members of the Polygonateae. Baker (1875) included *Maianthemum* in his tribe *Tovarieae* including *Speirantha* and *Theropogon* rather than his tribe *Convallarieae* that included *Polygonatum*. In fact, the base chromosome number of *Maianthemum* ($x = 18$) is shared with most of the genera and species of the *Ophiopogoneae* (Wang et al. 2013). The placement of *Maianthemum* within the Nolinoideae requires further investigation to resolve its position.

Molecular phylogenetic results support previous estimations of the monophyly of *Disporopsis*, *Heteropolygonatum* and *Polygonatum* based strictly on chloroplast data (Meng et al. 2014) though the relationships to one another differ (Figs. 2.2–2.5). Their delimitation is well-supported by their respective shared derived characters including perigone morphology and cytology. Conflicting topology of the axillary-flowered Polygonateae are shown in among the loci sampled (Figs. 2.2–2.5). Combined analyses placed *Heteropolygonatum* as the direct sister to *Polygonatum*. Nonetheless, analyses of ETS placed the morphologically less similar *Disporopsis* as sister. Analyses of the whole plastid and *petA* + ITS analyses placed *Heteropolygonatum* as the direct sister to *Polygonatum* (Floden and Schilling unpubl.). Plastid data (Meng et al. 2014) and plastid + ITS (Wang et al. 2016) showed a *Heteropolygonatum* + *Disporopsis* sister relationship to *Polygonatum*. Additional molecular studies involving nuclear loci are needed to resolve the relationships of the axillary-flowered Polygonateae.

This study provides the first phylogenetic analysis of species relationships within *Disporopsis*. The Indochinese *D. longifolia* was placed as the earliest lineage sister to all the other species. *Disporopsis longifolia* has many-flowered axillary fascicles (5–10), a wholly white perigone, a reduced corona, and white fruit. The remaining species of *Disporopsis* have few-flowered (1–3) axillary fascicles, perigones without color or that are fuscous maculate, a prominent corona that differs between the species, and purple fruits (Floden 2015, Conran and Tamura 1998). The remainder of *Disporopsis* was not well-resolved. The next lineage included *D. aspersa* sister *D. yui* with low support, a clade containing *D. bakeri* samples sister to one sample of *D. pernyi*, *D. bodinieri*, *D. fuscopicta*. The other *D. pernyi* was included in a large unresolved group including *D. jinfushanensis*, several accessions of *D. arisanensis*, *D. luzoniensis*, and several *D. undulata* accessions. Overall, the resolution of the species in *Disporopsis* by the markers employed was not well supported despite clear morphological differences that delimit the majority of the

species. While most species are easily separated based on morphology the Taiwanese *D. arisanensis* and the Philippine *D. luzoniensis* are nearly inseparable and might be better treated as a single species. The latter species was placed in with several samples of the former which also included the Sichuan, China endemic *D. undulata* samples. The relationships of the species within this ultimate clade which included *D. arisanensis*, *D. luzoniensis*, and *D. undulata* were unresolved by these molecular markers and this might be due to recent and rapid radiations. Nonetheless, the samples of *D. undulata* are easily separated into two morphological groups, but did not resolve into two clades based on the molecular data. Further molecular studies are needed to fully understand the relationships within *Disporopsis*.

Results presented here for provide the most complete sampling of *Heteropolygonatum* in a molecular phylogenetic study which includes 6/10 species (Fig. 2.5). These results support the recent transfer of several species to this genus (Floden 2014, Chao et al. 2013) and also reveal possible polyphyly of the most widespread species, *H. marmoratum*. *Heteropolygonatum alternicirrhosum*, the only species with cirrhose leaf apices, was the earliest diverging species followed by *H. roseolum*. Surprisingly, two morphologically distinctive species occurring in the same valley were sister to one another; the diminutive *H. anomalum* and the large pendulous stemmed *H. pendulum*. The sister clade to this species-pair were two samples of *H. ginfushanicum* which occurs 300 km eastward only on five disjunct mountain peaks (Floden 2014). The isolation of the populations of *H. ginfushanicum* might prove to be genetically isolated based on their distance from one another; an average distance of 200 km between populations. In the last clade *H. altelobatum* was placed in between two groups of *H. marmoratum* samples (Fig. 2.5). *Heteropolygonatum marmoratum* has the most expansive distribution occurring over large parts of the Yunnan-Guizhou plateau eastward to eastern Guizhou and the only species to occur outside of China (Floden 2014). It also has the highest number of documented populations of any of the species (Floden 2014). More importantly the two different groups of *H. marmoratum* recovered here differ in their perigone morphology, but further sampling across its total distribution is required to understand the diversity observed in the four samples here. Several species, not sampled here might alter the relationships in the genus: *H. ogisui* Tamura and Xu is morphologically similar to *H. anomalum* and might be conspecific with it; *H. xui* Bao and Tamura is syntopic with *H. ogisui*, but is morphologically distinct; and *H. parcefolium* (H. Lév.) Floden, which is known only from two collections, has characters similar to *H. anomalum*, *H. ginfushanicum*, and *H. marmoratum*, but is allopatric. Inclusion of the remaining *Heteropolygonatum* will need to be included to resolve the relationships in the genus.

The multiple-loci molecular phylogeny of *Polygonatum* is the most extensively (species sampled) and intensively (samples per species) effort undertaken for the genus thus far (Fig. 2.5). These results provide confirmation of the sectional divisions in *Polygonatum*, uncover polyphyly in several species, provide molecular placement of several previously unplaced species, and they reveal novel diversity in the genus. Nevertheless, the support values of the backbone of the phylogeny of *Polygonatum* are not well-supported (Fig. 2.5). The sections *sensu* Tamura et al. (1997) and Meng et al. (2014) are supported; *P.* sections *Polygonatum*, *Verticillata*, and *Sibirica*. Regarding sectional divisions, Lu et al. (2000) used leaf epidermal and seed testa characters which supported two distinct groups in *Polygonatum* though sampling was limited to a few species. Despite the low support values of the backbone of the *Polygonatum*, many of the

shallow branches reveal well-supported clades that inform their taxonomy and also species boundaries in several groups.

A surprising result of the molecular phylogeny is the unexpected placement of the relatively unknown *Polygonatum wardii*. Four samples of *P. wardii*, from a small area of China and adjacent India in alpine zones, are placed as a clade sister to *P.* section *Verticillata* (99). No previous classification schemes have included this species which has been unknown outside its type collection, but morphological comparisons suggested it might be placed within *P.* section *Verticillata*, possibly near the morphologically similar and geographically proximate *P. prattii*. In fact, it was placed in an unresolved *P.* section *Verticillata* based on the plastid *petA*, but in analyses of ETS and ITS *P. wardii* was placed sister to all of *P.* section *Verticillata*. In analyses of the mitochondrial *rps10* *P. wardii* was placed as sister to all other *Polygonatum*. This placement of *P. wardii* is interesting in light of its morphological characteristics. It has a slightly scabrous stem below the first leaf, leaves that are alternate through most of the stem, occasionally opposite at the mid-stem, and a terminal whorl of 3–5, a perigone that is yellow, an ovary and style that are subequal in length, large filaments unlike most members of *P.* section *Verticillata*, and large orange fruit like series *Grandiflora*. This suite of characters is unique to this single species, though some characters can be found in other species of *P.* sect. *Verticillata*. Its chromosome number and karyotype are unknown, but would be informative to the chromosome evolution in the genus.

The highly diverse *Polygonatum* section *Verticillata* is well-supported as a monophyletic group based on these data and is enlarged by the inclusion of several species formerly treated in *P.* section *Polygonatum* (*Alternata* of Baker (1875); Fig. 2.5). While some “natural” groups previously based on morphology and karyology are well supported, most support values within *P.* section *Verticillata* are low and their relationships to one another are not well resolved (Fig. 2.5). Series *Punctata* and *Grandiflora* are recovered with high bootstrap support, but series *Oppositifolia*, which includes verticillate and alternate leaved species, is not well supported. The futility of classification based on phyllotaxy in *Polygonatum* has been recently documented (Meng et al. 2014), but these data show that labile phyllotaxy is not only sectional, but also frequent within some series.

Polygonatum series *Grandiflora* is morphologically distinct and it was well supported as distinct by the molecular phylogenetic results (Fig. 2.5). Here it was placed sister to series *Punctata* with low support. Species of *P.* series *Grandiflora* share a large perigone that is often brightly colored, a style ca. 3 × longer than the ovary, tepal margins that are decurrent along the perigone tube, large orange fruits, seeds that are spherical and 2–3 mm in diameter, and germination which is immediate and epigeal (Floden pers. obs.). Taxonomic resolution within series *Grandifolia* is a problem that has not been thoroughly investigated (Jeffrey 1980, Rix and Rushforth 2016). Jeffrey (1980) treated taxa that were previously recognized as species as several varieties of *P. kingianum*, but more recently only a broadly defined *P. kingianum* without intraspecific taxa has been recognized (Rix and Rushforth 2016, Chen and Tamura 2000) despite the variation in morphology. Here AF prefers to recognize several species as distinct from *P. kingianum*. Two species, *P. huanum* and *P. uncinatum*, of several recognized here are shown as distinct by analyses of whole plastomes (Floden and Schilling unpubl.). Also, the southernmost species, *P. vietnamicum*, is placed sister to the other species and differs in its terete sigmoid

staminal filaments (Abramova 1986). The resolution within the remaining samples is too low to fully resolve the relationships of the species recognized herein, but there are morphological discontinuities and a large amount of molecular variation in the samples of *P. kingianum* used in this study. In addition, Li et al. (2017) found in a study of herbal medicines that *psbA-trnH* differed between their *P. kingianum* and their *P. uncinatum* samples, and a second locus only amplified for *P. kingianum* and not potential adulterant species. Also within this clade are documented instances of polyploidy that might support specific treatment (Wang et al. 1993, Zhao et al. 2014), but further herbarium, field and molecular study are needed to clarify the systematics of this group.

Molecular phylogenetic results provide additional data corroborating several recently described species (Floden 2015, Zhao and He 2014, Tamura et al. 2014), several additional novel species awaiting formal description, and have also confirmed the unexpected placement of two species in *Polygonatum* series *Oppositifolia* (Fig. 2.5). Series *Oppositifolia* has no strongly unifying morphological features except that most species do have opposite leaves (Baker 1875). Exceptions to this include the whorled-leaved *P. tessellatum* (Tang 1875) and the unexpected inclusion of two alternate leaved species; *P. brevistylum* and *P. cf. nervulosum*. Baker (1875) had placed both species in his *Alternata* (= section *Polygonatum*) based on their alternate phyllotaxy and since that time very few additional collections have been made to provide a comprehensive analysis of them. One sample of *P. brevistylum* from Myanmar was placed sister to an undescribed species in a clade with the recently described *P. autumnale*, and two samples of *P. cathcartii*. The other two *P. brevistylum* samples were sister to two samples of *P. cf. nervulosum*. The last two samples of *P. cf. nervulosum* from Bhutan were recovered in a different clade and were sister to one sample of *P. cathcartii*. All of the undescribed species in this group have been recently discovered or only known from a few non-fertile collections and have yet to be described formally.

Results of the molecular phylogenetic data have revealed unexpected polyphyly in the morphologically uniform *Polygonatum cathcartii* in *P. series Oppositifolia*. This species occurs in higher elevation sub-temperate to temperate cloud-forests along the axis of the Himalaya from Nepal into China (Chen and Tamura 2000, Noltie 1994). It variably grows as an epiphyte or terrestrially and the two forms have not been observed to grow syntopically (Zhao and He 2014, Floden pers. obs., P. Barney, N. Macer, B. Olsen, B. Wynn-Jones, and P. Zale pers. comm.). The molecular variation between samples studied here is equal to or greater than the differences observed between the morphologically distinct *P. oppositifolium* and *P. tessellatum*. These results might be due to chloroplast capture or narrow endemism due to isolation on high mountain peaks within largely subtropical zones that have prohibited gene flow. Similar polyphyly has been documented in *Gaultheria* from the same region and altitudes (Lu et al. 2010). Extensive, detailed field collections across the large area of distribution of *P. cathcartii* are needed to understand the diversity observed here.

Novel species in *Polygonatum* have revealed exceptional morphological characters and life histories. Many of recent novel species are placed in *P. section Verticillata* series *Oppositifolia* (Fig. 2.5). *Polygonatum autumnale* exhibits the only autumnal flowering period in the genus and its growth period is during the winter with summer dormancy during the monsoon season. It also has spurred filaments shared with two other recently described species; *P. campanulatum* not

sampled here, and *P. gongshanense*. The latter species was recovered in a clade containing a sample of *P. cathcartii* and several other undescribed species all with opposite leaves, though the others do not have spurred filaments. *Polygonatum angelicum* and *P. luteoverrucosum*, the only two species with conspicuously verrucose perigone surfaces, were paraphyletic with respect to one another which supports AF's hypothesis of the probable hybrid origin of the former (Floden 2015). This is likely due to the possibility of *P. angelicum* being of hybrid origin with *P. luteoverrucosum* as one parent and *P. cathcartii* the other (Floden 2015). No samples of *P. cathcartii*, even those proximal to populations of *P. angelicum*, were recovered with these in the combined analyses. Also within series *Oppositifolia* are two additional undescribed taxa that are highly morphologically distinct from all known species and from each other. One, *P. sp.* NM sn, has alternate leaves with three distinct veins, urceolate perigones, and is a large evergreen plant. Early morphological assessments suggested affinities to series *Punctata* though the appearance of the leaves suggested a *Disporopsis*. In the combined analyses it is sister to *P. brevistylum* and *P. cf. nervulosum*, but variously placed in series *Oppositifolia* in the individual analyses. The other undescribed species is recovered sister to the *P. cf. brevistylum* from Myanmar and *P. cathcartii*. This species has only two opposite leaves with a single yellow inflated perigone borne at the apex of the short stem when mature. The results of these recent collections and the molecular phylogeny reveal that there remains a significant amount of diversity in *Polygonatum*, especially within the eastern Himalaya which remains underexplored.

Polygonatum series *Punctata* has recently been expanded from the former single accepted species, *P. punctatum*, to include multiple allopatric species (Floden 2014, 2015). *Polygonatum* series *Punctata*, as defined in Floden (2013, 2014, and 2015) is a cohesive group with distinctive perigone morphology and karyotype. The results of the molecular phylogenetic data supported the recognition of multiple species in the group and placed *P. annamense* as the first-branching lineage (Fig. 2.5). *Polygonatum costatum* was placed as part of a polytomy with two other clades; one containing the Himalayan *P. punctatum*, and the other the Indochinese *P. mengtzense* and *P. urceolatum*. The morphologically similar *P. mengtzense* and *P. urceolatum* were placed as sister species though there is discernible molecular variation in the latter. In the ETS results *P. mengtzense* was surprisingly sister to the remaining species and for ITS their relationships were unresolved. Significantly, a single sample included only in the ITS analyses from the Gaoligongshan, Yunnan, China (GSBS 23773) was recovered as sister to *P. punctatum* though that species is not known from this region of China. This sample was morphologically closer to *P. punctatum*, but it is sterile and difficult to assess its identity accurately. The Gaoligongshan, with its many endemics is deserving of additional sampling. Consistent with the results of Floden (2013) the variants of *P. mengtzense* did not show any molecular variation across the loci sampled here even though they are morphologically separable and occur at different elevations.

In contrast to some clades within section *Verticillata* the remaining species relationships are not well-resolved and some samples of what are identified as a single species do not form sister pairs (Figs. 2.5). The absence of molecular resolution is paralleled by extensive morphological diversity. The variation in perigone form and color, plant size, rhizome shape, and leaf characters in this group does not afford an easy classification scheme based on morphology. *Polygonatum verticillatum* from Europe was not sister to any samples from eastern Asia that have been identified as *P. verticillatum*. This corroborates whole plastid genome analyses of Floden and Schilling (unpubl.) in which *P. verticillatum* from Europe was shown to be distinct from Sino-

Himalayan samples. The geographically isolated central Asian *P. roseum* is placed sister to the European *P. verticillatum*. Samples identified as *P. verticillatum* from the Sino-Himalaya were polyphyletic and thus in need of further studies. Based on results here, AF prefers to recognize several taxa from the Sino-Himalaya as distinct from *P. verticillatum*. First, there is *P. jacquemontianum* from higher elevations and often has only three leaves per leaf whorl which also differs in having two-flowered inflorescences with pink flowers. Additional species that should be considered separate from the former broadly defined *P. verticillatum* are *P. leptophyllum*, recognized by Noltie (1994), and *P. kansuense* following Jeffrey (1980). Even beyond the recognition of three entities in this group distinct from *P. verticillatum*, the taxon and molecular sampling presented here is insufficient to provide a full resolution of the taxonomy though it does reveal that the diversity in this group has been greatly underestimated.

Within *Polygonatum* section *Verticillata* there were further instances of polyphyly of putatively conspecific samples (Fig. 2.5). Surprisingly two samples of the morphologically homogeneous alpine *P. hookeri* were not placed as sister to one another. One sample from Nepal is placed sister to a sample of *P. graminifolium* also from Nepal, and the other from Yunnan is sister to a sample of *P. graminifolium* from Yunnan. The *P. graminifolium* samples come from lower elevations and unlike the samples of *P. hookeri* are morphologically distinctive from one another. The Nepalese sample of *P. graminifolium*, which is purportedly from the area of the type locality, is very much like *P. kansuense* with scabrous stem angles, whereas the Yunnan plant matches the types of *P. pumilum* Hua which is currently considered to be a synonym of *P. hookeri*. Similar instances of non-monophyly have been observed in *Gaultheria* from the same region (Lu et al. 2010). In the case of *Gaultheria* the morphological similarities in non-monophyletic species was attributed to chloroplast capture or morphological convergence. Understanding the results for *Polygonatum* requires further investigation to resolve the relationships of *P. hookeri*, *P. graminifolium*, and related species to determine whether reticulate evolution or narrow endemism is the explanation.

An extreme case of polyphyly is documented here for the group of verticillate-leaved samples with cirrhose leaf apices that have been placed in *Polygonatum cirrhifolium* (Fig. 2.5). The only sample considered here to be conspecific with the type of *P. cirrhifolium* was placed sister to series *Grandiflora*, *Oppositifolia*, and *Punctata* though this may be due to missing data in the matrix due to sequencing difficulties (only partial ITS and partial *petA* could be sequenced for this sample). Otherwise all other cirrhose leaved species were distributed in four separate clades within section *Verticillata*. The clades of cirrhose leaved species vary in their cytology, morphology, and a range of perigone shapes and colors. What appear to be morphologically similar accessions, treated here as *P. trinerve*, are recovered in two separate clades with one sample sister to *P. hirtellum* and an as yet unnamed species. The other samples of *P. trinerve* are sister to the diminutive non-cirrhose *P. prattii*. Clarification of the taxonomy of the cirrhose leaved species from the Sino-Himalaya will require thorough sampling across the Himalaya into the Hengduan of southwest China and a larger sampling of molecular loci to resolve the taxonomic issues revealed here and in Meng et al. (2014).

Samples of the recently elevated monotypic *Polygonatum* section *Sibirica* were placed in a clade that was distinct from other verticillate-leaved species, but rather was sister to *P.* section *Polygonatum*, and this placement corroborates the findings of Meng et al. (2014) (Fig. 2.5). The

moderate support for this placement (70/1) is likely a reflection of the molecular variation observed in the ITS and the *petA-psbJ* regions for the samples of this species. This species differs from other species with verticillate leaves in the ovary:style ratio. *Polygonatum sibiricum* has a style that is 2–3 × longer than the ovary (Chen and Tamura 2000) and in other verticillate species the style and ovary are usually subequal in length (excluding the highly distinctive series *Grandiflora*). This character is more similar to species of *P.* section *Polygonatum*. Although *P. sibiricum* has been reported from the Himalaya (Chen and Tamura 2000, Noltie 1994, Hooker 1850), it is likely that it does not occur there; this impression is reinforced by examination and inclusion of molecular data of samples from Himalaya that were attributed to *P. sibiricum*, but were clearly misidentified.

The molecular phylogenetic results of the nuclear ITS region provides the first molecular examination of the Central Asian *Polygonatum sewerzowii*. This species is morphologically very similar to *P.* section *Sibirica*. It and *P. sibiricum* share similar perigone morphology and phyllotaxy, but *P. sewerzowii* has alternate leaves scattered between whorls and the perigone is tubular rather than constricted near the middle (Abramova 1975). The single sample only amplified and could be sequenced for partial ITS which resulted in weakly supported placement as the earliest diverging lineage in *Polygonatum* rather than placed with *P.* section *Sibirica* or in section *Verticillata*. Abramova (1975) erected a monotypic series for *P. sewerzowii* which differs from *P. sibiricum* in its chromosome number; $x = 13$ vs. $x = 12$, respectively (Abramova 1975, Zhao et al. 2014). Based on the limited molecular data obtained the placement was not able to be resolved though it might be sister to *P. sibiricum* or resolved as a second monotypic section.

Polygonatum section *Polygonatum* is a monophyletic group of species with consistently alternate leaves, greenish-white perigones, blue fruits, and base chromosome numbers from $x = 9–11$ (Tamura et al. 1997). Lack of species resolution in section *Polygonatum* is not as problematic as in section *Verticillata* though both share relatively low support values for the major nodes. In fact, many of these species are morphologically well-defined and their delimitation is easily facilitated by numerous characters, particularly the shape and surface characters of the staminal filaments (Tamura 1997, 1993, 1991). In the molecular phylogenetic results the recently reinstated *Polygonatum yunnanense* (Floden 2012) was placed sister to *P. nodosum*, under which it had been subsumed, and these were sister to *P. govanianum* from the NW Himalaya (Fig. 2.5). This result highlights the need to separate the Asian species, *P. govanianum*, which was formerly synonymized under the European *P. multiflorum* (Floden and Schilling unpubl.). The two North American species in the combined analyses showed an unresolved relationship of *P. biflorum* and *P. pubescens*. *Polygonatum lasianthum* was until recently considered to be a single non-variable species. Morphological assessments by Tamura (2009) proposed the recognition of *P. amabile* and two varieties of *P. lasianthum*. The results here placed *P. amabile* sister to several samples of *P. lasianthum* var. *coreanum* from South Korea. The nominal variety of *P. lasianthum* from Japan was not sampled here, but morphology of the two varieties and their allopatric distributions might warrant specific treatments for both. The widespread southeastern Chinese *P. filipes* was sampled from the eastern and western portions of its distribution. The sample from the western end, recently described as *P. sinopubescens* (An et al. 2016) was nested within the four accessions from the eastern area of its distribution. These results do not support its recognition at species status, but rather these are novel distributional records at the edge of range of *P. filipes*. Amongst these samples there is variation in the length and pubescence density

of the staminal filaments and the presence or absence of stem trichomes (Floden pers. obs.). This has also been observed throughout the range of *P. filipes* in herbarium specimen examination. Resolving the intraspecific relationships in *P. lasianthum* and *P. amabile*, and assessing the variation in *P. filipes* will require additional molecular and taxonomic sampling.

Understanding intraspecific variation in widespread and morphologically variable species of *Polygonatum* section *Polygonatum* will require denser sampling than that produced here. In some species where multiple samples were assessed there was some structure correlated with geographic origin. In *P. orientale* samples from the northern side of the Caucasus form a clade sister to those from the south (Fig. 2.5). Likewise, samples of the Taiwanese endemic *P. arisanense* differ in their growth habit (pers. obs) and polyploidy has been documented (Hsu 1971) though accurate documentation of the distribution of the erect vs leaning habit is not known. One sample with distinctly leaning stems and matte leaves is sister to three samples that are strictly upright lustrous leaf surfaces. In contrast to the results of Park et al. (2011), the widespread *P. odoratum* is not paraphyletic, but does exhibit strong phylogenetic structure with regards to their geographic origin (Fig. 2.5). Samples from the Caucasus form a clade sister to remaining samples with the European accessions forming a clade (in which the purported hybrid “Prince Charming” is included), and a second clade includes the remainder, all of which are from eastern Asia. In this latter group there are three morphologically distinct forms. One from the northern part of China is very distinct and has papillose abaxial leaf surfaces and a large inflated perigone. This is sister to the large northeastern Chinese, Russian, and Japanese *P. odoratum* v. *maximowiczii* also with a papillose-scabrous abaxial leaf, and finally *P. odoratum* var. *pluriflorum*. The European forms of *P. odoratum* are different in their floral odor from Asian samples, only some of which have a detectable floral odor (Floden pers. obs.). Though these results contrast with Park et al. (2011) additional molecular and morphological studies should investigate *P. odoratum* and its varieties to better understand their taxonomy.

The molecular phylogenetic results for *Polygonatum* section *Polygonatum* revealed a polyphyletic *P. cyrtonema* (Fig. 2.5). Its polyphyly was expected based on morphological assessments and first reported based phylogenetic results from data from the chloroplast (Meng et al. 2014). Here it is recovered in two clades with structuring occurring within those clades that might support sister species pairs. One of the clades is positioned as an early diverging group in *P. sect. Polygonatum* and the other sister to the European *P. multiflorum*. The morphology within smaller groups within the larger clades is consistent and the geographic distribution is well-correlated with the phylogenetic results. In individual clades there is substantial molecular variation and also morphology that suggests that each clade is composed of more than a single species. For instance, within the first diverging clade there are three different staminal filament shapes of different sizes and surface characters. One sample, *USNA60726J*, is sister to a clade of samples and has large cauducous bracts whereas the sister clade has persistent subulate bracts (Floden 2012). It is unclear what available names are appropriate for those not representing plants obtained from near the type locality (*FO15 sn*) which was placed in a different clade. The breadth of molecular and morphological variation under the current circumscription of *P. cyrtonema* deserves further and broader sampling to unravel the diversity that is hindered by strictly herbarium based studies. Often herbarium samples have been collected in fruiting stages or too early in the season to assess staminal filament shape and surface characters.

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Appendix D

Table 2.1. Voucher information for DNA samples produced for this study. Vouchers marked ** are photographs only.

Species	Voucher	Herb	DNA#	ETS	ITS	<i>petA</i>	<i>rps10</i>
<i>Aspidistra hainanensis</i> Chun & How	Floden	TENN	4260				
<i>Aspidistra</i> sp	Floden sn	TENN	4053				
<i>Convallaria majalis</i> L.	TCM10-282	TENN	3898				
<i>C. majalis</i>	Floden	TENN	4596				
<i>Disporopsis arisanensis</i> Hayata	PBR	TENN	3933				
<i>D. arisanensis</i>	MWT08216A	TENN	3951				
<i>D. arisanensis</i>	BSWJ3388	TENN	3213				
<i>D. arisanensis</i>	BSWJ1894	TENN	3214				
<i>D. aspersa</i> (Hua) Engler	FO sn	TENN	4456				
<i>D. bakeri</i> Floden	Floden	TENN	3953				
<i>D. bakeri</i>	Floden	TENN	3956				
<i>D. bodinieri</i> (H. Lév.) Floden	Floden et al. 1924	TENN	3636				
<i>D. fuscopicta</i> Hance	Floden sn	TENN	4475				
<i>D. jinpushanensis</i> Z.Y. Liu	MD10-117	TENN	3487				
<i>D. longifolia</i> Craib	AG2013	TENN	4189				
<i>D. longifolia</i>	BO sn	TENN	3806				
<i>D. longifolia</i>	A1VT-340	TENN	3215				
<i>D. longifolia</i>		TENN	4189				
<i>D. luzoniensis</i> (Merr.) J.M.H. Shaw	BSWJ3891	TENN	3211				
<i>D. pernyi</i> (Hua) Diels	CY S-75	TENN	3210				
<i>D. pernyi</i>	Waddick sn	TENN	3761				
<i>D. sp.</i>	Floden sn	TENN	3950				
<i>D. undulata</i> Tamura & Ogisu	Floden	TENN	3852				
<i>D. undulata</i>	Floden	TENN	3853				
<i>D. undulata</i>	Erlang	TENN	3954				
<i>D. undulata</i>	RHR CH047	TENN	3955				
<i>D. undulata</i>	CY	TENN	3212				
<i>D. undulata</i>	CDR2057	TENN	3952				
<i>D. undulata</i>	DJHC735	TENN	3209				
<i>D. yui</i> Floden	B53	TENN	3456				
<i>Heteropolygonatum altelobatum</i> (Hayata) Y.H. Tseng, H.Y. Tzeng and C.T. Chao	**	-	3844				
<i>H. alternicirrhosum</i> (Hand.-Mazz.) Floden	X	TENN	3736				
<i>H. anomalum</i> (Hua) Floden	**	TENN	3184				
<i>H. ginfushanicum</i> (F. T. Wang and T. Tang) M. N. Tamura et al.	FO 2856	TENN	4420				
<i>H. marmoratum</i> (H. Lév.) Floden	Floden 2458	TENN	3708				
<i>H. marmoratum</i>	BO sn	TENN	4114				
<i>H. marmoratum</i>	Floden 2725	TENN	4232				
<i>H. marmoratum</i>	BO sn	TENN	4381				
<i>H. pendulum</i> (Z. G. Liu and X. H. Hu) M. N. Tamura & Ogisu	**	-	3981				
<i>H. roseolum</i> M.N. Tamura & Ogisu	**	-	3637				
<i>Liriope</i> sp. nov.	CPC 9.5.01.2	TENN	4054				
<i>Maianthemum amplexicaule</i> (Nutt.) W.A.Weber	Floden & Schilling2784	TENN	4200				

Table 2.1. Continued.

<i>Species</i>	<i>Voucher</i>	<i>Herb</i>	<i>DNA#</i>	<i>ETS</i>	<i>ITS</i>	<i>petA</i>	<i>rps10</i>
<i>M. racemosum</i> (L.) Link	Floden & Schilling3006	TENN	4595				
<i>M. stellatum</i> (L.) Link	Floden	TENN	4639				
<i>M. stenolobum</i> (Franchet) S. C. Chen and Kawano	FBP 2758	TENN	4494				
<i>Ophiopogon bockianus</i> Diels	CPC	TENN	4069				
<i>O. clavatus</i> C. H. Wright ex Oliver	MD10-173	TENN	4070				
<i>O. clavatus</i>	Floden sn	TENN	4477				
<i>O. sp.</i>	FMWJ13202	TENN	4067				
<i>O. sp.</i>	FMWJ13427	TENN	4261				
<i>Peliosanthes macrostegia</i> Hance	**	TENN	4476				
<i>Polygonatum amabile</i> Watanabe	KK sn	TENN	3171				
<i>P. amabile</i>	**		4599				
<i>P. amabile</i>	**		4600				
<i>P. angelicum</i> Floden	Floden 2844	TENN	4257				
<i>P. angelicum</i>	FBP2765	CAL	4190				
<i>P. angelicum</i>	**		4271				
<i>P. annamense</i> Floden	**	MO	3175				
<i>P. arisanense</i> Hayata	BSWJ1639	TENN	3757				
<i>P. arisanense</i>	BSWJ271	TENN	4043				
<i>P. arisanense</i>	A1TW038	TENN	3744				
<i>P. arisanense</i>	MTW08307	TENN	3949				
<i>P. autumnale</i> Floden	PBR sn	CAL	4055				
<i>P. biflorum</i> (Walt.) Ell.	Floden sn	TENN	3664				
<i>P. biflorum</i> AR	Floden sn	TENN	3996				
<i>P. biflorum</i> FL	Floden sn	TENN	3808				
<i>P. brevistylum</i> Baker	FBP 2761	TENN	4164				
<i>P. brevistylum</i>	**		4169				
<i>P. brevistylum</i>	**		4351				
<i>P. cf. brevistylum</i>	**		4272				
<i>P. bulbosum</i> H. Lév.	Floden sn	TENN	3162				
<i>P. cathcartii</i> Baker	BSWJ2421	TENN	4116				
<i>P. cathcartii</i>	**		4187				
<i>P. cathcartii</i>	BSWJ2429	TENN	3859				
<i>P. cathcartii</i>	**		4166				
<i>P. cathcartii</i>	**		4170				
<i>P. cathcartii</i>	Li et al 12385	CAS	4391				
<i>P. cathcartii</i>	Floden 2735	TENN	3859				
<i>P. cf. nervulosum</i>	Miehe 00-002-06	E	4109				
<i>P. cf. nervulosum</i>	Grierson & Long 4072	E	4112				
<i>P. cf. nervulosum</i> Baker	FBP 2762	TENN	4171				
<i>P. cf. nervulosum</i>	FBP	TENN	4259				
<i>P. cirrhifolium</i> (Wall.) Royle	JRSA69	E	4048				
<i>P. costatum</i> Floden	**	MO	3172				
<i>P. cryptanthum</i> H.Lév. & Vaniot	Floden 1437	TENN	3169				
<i>P. cryptanthum</i>	Floden 2727	TENN	3992				
<i>P. cryptanthum</i>	USNA56584h	TENN	3661				

Table 2.1. Continued.

Species	Voucher	Herb	DNA#	ETS	ITS	petA	rps10
<i>P. curvistylum</i> Hua	CLD761		3224				
<i>P. cyrtonema</i> Hua	FO15 sn	TENN	4473				
<i>P. cyrtonema</i>	Floden 3064	TENN	3972				
<i>P. cyrtonema</i>	Floden 2448	TENN	3973				
<i>P. cyrtonema</i>	CPC30041	TENN	3742				
<i>P. cyrtonema</i>	**		4382				
<i>P. cyrtonema</i>	USN60726J		3758				
<i>P. cyrtonema</i>	Floden 2400	TENN	3739				
<i>P. cyrtonema</i>	**		3770				
<i>P. cyrtonema</i>	**		4419				
<i>P. cyrtonema</i>	DJH		4430				
<i>P. cyrtonema</i>	CPC2.5.01.4	TENN	4273				
<i>P. darisii</i> H.Lév.	Floden 2907	TENN	3665				
<i>P. desoulavyi</i> Komarov	**	TENN	4231				
<i>P. falcatum</i> A. Gray	Floden 3066	TENN	4046				
<i>P. falcatum</i>	DJHC970662	TENN	3300				
<i>P. falcatum</i> “Wedding Bells”	Floden 2411	TENN	4129				
<i>P. falcatum</i>	BSWJ1077	TENN	4044				
<i>P. falcatum</i>	BSWJ1501	TENN	3788				
<i>P. filipes</i> Merrill ex C. Jeffrey and McEwan	Floden 2119	TENN	3170				
<i>P. filipes</i>	Floden 2737	TENN	3786				
<i>P. filipes</i>		TENN	3991				
<i>P. filipes</i>	FO 2857	TENN	4418				
<i>P. franchetii</i> Hua	**		4421				
<i>P. geminiflorum</i> Decne.	Edrom		3975				
	Nurseries						
<i>P. geminiflorum</i>	Floden 2738	TENN	4126				
<i>P. gongshanense</i> L.H. Zhao & X.J. He	**	TENN	4115				
<i>P. gongshanense</i>	GSBS 30763	CAS	4388				
<i>P. govanianum</i> Royle	**		3856				
<i>P. graminifolium</i> Hooker	G-WPH 803	**	3221				
<i>P. graminifolium</i>	**		3222				
<i>P. hirtellum</i> Handel-Mazzetti	Floden 2745		4125				
<i>P. hookeri</i> Baker	Floden 2404	TENN	3732				
<i>P. hookeri</i>	Floden 2724	TENN	4184				
<i>P. hookeri</i>	Manaslu 08	E	4051				
<i>P. huanum</i> H. Lév.	Floden 2461	TENN	3704				
<i>P. humile</i> Fisch. ex Maxim.	JW	TENN	3780				
<i>P. humile</i>	A1C246	TENN	3763				
<i>P. humile</i>	TCM-681		3769				
<i>P. inflatum</i> Komarov	Cheju	TENN	3978				
<i>P. inflatum</i>	A1K068	TENN	3764				
<i>P. inflatum</i>	CY	TENN	3762				
<i>P. infundiflorum</i> Y.S.Kim, B.U.Oh and C.G.Jang	Floden 2439	TENN	3743				
<i>P. involucratum</i> (Franch. & Sav.) Maxim.	A1K122	TENN	3748				

Table 2.1. Continued.

Species	Voucher	Herb	DNA#	ETS	ITS	<i>petA</i>	<i>rps10</i>
<i>P. involucreatum</i>	Floden 2398	TENN	3166				
<i>P. involucreatum</i>	CY	TENN	3740				
<i>P. involucreatum</i>	A1K011	TENN	3747				
<i>P. involucreatum</i>	Floden 2748	TENN	4134				
<i>P. jacquemontianum</i> Royle	1074472	CAS	4395				
<i>P. kansuense</i> Maxim. ex Batalin	Floden 2427	TENN	3976				
<i>P. kansuense</i>	SSW555	TENN	3999				
<i>P. kingianum</i> Collett and Hemsley	CY sn	TENN	3182				
<i>P. kingianum</i>	BO13	TENN	4276				
<i>P. kingianum</i>	BO sn	TENN	4383				
<i>P. kingianum</i>	CY sn	TENN	4211				
<i>P. kingianum</i>	BO sn	TENN	4219				
<i>P. kingianum</i>	BSWJ6562	TENN	3178				
<i>P. kingianum</i>	BSWJ6545	TENN	3994				
<i>P. kingianum</i>	CY sn	TENN	4379				
<i>P. lasianthum</i> var. <i>coreanum</i> Nakai	HC970391	TENN	3163				
<i>P. lasianthum</i> var. <i>coreanum</i>	Floden 2451	TENN	3768				
<i>P. lasianthum</i> var. <i>coreanum</i>	A1K-162	TENN	3765				
<i>P. latifolium</i> (Bosc ex Poir.) Pursh	BM307	TENN	3734				
<i>P. latifolium</i>	TCM10-234	TENN	3795				
<i>P. latifolium</i>	TCM10-221	TENN	3737				
<i>P. latifolium</i>	TCM10-164	TENN	3794				
<i>P. leptophyllum</i> D. Don	Bajhang09	E	4052				
<i>P. luteoverrucosum</i> Floden	FBP2764	CAL	4167				
<i>P. luteoverrucosum</i>	**		4352				
<i>P. macranthum</i> (Maxim.) Koidz.	CPC97.11.4	TENN	3783				
<i>P. macranthum</i>	Floden 3091	TENN	3779				
<i>P. macranthum</i>	TCM12-775	TENN	4061				
<i>P. macropodium</i> Turczaninow	Floden 3064	TENN	3759				
<i>P. megaphyllum</i> P.Y. Li	SQAE 295a	E	4110				
<i>P. mengtzensense</i> F. T. Wang & T. Tang	HWJ861	TENN	3177				
<i>P. mengtzensense</i>	Floden 1890	TENN	3805				
<i>P. mengtzensense</i>	FMWJ 1869	TENN	3301				
<i>P. mengtzensense</i>	**		3861				
<i>P. mengtzensense</i>	FMWJ 1851	TENN	3771				
<i>P. multiflorum</i> (L.) All.	TCM12-320	TENN	4057				
<i>P. multiflorum</i>	TCM	TENN	4216				
<i>P. multiflorum</i>	TCM10-380	TENN	4209				
<i>P. multiflorum</i>	TCM11-164	TENN	3706				
<i>P. multiflorum</i>	TCM11-229	TENN	3804				
<i>P. multiflorum</i>	TCM12-272	TENN	3791				
<i>P. nodosum</i> Hua	FO 2868	TENN	4455				
<i>P. odoratum</i> (Mill.) Druce	TCM12-293	TENN	3790				
<i>P. odoratum</i> "Prince Charming"	Floden 2437	TENN	3746				
<i>P. odoratum</i>	Ruksans	TENN	3995				
<i>P. odoratum</i>	Floden 2450	TENN	4064				
<i>P. odoratum</i>	TCM10-288	TENN	4213				

Table 2.1. Continued.

Species	Voucher	Herb	DNA#	ETS	ITS	petA	rps10
<i>P. odoratum</i>	TCM11-352	TENN	4063				
<i>P. odoratum</i>	Floden 1436	TENN	3164				
<i>P. odoratum</i>	Floden 2401	TENN	3993				
<i>P. odoratum</i>	Floden 2420	TENN	4131				
<i>P. odoratum</i>	TCM12-235	TENN	3801				
<i>P. odoratum</i>	TCM12-273	TENN	3946				
<i>P. odoratum</i>	TCM12-295	TENN	3773				
<i>P. odoratum</i>	TCM12-165	TENN	3947				
<i>P. odoratum</i> v. <i>maximowiczii</i> (F. Schmidt) Koidz.	MW	TENN	3738				
<i>P. odoratum</i> v. <i>maximowiczii</i>	Floden sn	TENN	3784				
<i>P. odoratum</i> v. <i>pluriflorum</i> (Miq.) Ohwi	PBG507701	TENN	3767				
<i>P. oppositifolium</i> (Wall.) Royle	Floden 2733	TENN	3491				
<i>P. oppositifolium</i>	Floden 2415	TENN	3492				
<i>P. oppositifolium</i>	**	TENN	4262				
<i>P. orientale</i> Desf.	TCM12-194	TENN	3776				
<i>P. orientale</i>	TCM	TENN	3728				
<i>P. orientale</i>	TCM	TENN	4472				
<i>P. orientale</i>	SB	TENN	3733				
<i>P. orientale</i>	TCM12-199	TENN	3802				
<i>P. orientale</i>	TCM12-317	TENN	3803				
<i>P. orientale</i>	TCM12-286	TENN	3807				
<i>P. prattii</i> Baker	Floden sn	TENN	3223				
<i>P. prattii</i>	CBE	TENN	3998				
<i>P. prattii</i>	DR	TENN	3729				
<i>P. prattii</i>	BO sn	TENN	4210				
<i>P. prattii</i>	Floden 3065	TENN	4626				
<i>P. prattii</i>	Floden sn	TENN	4377				
<i>P. pubescens</i> (Willd.) Pursh TN	Floden 2376	TENN	4127				
<i>P. pubescens</i> MN	JL sn	TENN	4186				
<i>P. punctatum</i> Royle ex. Kunth	BSWJ2395	TENN	3707				
<i>P. punctatum</i>	**	TENN	3494				
<i>P. roseum</i> (Led.) Kunth	Sodombekov and Rogova KPL00644	TENN	4229				
<i>P. roseum</i>	**	TENN	3167				
<i>P. sewerzowii</i> Regel	Belolipov UPL00010	TENN	4230				
<i>P. sibiricum</i> Redouté	Floden 2489	TENN	3775				
<i>P. sibiricum</i>	Floden 2441	TENN	3756				
<i>P. sibiricum</i>	GB sn	TENN	4353				
<i>P. singalilense</i> H. Hara	LKSRD 63	E	4111				
<i>P. singalilense</i>	Floden 2731	TENN	4117				
<i>P. sp.</i>	Bajhang 09	E	4049				
<i>P. sp.</i>	**		4429				
<i>P. sp.</i>	**		4278				
<i>P. sp.</i>	A1C378	TENN	3787				
<i>P. sp.</i>	**		4163				

Table 2.1. Continued.

<i>Species</i>	<i>Voucher</i>	<i>Herb</i>	<i>DNA#</i>	<i>ETS</i>	<i>ITS</i>	<i>petA</i>	<i>rps10</i>
<i>P. sp.</i>	<i>Floden 2778</i>	TENN	4203				
<i>P. sp.</i>	<i>USNA</i>	TENN	3761				
<i>P. sp.</i>	<i>Floden 2442</i>	TENN	3792				
<i>P. sp.</i>	<i>Floden 2433</i>	TENN	3741				
<i>P. sp.</i> Emei	**		3772				
<i>P. sp.</i> Daocheng	**		3777				
<i>P. sp.</i>	**		4350				
<i>P. sp.</i>	<i>GSBS 23773</i>	CAS	4389				
<i>P. sp.</i>	<i>Floden 2746</i>	TENN	3793				
<i>P. sp.</i>	<i>BSWJ2417</i>		3860				
<i>P. sp.</i>	<i>CC6472</i>	**	3774				
<i>P. sp.</i>	<i>Floden 2122</i>	TENN	3165				
<i>P. sp.</i>	<i>CPC01.5.27.1D</i>	TENN	3781				
<i>P. sp.</i> “Himalayan Giant”	<i>Floden 2900</i>	TENN	4597				
<i>P. sp. nov.</i>	**		4274				
<i>P. sp. nov.</i>	**		4275				
<i>P. sp. nov.</i>	**		4168				
<i>P. sp. nov.</i>	**		4652				
<i>P. stewartianum</i> Diels	<i>CLD325</i>	TENN	3705				
<i>P. tessellatum</i> F. T. Wang & T. Tang	**		4279				
<i>P. tessellatum</i>	<i>Floden 2848</i>	TENN	3857				
<i>P. trinerve</i> Hua	<i>YW sn</i>		3799				
<i>P. trinerve</i>	**		3977				
<i>P. trinerve</i>	**		3789				
<i>P. uncinatum</i> Diels	<i>Floden 2914</i>	TENN	3659				
<i>P. uncinatum</i>	<i>B5</i>	TENN	4113				
<i>P. urceolatum</i> (J.M.H.Shaw)	**		3493				
<i>P. urceolatum</i>	<i>Floden 2416</i>	TENN	3855				
<i>P. urceolatum</i>	<i>Floden 2911</i>	TENN	3662				
<i>P. urceolatum</i>	<i>Floden 2747</i>	TENN	3663				
<i>P. verticillatum</i>	<i>PZM sn</i>		4641				
<i>P. cf. verticillatum</i> (L.) All.	<i>DNEP3 BX87</i>	E	4050				
<i>P. cf. verticillatum</i>	<i>Manaslu 08</i>	E	4047				
<i>P. verticillatum</i>	<i>Floden sn</i>		3862				
<i>P. verticillatum</i>	**		3800				
<i>P. verticillatum</i>	<i>Floden 2406</i>	TENN	3735				
<i>P. vietnamicum</i> Abramova	<i>Floden 2873</i>	TENN	3845				
<i>P. wardii</i> F. T. Wang & T. Tang	<i>YW</i>		4378				
<i>P. wardii</i>	**		4433				
<i>P. wardii</i>	**		4396				
<i>P. wardii</i>	**		4428				
<i>P. yunnanense</i> H. Lév.	<i>Floden 2839</i>	TENN	3660				
<i>P. yunnanense</i>	<i>Floden 2838</i>	TENN	3168				
<i>P. yunnanense</i>	<i>Floden 2120</i>	TENN	4130				
<i>P. yunnanense</i>	<i>Floden 2796</i>	TENN	4214				
<i>P. yunnanense</i>	**	TENN	3974				

Table 2.1. Continued.

<i>Species</i>	<i>Voucher</i>	<i>Herb</i>	<i>DNA#</i>	<i>ETS</i>	<i>ITS</i>	<i>petA</i>	<i>rps10</i>
<i>P. yunnanense</i>	FO 2855	TENN	4417				
<i>P. zanolanscianense</i> Pamp.	**	TENN	3997				
<i>P. zanolanscianense</i>	PBG	TENN	4132				
<i>P. zanolanscianense</i>	Floden 2750	TENN	3766				
<i>Reineckea carnea</i> Andrews	CBCH324	TENN	3934				
<i>Rohdea japonica</i> (Thunb.) Roth	Floden sn	TENN	4068				
<i>Speirantha gardenia</i> (Hook.) Baill.	Floden	TENN	3901				
<i>Tupistra delavayi</i> Franch.	**	TENN	4479				
<i>T. sp.</i>	Floden sn	TENN	4065				
<i>T. tonkinensis</i> Baill.	FMWJ	TENN	4066				



Figure 2.1. Representatives of *Polygonatum* and *Heteropolygonatum* species showing the breadth of morphological diversity in the genus. From top Left: Row 1; *P. jacquemontianum*, *P. trinerve*, *P. nervulosum* (photo by J. Nilson), *P. cf. nervulosum*, *P. luteoverrucosum*, Row 2; *P. wardii* (photo by Y. Wang), *P. huanum*, *P. kingianum*, *P. kansuense*, *P. oppositifolium*, Row 3; *P. cathcartii*, *P. humile*, *P. cryptanthum*, *P. sibiricum*, and *H. anomalum*.

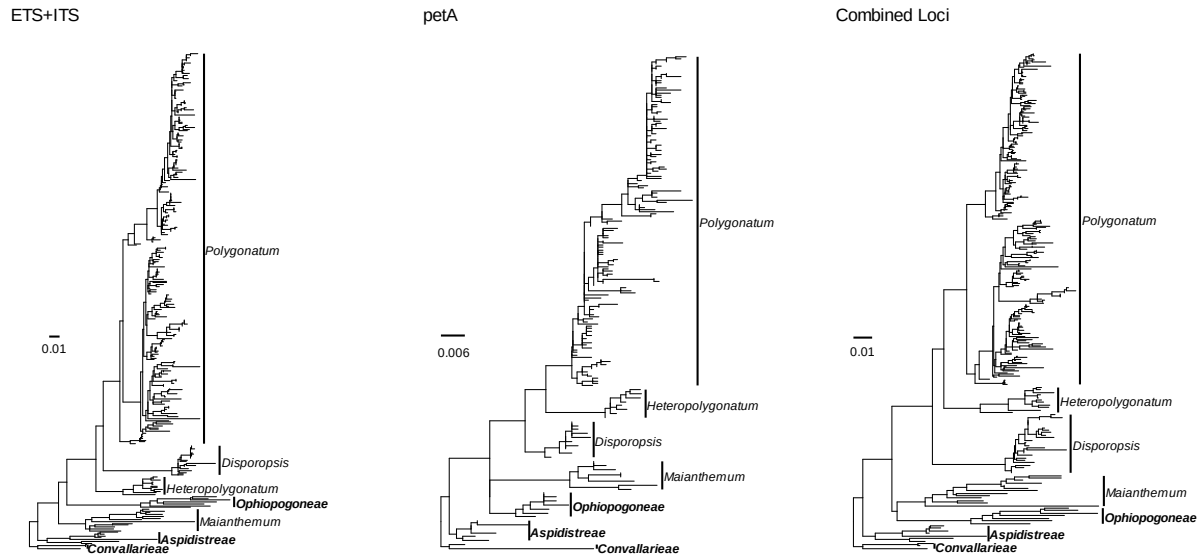


Figure 2.2. Relationships of the Polygonateae across loci from maximum likelihood inferences with 1000 bootstrap replications. These phylograms show the placement of *Maianthemum* with respect to the other genera of the Polygonateae and the placement of *Disporopsis* and *Heteropolygonatum* to *Polygonatum*. Locus or loci used in inference are indicated at the top left of each phylogram.

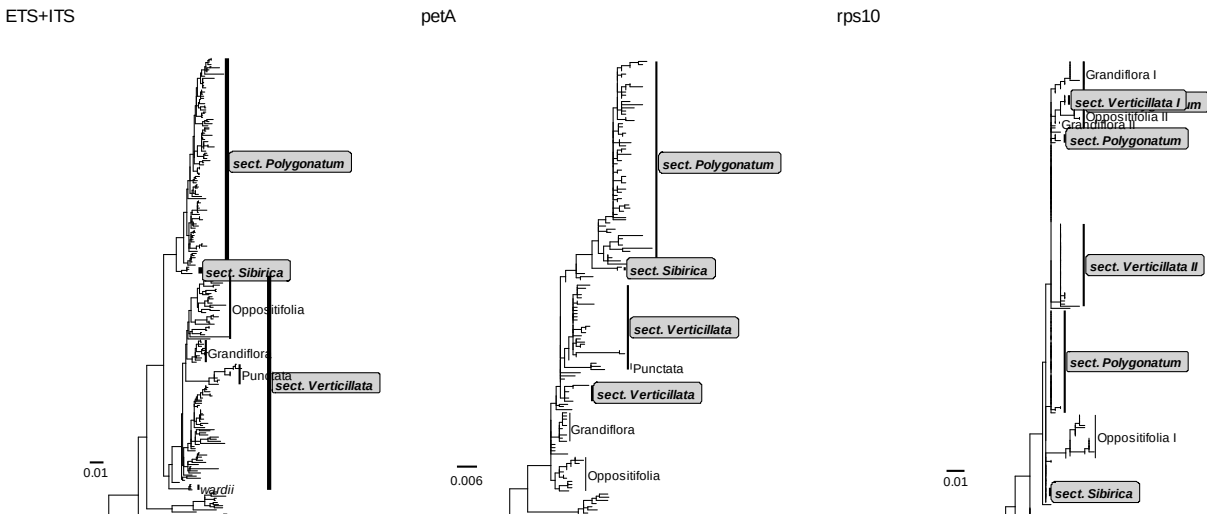


Figure 2.3. Sectional and series divisions in *Polygonatum* based on maximum likelihood analyses with 1000 bootstrap replications. Sections of *Polygonatum* are in grey boxes, and series in plain text. Locus or loci used in inference are indicated at the top left of each phylogram.

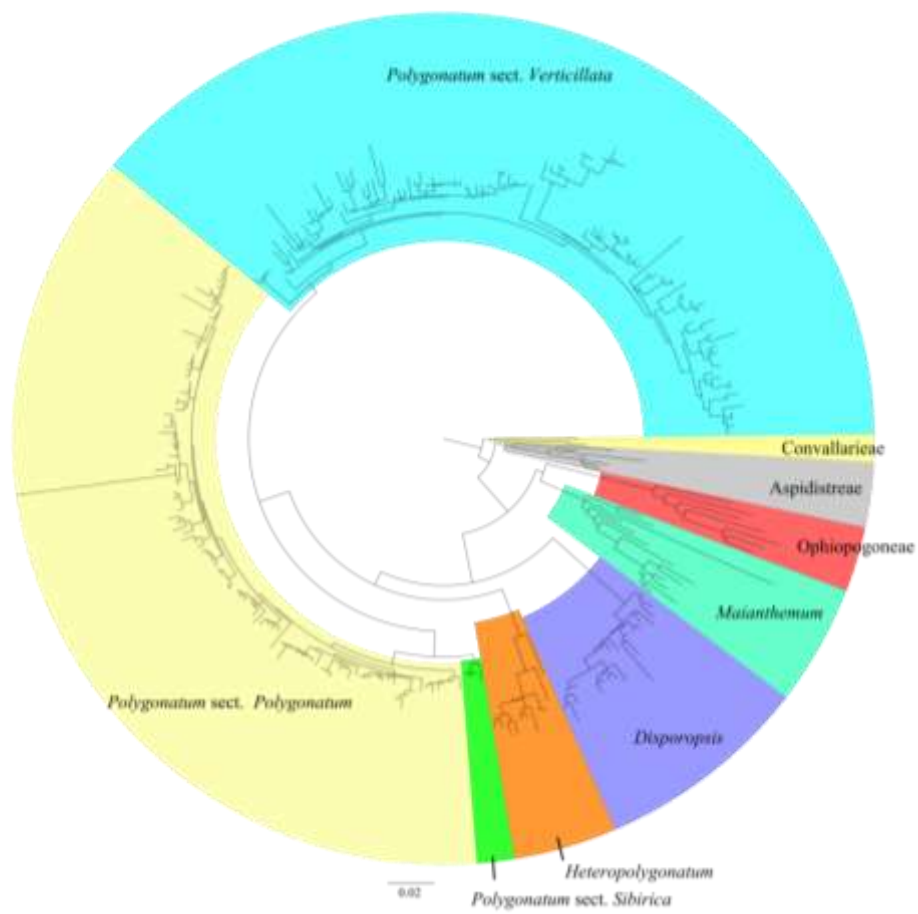


Figure 2.4. Circular phylogram of the total evidence maximum likelihood inference showing the generic relationships and sectional divisions in *Polygonatum*.

Figure 2.5. Total evidence cladogram of *Polygonatum* of the best tree from Maximum Likelihood analyses using 1000 bootstrap replications with Bayesian branch supports included. Branch supports are indicated by: thicker black branches indicate strong to moderate bootstrap support and posterior probabilities ($bs \geq 60$, $pp \geq 80$); grey and white bars indicate moderate to strong support ($bs \geq 60$); and solid grey bars indicate moderate to strong supported posterior probabilities.

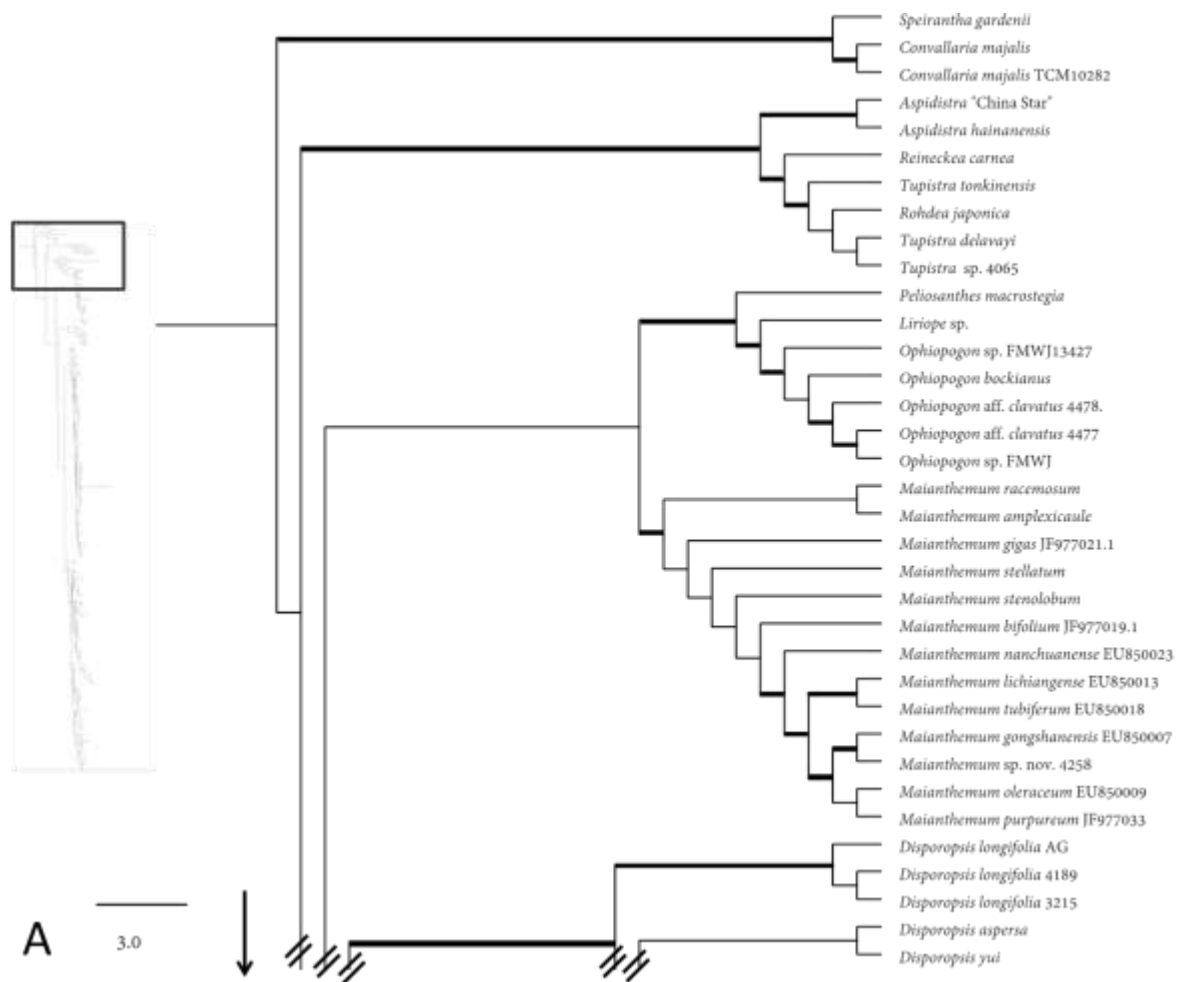


Figure 2.5. Continued.

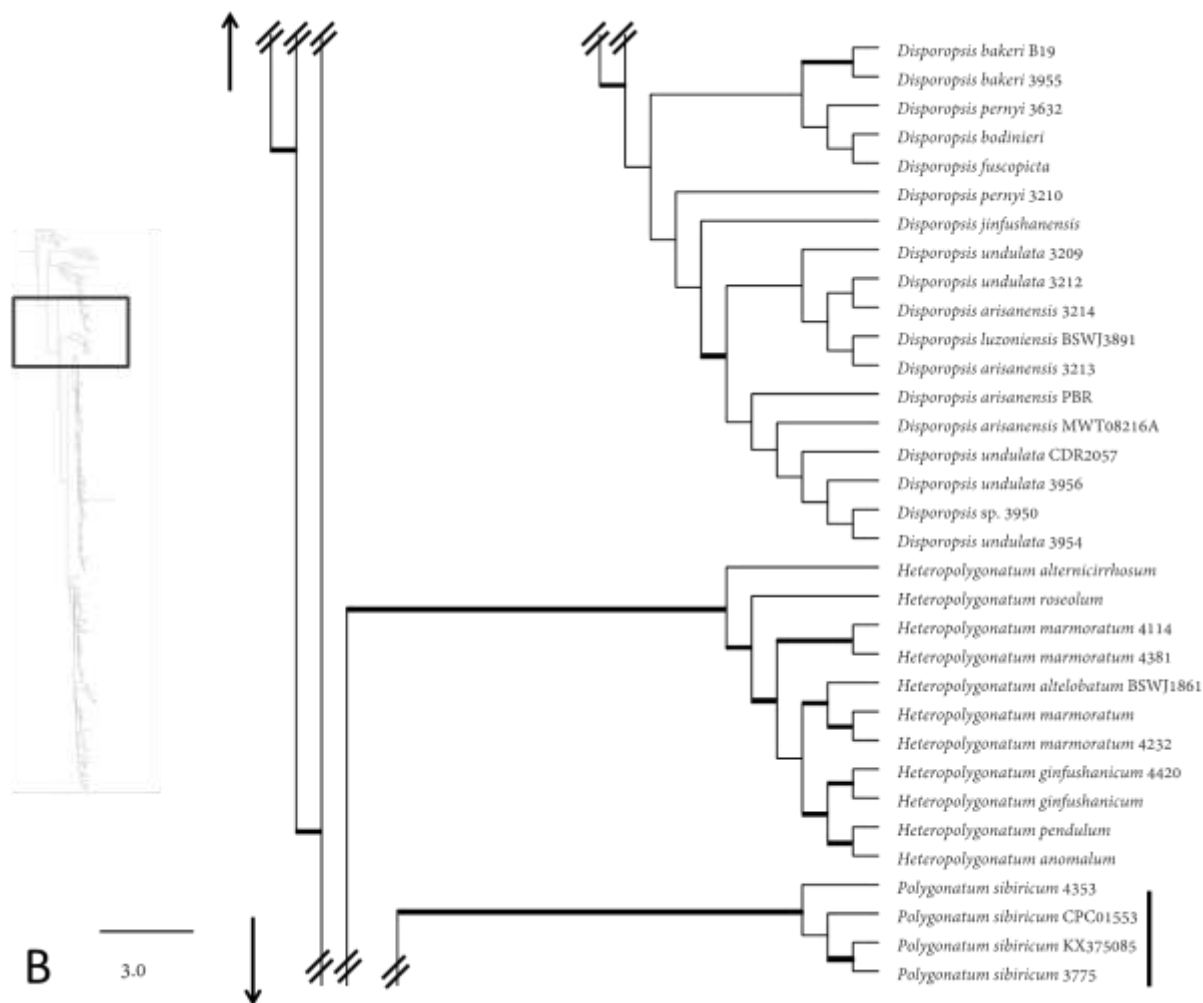


Figure 2.5. Continued.

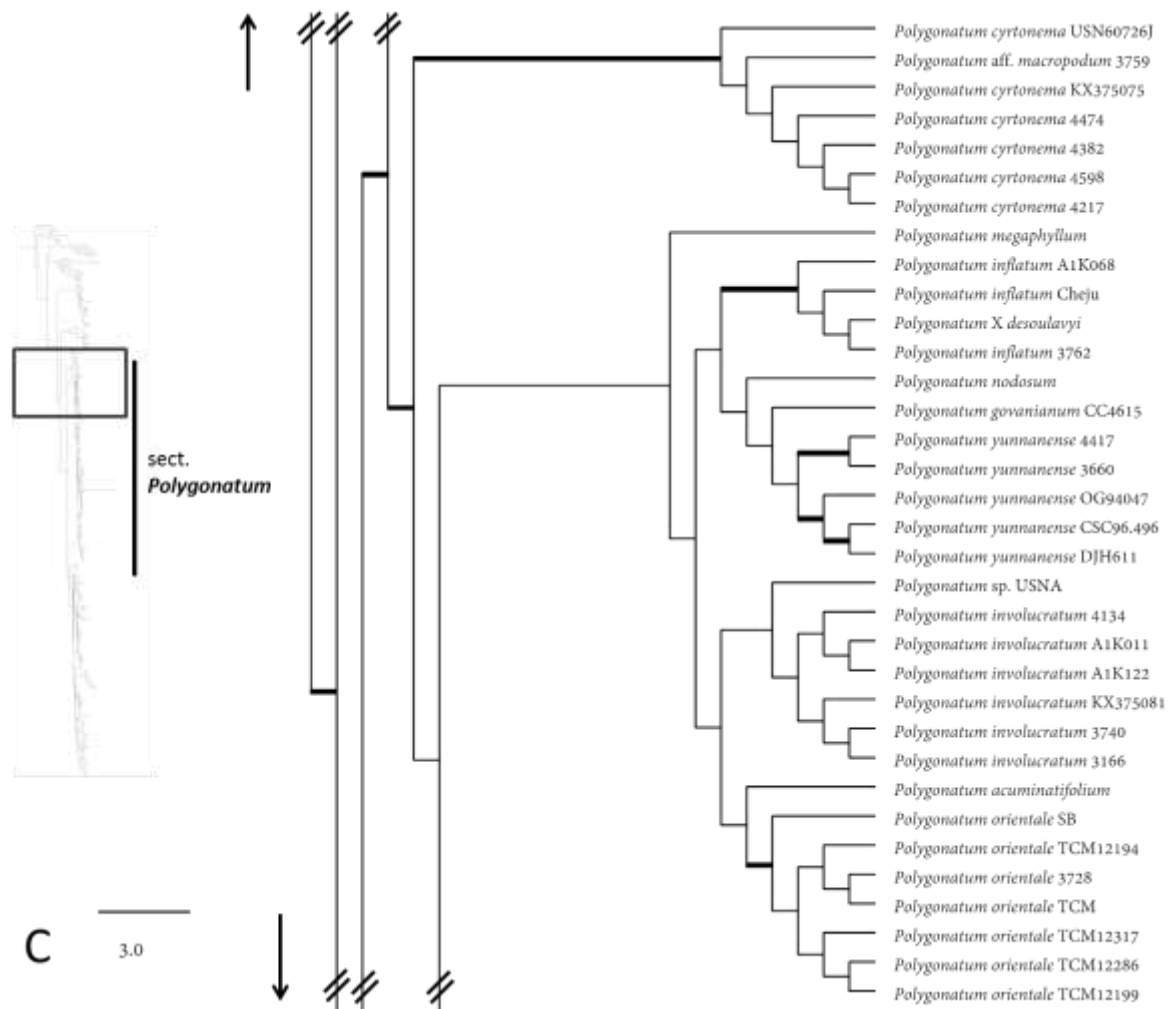


Figure 2.5. Continued.

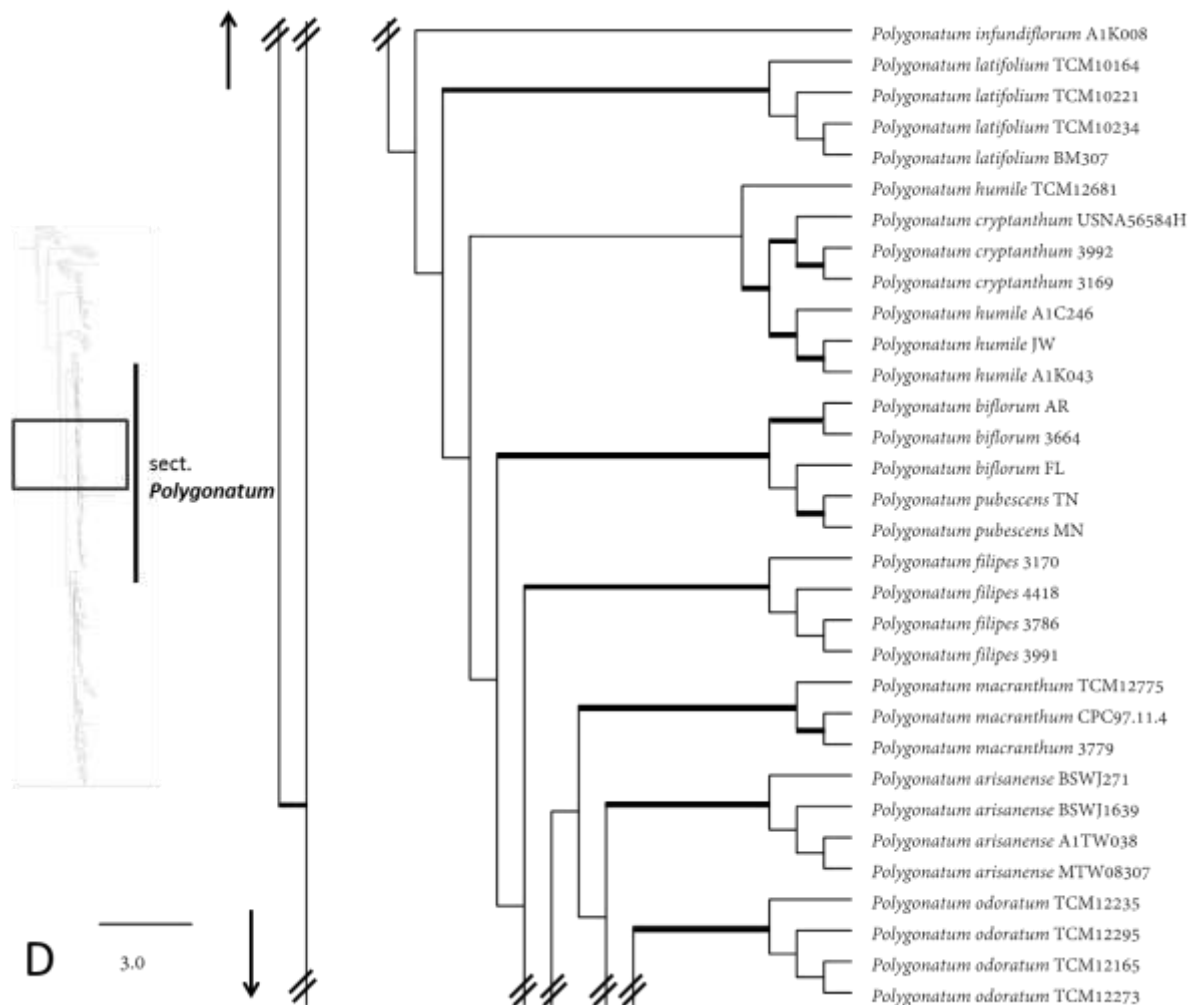


Figure 2.5. Continued.

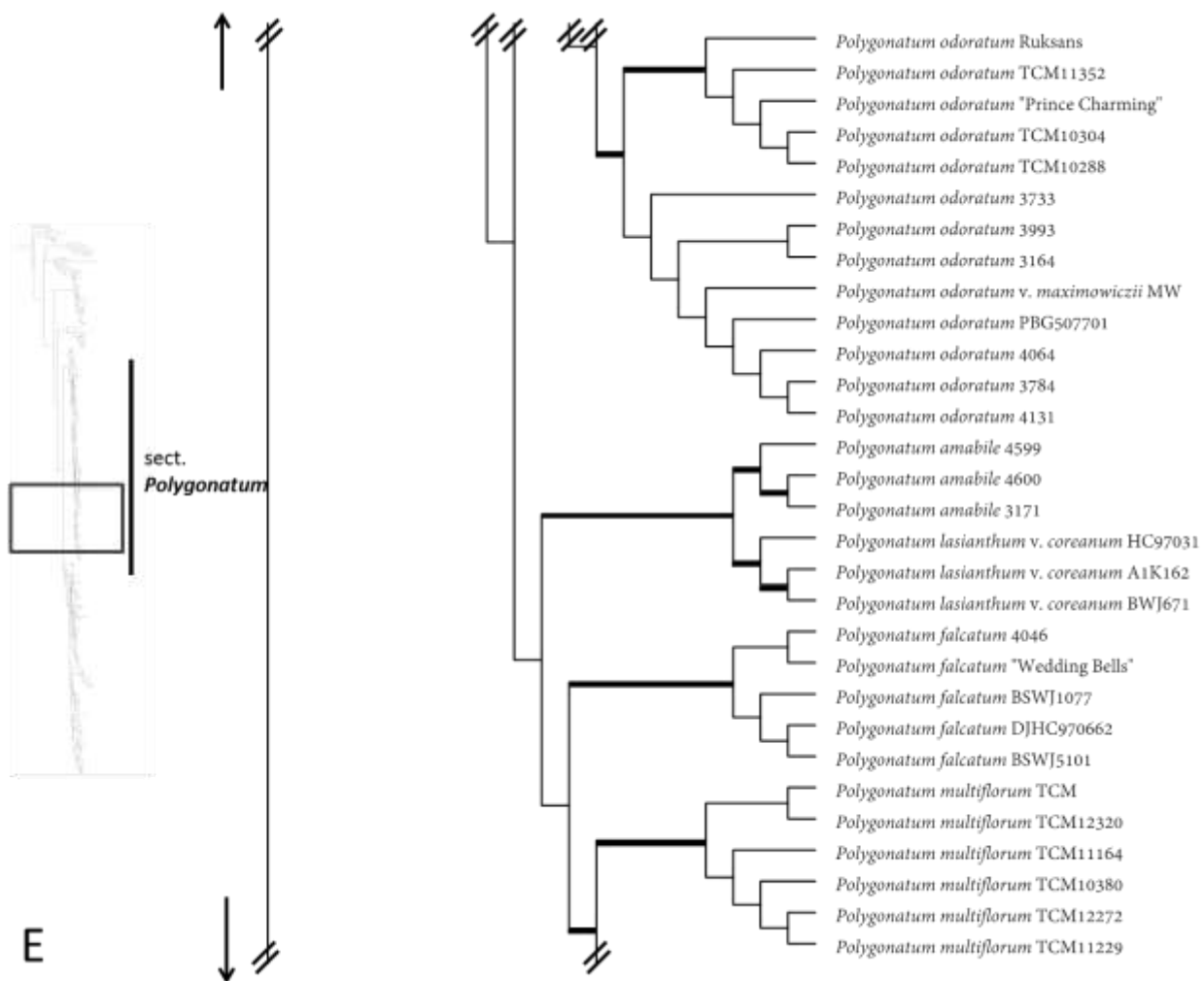


Figure 2.5. Continued.

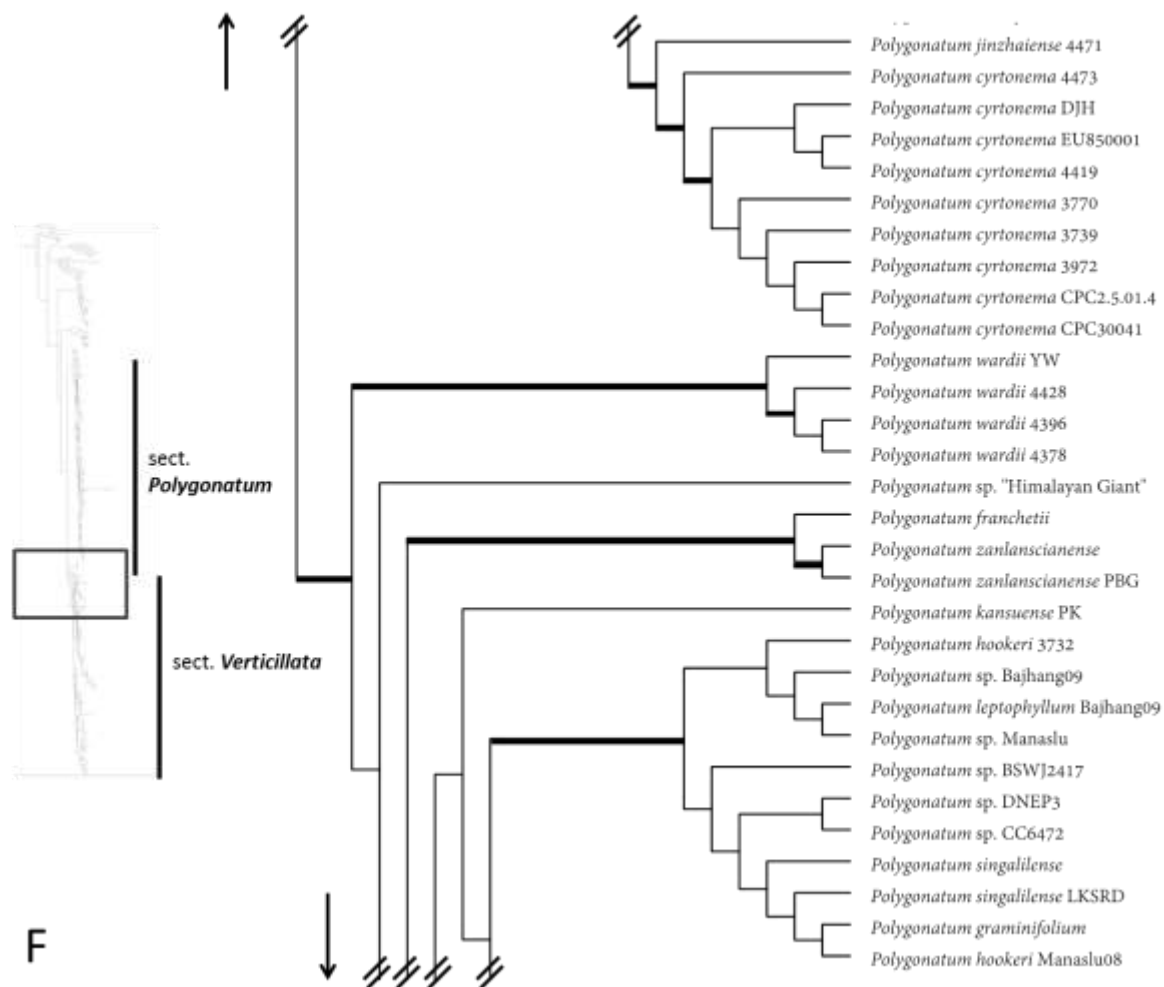


Figure 2.5. Continued.

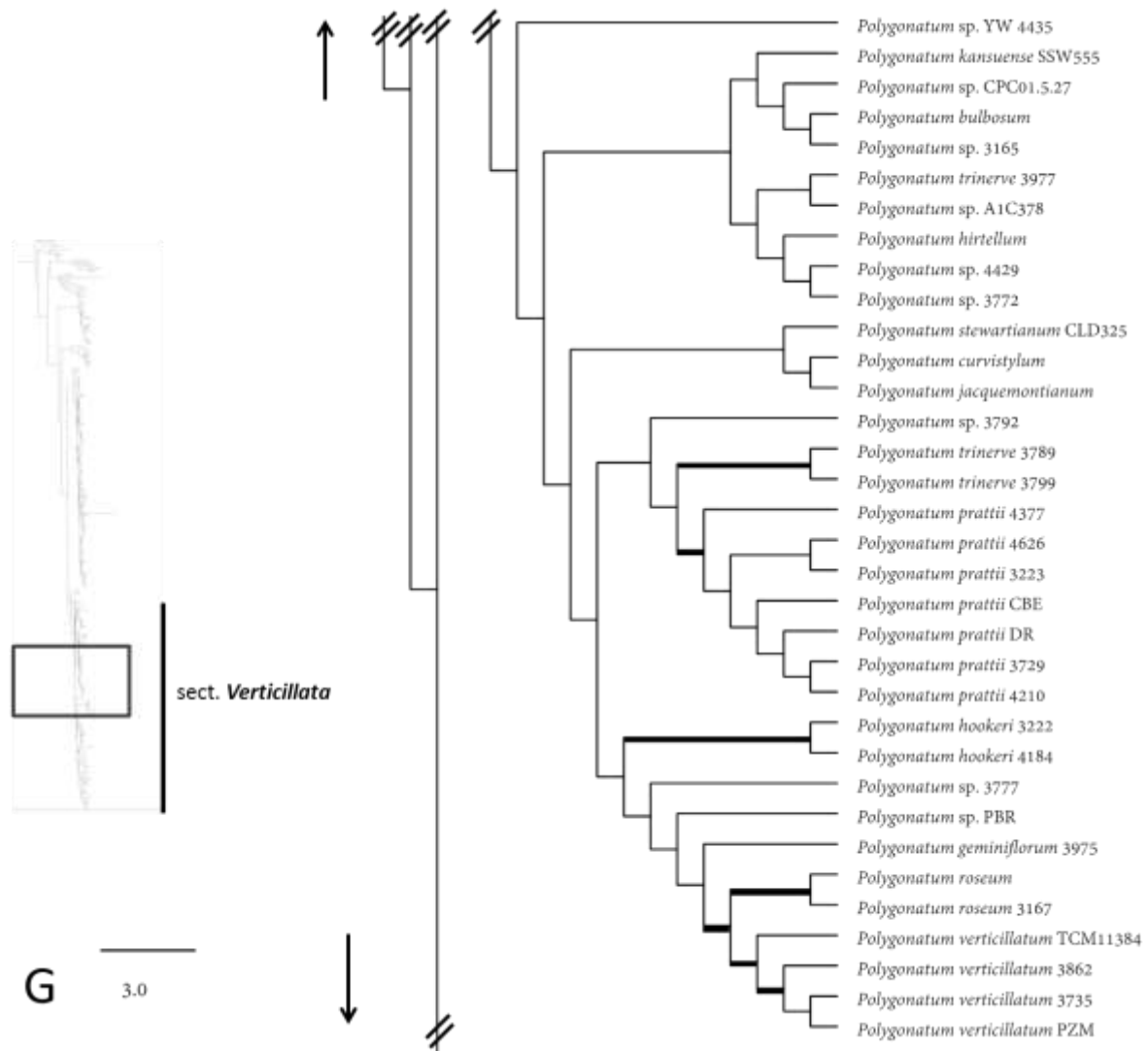


Figure 2.5. Continued.

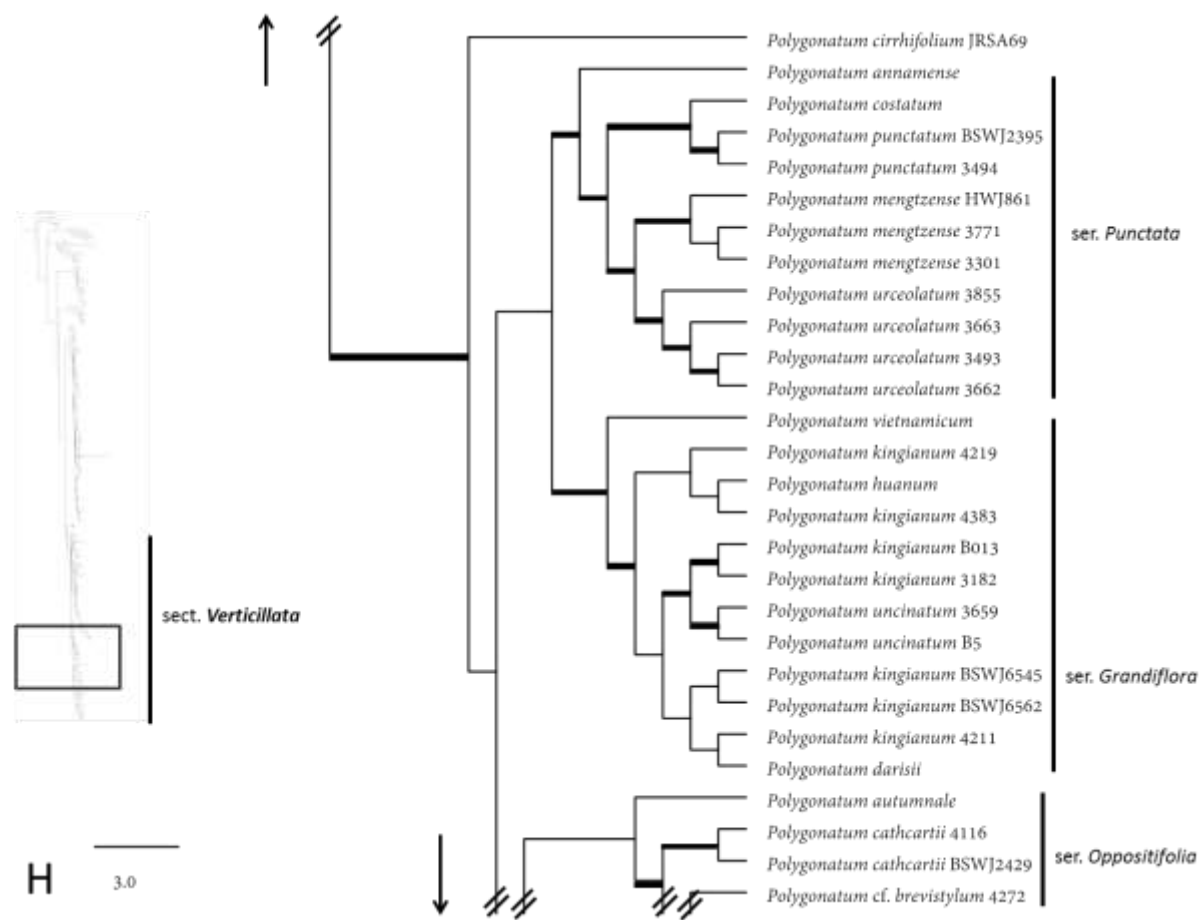


Figure 2.5. Continued.

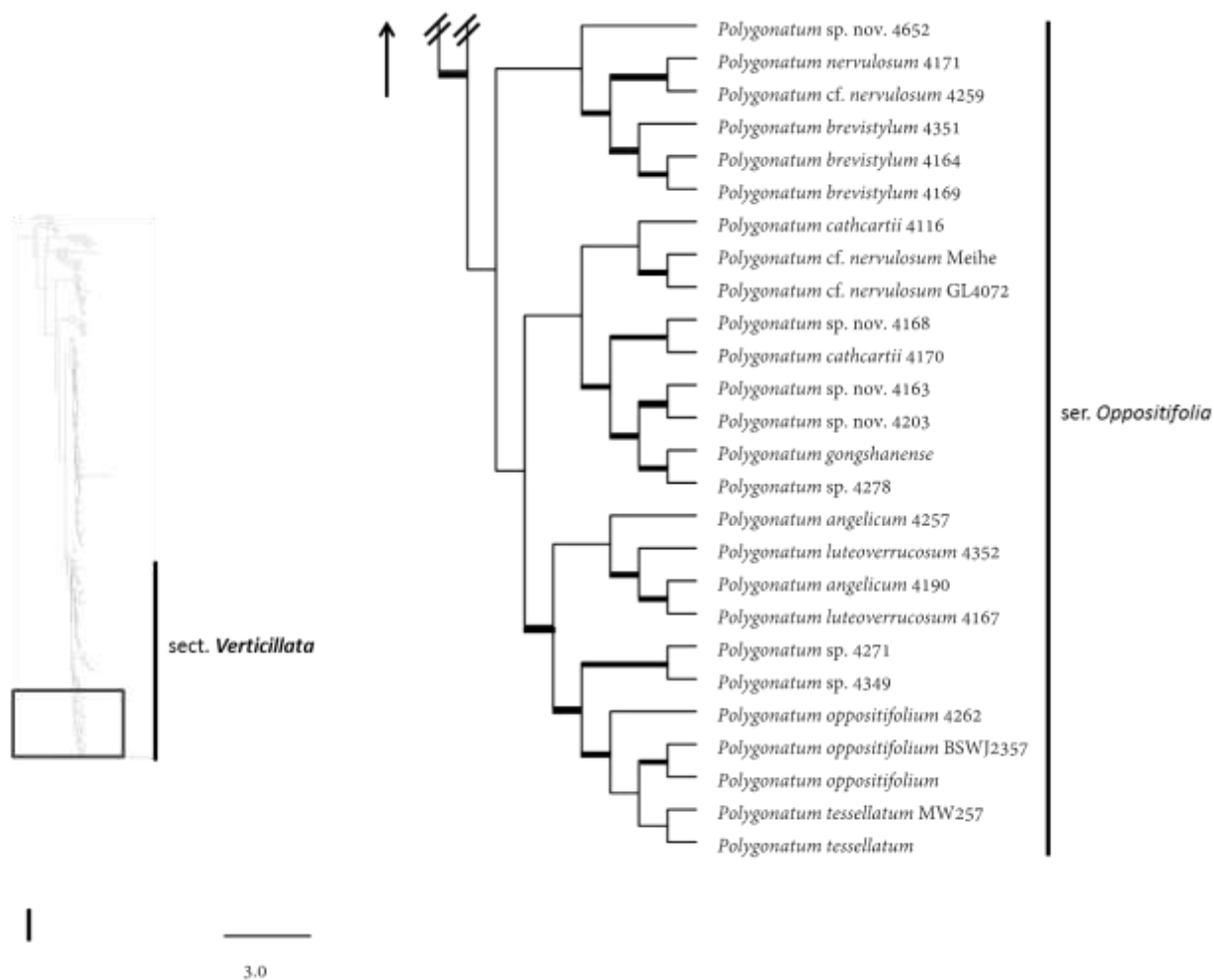


Figure 2.5. Continued.

Chapter 3

Cytological evolution in the Polygonateae (Asparagaceae: Nolinoideae).

Abstract

The Polygonateae (Asparagaceae: Nolinoideae) contains three genera including *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* that exhibit a range of base chromosome numbers and karyotype differences. Polyploidy is documented in *Heteropolygonatum* and *Polygonatum* with unequal distribution among clades. The assumed evolutionary trend in *Polygonatum* has been ascending dysploidy, with karyotypes increasing in chromosome number and asymmetry. Here we provide the first estimation of chromosome evolution in the tribe using a multilocus phylogeny with the program ChromEvol to test hypotheses of the evolutionary trend in the genus. The results reveal that descending dysploidy events are estimated to have occurred more frequently than ascending dysploidy events. We also provide counts for seven species not previously reported and the first tetraploid report for *P. sibiricum*.

Introduction

Changes in chromosome structure and number play an essential role in evolution (Levin 2002). The karyotype of a species includes chromosome number and size, positions of primary and secondary constrictions, and their degree of symmetry (Leitch and Leitch 2013). Mutations and chromosome changes provide sources for natural variation and changes in the chromosome number and karyotype (dysploidy), the doubling of an entire genome (polyploidy), and rearrangements in the karyotype can have profound effects on the phenotype, recombination, and potentially lead to reproductive isolation (Stebbins 1971). The cytotaxonomy of a tribe or genus can provide important systematic data to understand macro-evolutionary patterns.

The genera of the Polygonateae (Asparagaceae), *Disporopsis*, *Heteropolygonatum*, and *Polygonatum* (Conran and Tamura 1998, Tamura et al. 1997) have comparatively large chromosomes which often reach or exceed 10µm in length (Tamura 1990, 1991, 1993) making them relatively easy to study. There is conspicuous variation in karyotype in the subtribe due to differences in the comparative sizes of chromosomes (Zhao et al. 2014). *Disporopsis* and *P. sect. Polygonatum* have mostly symmetric and monomodal karyotypes with homogeneous chromosome sizes (Tamura 1995). In contrast, *Heteropolygonatum*, *P. sect. Sibirica*, and some of *P. sect. Verticillata* have asymmetric and typically bimodal karyotypes (Bao et al. 1998, Deng et al. 2009, Kawano et al. 1967, Tamura 1990, 1991, 1993, 1997, Yamashita and Tamura 2001, Zhao et al. 2014). The bimodal karyotype is signified by the presence of two distinct size classes of chromosomes without intermediates and its occurrence has been attributed to fission-fusion events (Schubert and Lysak, 2011) or allopolyploidy events involving parents with two different chromosome sizes (McKain et al. 2012). Fission, the splitting of chromosomes, leads to a higher base number and potentially to more asymmetry whereas fusion, the joining of chromosomes, leads to larger-sized chromosomes.

Studies of the cytotaxonomy in the Polygonateae have documented polyploidy, a variable base number ($x = 9-15$), and correspondingly variable karyotypes between and even within species (Zhao et al. 2014, Deng et al. 2009, Tamura, 1997, 1993, 1991a, b, Abramova 1975, Therman 1950, 1953a, 1953b, Suomalainen 1947). The three taxonomic sections within *Polygonatum* have each been documented to have different base chromosome number or numbers, and this has received support from molecular phylogenetic studies (Floden and Schilling unpubl., Meng et al., 2014, Wang et al. 2016). *Polygonatum* displays a prominent range of base chromosome numbers: *P. sect. Polygonatum* ranges from $x = 9-11$, *P. sect. Sibirica* has a base of $x = 12$, and

P. sect. Verticillata range from $x = 13$ – 15 (though other numbers have been reported for each section it is suspected that these aberrant counts are in error) (see Zhao et al. 2014 and citations therein, and supp. material). The base chromosome number and karyotype in *Polygonatum* are associated with morphologically cohesive groups of species; e.g. *P. series Punctata* has a distinctive karyotype with a secondary constriction on the longest pair that is a synapomorphy for this clade (Floden 2014a). In contrast, *Disporopsis* and *Heteropolygonatum* both have constant base numbers, $x = 20$ and $x = 16$, respectively, and similar karyotypes among species (Floden 2014b, Yamashita and Tamura 2001, Bao et al. 1998, Tamura et al. 1997, Hong and Zhu 1990).

Molecular phylogenetic work including all of the genera of the traditionally defined Polygonateae has been insufficient in taxon and molecular sampling to resolve generic relationships. Several studies of the broad Asparagaceae have utilized only chloroplast (CP) data (Rudall et al. 2000), ribosomal nuclear data and CP (Kim et al. 2010, Meng et al. 2008), or mitochondrial and CP (Seberg et al. 2012). In each of these the sampling within the Polygonateae did not include *Heteropolygonatum* and sampling within genera of the Polygonateae was often limited to a single representative species. The molecular data of Rudall et al. (2000) did not resolve the relationships of *Disporopsis*, *Polygonatum*, and *Maianthemum* relative to *Semele*, *Danae*, and *Ruscus*, but the inclusion of morphological characters resolved the traditionally defined Polygonateae. The results of Seberg et al. (2012) did not recover a monophyletic Polygonateae; *Ophiopogon* was sister to *Polygonatum*. In the CP and combined analyses of Kim et al. (2010) *Disporopsis* was not recovered as a member of the Polygonateae. Meng et al. (2014) used only CP data and *Maianthemum* (94 bootstrap (bs)) was placed sister to a clade containing *Heteropolygonatum* and *Disporopsis* (58 bs) that were sister to *Polygonatum* (86 bs). More recently, based on phylogenetic results using mitochondrial, nuclear ribosomal, and CP data (Floden and Schilling unpubl.), *Maianthemum* was placed sister to the Ophiopogoneae, to which it is more similar in chromosome number and karyotype. Here we define the Polygonateae to include only the axillary-flowered genera *Disporopsis*, *Heteropolygonatum*, and *Polygonatum*.

A common evolutionary trend within a group of plants is to have an increase in chromosome size or karyotype diversity, measured by asymmetry (Stebbins 1971). Ascending dysploidy (fission) or descending dysploidy (fusion) both results in chromosome number changes to the karyotype, but not necessarily in the genomic content of a species. In *Polygonatum* the accepted evolutionary trend has been that the morphologically more “simple” alternate-leaved species are positioned at the base of the phylogeny and more recent diversification in the genus has been towards morphological and cytological complexity through ascending dysploidy (Wang et al. 1986, Deng et al. 2009, Zhao et al. 2014). Molecular phylogenetic results have placed both *Disporopsis* and *Heteropolygonatum* as the sister genus to *Polygonatum* (Floden and Schilling unpubl.) or placed a *Disporopsis* + *Heteropolygonatum* clade sister to *Polygonatum* (Wang et al. 2016, Meng et al. 2014). Additionally, these inferences have placed *P. section Sibirica* ($x = 12$) as the sister to *P. sect. Polygonatum* which has lower chromosome numbers ($x = 9$ – 11). The higher base numbers of *Disporopsis* and *Heteropolygonatum* are sister to *Polygonatum* which has lower base chromosome numbers, from which it might be inferred that descending dysploidy resulting in larger chromosome sizes predominates.

The presence of three or more complete chromosome complements is known as polyploidy and is a major mechanism of adaptation and potential speciation in plants. Intraspecific polyploidy in *Polygonatum* is well documented (see Zhao et al. 2014 and references therein), but the presence of polyploidy in *Polygonatum* is not uniform across taxonomic sections. Polyploids have been reported frequently in *P.* sect. *Verticillata*, infrequently in *P.* sect. *Polygonatum*, and not all in *P.* sect. *Sibirica*. In *P.* sect. *Verticillata* polyploids are notably frequent in southwestern China along the margin of the Tibetan plateau uplift (Nie et al. 2005, Zhao et al. 2014). It is also worthy to note that polyploidy is largely absent in *P.* section *Polygonatum* in Asia where the greatest diversity of species exists (see Zhao et al. 2014), but in North America polyploids are well-documented in *P. biflorum* and *P. pubescens* though these have been considered to be of autopolyploid origin (Ownbey 1944). In *Heteropolygonatum* several species are documented to be polyploids (Floden 2014b, Yamashita and Tamura 2001, Bao et al. 1998, Tamura et al. 1997) though chromosome numbers are unknown for *H. ginfushanicum*, *H. parcefolium* and *H. anomalum* (Floden 2014b).

Recent modeling programs that use molecular phylogenies to model cytological evolution have renewed interest in testing hypotheses of karyotype evolution across families and genera (Glick and Mayrose 2014, Mayrose *et al.* 2010). ChromEvol (Glick and Mayrose 2014, Mayrose *et al.* 2010) is based on a continuous time Markov process to model changes under the assumption that changes occur anagenetically, along the branches and to estimate ancestral chromosome numbers at internal nodes. ChromEvol uses several models of chromosome evolution and can account for intraspecific cytotype variation and polyploidy proportions. These models will be used to take advantage of recently obtained molecular phylogenetic data to provide the first analysis of chromosome evolution of the Polygonateae.

The goals of the current study are to use modeling methods to understand the history of cytological evolution in the Polygonateae with a focus on the more diverse *Polygonatum*. Numerous cytological studies have documented counts for the majority of species in the genera. Here a living collection assembled for systematic and phylogenetic studies is used to report chromosome numbers for species not previously documented, counts for several recently described species, and additional counts for many other species. From these data we seek to address several questions regarding the evolutionary hypotheses of chromosome evolution in *Polygonatum* and its sister genera: 1) Which of the modes of karyotype evolution are predominate in the evolution of the Polygonateae, descending or ascending dysploidy?, and 2) has intraspecific polyploidy resulted in speciation events and reproductive isolation? This study provides the largest dataset thus far of counts for the genus and a high species sampling phylogeny to test chromosome evolution across these genera.

Materials and Methods

Chromosome Counts

Chromosome numbers used in the inference of chromosome evolution of the Polygonateae were sourced from Zhao et al. (2014) and references therein, Deng et al. (2009), Tamura (1991, 1993, 1997), and the Index to Plant Chromosome Numbers (<http://www.tropicos.org/Project/IPCN>; March 2017). When multiple chromosome numbers had been documented for a species then two strategies were used to include the species. First, the most frequently reported count was used for that species. Second, counts produced for this study were used when vouchers for other reported

counts could not be obtained. Plants cultivated by AF were extensively used to document chromosome numbers. Methods for mitotic chromosome counts followed the protocol in Floden (2014a). Chromosome counts produced for this study are presented in Table 1. Samples for which no chromosome count has been documented, or the species identification was unclear because of documented polyphyly in recent molecular studies (Floden and Schilling unpubl.) were coded as “X” in ChromEvol. For example, *P. verticillatum* was shown to be highly polyphyletic with samples from Europe and the Caucasus remaining as *P. verticillatum*. Samples formerly included in *P. verticillatum* from the Himalaya were also polyphyletic and also in the region they exhibit a range of base chromosome numbers and polyploids of those numbers (Himalaya (Mehra and Pathania 1960, Mehra and Sachdeva 1976, Pandita and Mehra 1982, Sachdeva and Malik 1977, Sachdeva et al. 1986). Thus the variation in chromosome number and polyploidy cannot be assessed from a molecular voucher without living material. Some accessions from the Himalaya are then also coded as “X” in the dataset for inference of chromosome evolution in ChromEvol.

Taxon sampling and phylogenetic analyses

Here we included 67/ca. 80 species of *Polygonatum*, 6/10 *Heteropolygonatum*, and 3/10 *Disporopsis*, and we included two novel species of *Polygonatum* (Table 1). This dataset was a subset of a larger matrix of 285 taxa used in a phylogenetic reconstruction of *Polygonatum* based on data from four molecular loci: nuclear ribosomal ETS and ITS, the plastid *petA*, and the mitochondrial *rps10* (Floden and Schilling unpubl.). For most species only a single sample was included when no variation in the molecular data of conspecific samples was observed. When polyploidy was documented for conspecific accessions from which the molecular data was obtained and there was also a diploid representative both samples were included. When conspecific accessions did not group together these were included even in the absence of documented polyploidy. The resultant dataset was comprised of 113 taxa including the outgroups. Sequences were concatenated using “Concatenate sequences or alignments” function in Geneious 7.1.9 (Biomatters Ltd., New Zealand) and the alignment was performed in Mafft v7.023b (Katoh et al. 2002) under default parameters. Alignments were then manually adjusted to reduce indels in Geneious. The resulting complete alignment was then coded for indels using FASTGAP2.1 (Borchsenius 2009) which uses the simple indel coding of Simmons and Ochoterena (2000). The phylogenetic inferences were done using RaxML 7.2.8 (Stamatakis 2006) using GTR- Γ -I model based on AIC scores from MrModeltest v2 (Nylander 2004). Statistical support was assessed by 1000 ML bootstrap replicates (Felsenstein 1985) with a search for the best scoring tree. The maximum likelihood phylogram from the combined matrix was transformed into an ultrametric tree with the R 3.3.3 (R Development Core Team 2017) function “chronos” implemented in the APE package v. 4.1 (Paradis et al. 2004).

ChromEvol

For phylogenetic inferences of ancestral chromosome numbers we used ChromEvol 2.0 (Glick and Mayrose 2014, Mayrose et al. 2010) which infers anagenetic changes in a phylogeny. This program implements the eight original models of chromosome evolution (Mayrose et al. 2010) with two additional models which provide the ability to infer the monoploid number of a group, to also categorize whether a terminal taxon is diploid or polyploid, and also supports intraspecific chromosome number variation. It assumes the four types of chromosome change events are possible: ascending dysploidy (with rate λ), descending dysploidy (rate δ), whole

genome duplication (WGD) (rate ρ), and demi-polyploidy (rate μ). We first tested the “All_Models” option with 1000 replications to assess the particular model that best fits our data. The best scoring model was then re-run with 10,000 replications with “Optimize_Model” designated. Output was viewed and manipulated in R 3.3.3 using the package ChromEvol (Cusimano, 2013).

Results

Chromosome counts

Sixty-five new chromosome counts from samples of *Heteropolygonatum* and *Polygonatum* were made for this study which included new counts for seven species, the first tetraploid count reported for *P. sibiricum* ($2n = 4x = 48$), a first diploid count for *H. marmoratum* ($2n = 2x = 32$), and several new counts for species only reported once previous to this study (Fig. 3.1, Table 3.1, Supp. material). *Polygonatum angelicum*, *P. brevistylum*, *P. luteoverrucosum*, *P. cf. nervulosum*, *P. sp. nov.* 4275, and *P. sp. PZM018* were all determined to be $2n = 2x = 30$.

Phylogeny

The results of the phylogenetic analyses recovered Convallarieae, followed by the Aspidistreae, and Ophiopogoneae sister to a non-monophyletic Polygonateae (Fig. 3.2). The relationship of the remaining Polygonateae with axillary flowers was *Disporopsis* followed by *Heteropolygonatum* + *Polygonatum*. Within *Polygonatum* all three sections were recovered with high support and *P. series Grandiflora*, *P. series Oppositifolia*, and *P. series Punctata* within *P. sect. Verticillata* were well supported.

ChromEvol

The initial simulation in ChromEvol resulted in an AIC score that supported constant-rate model was the best explanation of the empirical data (AIC = 371.5). The results of the optimized model of ChromEvol inference of chromosome evolution can be seen in Figure 3.2. The summary of the output of the analyses reported 34 gain events, 53 loss events, and 10 duplication events with high posterior probabilities. There were no demi-duplication events in which are chromosome increases 1.5 times the base number (Glick and Mayrose 2014, Mayrose et al. 2010). Descending dysploidy was determined to be the most likely evolutionary scenario across the phylogeny. The inferred ancestral chromosome number for the group of tribe Nolinoideae studied here was $n = 19$ (Fig. 3.2). The Aspidistreae and Convallarieae were $n = 19$ with a single base number loss in the Ophiopogoneae + *Maianthemum* clade ($n = 18$). The next successive branch was $n = 18$ with a gain along the branch that lead to *Disporopsis* ($n = 20$). For the node at the split between *Heteropolygonatum* and *Polygonatum* the inferred chromosome number was $n = 16$. Within *Heteropolygonatum* there was a single duplication for one sample of *H. marmoratum*. The ancestral chromosome number for *Polygonatum* was inferred to be $n = 14$. The ancestral chromosome for the branch at the split between *P. sect. Sibirica* and *P. sect. Polygonatum* was $n = 12$ with a duplication in one tetraploid sample of *P. sibiricum*. The ancestral chromosome number was $n = 11$ for *P. sect. Polygonatum* and several losses to $n = 10$ and $n = 9$ and also several gains from those numbers within clades. The ancestral chromosome number for *P. sect. Verticillata* was $n = 14$ with several gains to $n = 15$ with losses of a single base to $n = 14$ in several clades of verticillate-leaved species. *Polygonatum series Grandiflora*, *P. series Oppositifolia*, and *P. series Punctata* were all $n = 15$ with only one duplication in *P. series Grandiflora*.

Discussion

First chromosome counts are documented here for seven species of *Polygonatum* with numerous additional counts which brings to 58 out of 83+ species of the genus for which chromosome number information is available (Fig. 3.1). *Polygonatum angelicum*, *P. brevistylum*, *P. luteoverrucosum*, and *P. cf. nervulosum* were all $2n = 2x = 30$ (Fig. 3.1). These reports for *P. brevistylum* and *P. cf. nervulosum* clearly support their inclusion in *P. sect. Verticillata* rather than in *P. sect. Polygonatum* in which Baker (1875) had included them. An accession of *P. sibiricum* proved to be the first reported tetraploid for the species with $2n = 2x = 48$ (Fig. 3.1). The chromosome number data, combined with results from comprehensive molecular phylogenetic analyses, provide the opportunity to make the first analytical estimation of chromosome evolution in the genera of the Polygonateae (Fig. 3.2). Descending dysploidy was inferred to be the predominant mode of chromosome evolution in the subtribe. The Convallarieae and the Aspidistreae were both inferred to have $n = 19$ as their ancestral number. The results of the phylogenetic analyses also corroborate the exclusion of *Maianthemum* from the remaining Polygonateae. *Maianthemum* has a base of $n = 18$ like most of tribe Ophiopogoneae (though counts of $n = 17$ have been reported for some *Ophiopogon* species (Wang et al. 2013)). These results show that the relatively high primary based numbers of $x = 15$ in *Polygonatum* are not the results of a polyploidy result within the genus.

Descending dysploidy, with inferred fusion of chromosomes, was the predominant mode of chromosome evolution inferred in the axillary-flowered Polygonateae. Studies of karyology of these genera are extensive and have concluded that ascending dysploidy was the observed evolutionary pattern (Chen 1989, Conran and Tamura 1998, Fang 1984, Hasegawa 1933, Tamura 1995, Wang et al. 1987, 1991, Yang et al. 1988, 1992, Zhao et al. 2014). The ancestral chromosome number for *Disporopsis* was inferred by the result of the ChromEvol analysis to have been $n = 20$, likely derived from an ancestor which had $n = 18$. Conran and Tamura (1998) suggested the possibility of a tetraploid origin of *Disporopsis* though the results here suggest an ascending dysploidy event. Within *Disporopsis* there is limited variation in karyotype and only a single base number has been reported (Hong and Zhu 1990). *Heteropolygonatum* was estimated to have had an ancestral chromosome number of $n = 16$, and there was one instance of polyploidy reported for a sample of *H. marmoratum* (Figs. 3.1–3.2). A conspecific sample was found to be diploid and not sister to the polyploid sample. The polyploid and diploid also have different floral morphology and the phylogenetic evidence and chromosome evolution would be consistent with treating these as distinct species (Floden 2014b).

The ancestral chromosome number for *Polygonatum* was estimated to be $n = 14$ (Fig. 3.2) which provides evidence that two larger clades have followed different chromosomal evolutionary histories. Descending dysploidy was observed in *Polygonatum sect. Polygonatum*, and also in its sister *P. sect. Sibirica* from an inferred starting base of $n = 12$. A single polyploidy event was shown for one *P. sibiricum*, which was a tetraploid, the first documented count at this level for the species. The tetraploid in cultivation is surprisingly a smaller plant than the diploid forms cultivated by AF. The monotypic *P. sect. Sibirica* has an asymmetric karyotype in contrast to its sister clade, *P. sect. Polygonatum*, which is characterized by its symmetric karyotype. In *P. sect. Polygonatum* there were successive reductions down to $n = 9$, e.g. *P. filipes* in a clade of mostly $n = 10$ and $n = 11$, and within the ultimate clades there were occasional ascending dysploidy events, e.g. *P. cryptanthum* placed sister to a clade of mostly $n = 10$ and $n = 11$. Suomalainen

(1947) inferred that in *P. sect. Polygonatum* a base number of $n = 9$ was derived from $n = 10$ (which was the only other number reported at the time) by means of mismatched pairing or unequal exchange. Conversely, *P. sect. Verticillata* exhibited ascending dysploidy which has been posited as the mechanism for all of *Polygonatum* due to the putatively derived characters including phyllotaxy and their more asymmetric karyotypes (Deng et al. 2009, Wang et al. 1987). The first clade of *P. sect. Verticillata*, comprising most verticillate-leaved species, contained several clades of $n = 15$ and several of $n = 14$. Ascending dysploidy events were observed in the clade containing *P. series Grandiflora*, *P. series Oppositifolia*, and *P. series Punctata*. No chromosome losses were observed in this clade, though duplications, polyploidy events were observed only in *P. series Grandiflora*. Several novel species and a few with undocumented chromosome counts were also included in the inference. *Polygonatum wardii*, sister to *P. sect. Verticillata*, is presumably $n = 14$ based on its phylogenetic placement. The novel species were all placed in *P. series Oppositifolia* and are all presumably $n = 15$.

Genome duplication events reported here in *Polygonatum* did not always appear to be associated with speciation events. In *P. sect. Polygonatum* a single polyploid, *P. biflorum*, was placed sister to another *P. biflorum* and these two sister to *P. pubescens*. This polyploid is from the southern extension of its distribution in Florida, USA. Despite Ownbey's (1944) assertion that if plants from this region were tetraploid they should be treated as a distinct species, our molecular data does not support this. Sampling of *P. biflorum* from across the total distribution does not reveal any molecular changes in nuclear ribosomal ETS and ITS, or in several chloroplast loci (Floden unpubl.). Within *P. section Verticillata* polyploidy is documented in several species from the Hengduan Mountains of southwest China (Tamura 1993), and in the Himalaya (Mehra and Pathania 1960, Mehra and Sachdeva 1976, Pandita and Mehra 1982, Sachdeva and Malik 1977, Sachdeva et al. 1986). The Himalayan polyploid reports are mostly for samples from a phylogenetically diverse lineage that were formerly placed within a broad circumscription of *P. verticillatum* (Floden and Schilling unpubl.). It is also not clear whether presumably conspecific samples are the same species, e.g. *P. kansuense* was placed in two separate clades and one of those was documented to be a polyploid. Morphologically they appear the same and they have the same base chromosome number. Ascending dysploidy and a duplication event to $n = 15$ ($2n = 60$) were estimated for *P. bulbosum* and *P. sp. Daocheng* from the Hengduan Mountains, China, and these were placed within a clade where the base number was mostly $n = 14$. Because of the extreme environments occupied in the Himalaya by *Polygonatum*, it might be speculated that polyploidy has been a key part of their adaptive success (Grant 1981, Levin 1983).

From chromosome studies and inference of ancestral chromosome numbers we further documented polyploidy in this subtribe of the Nolinoideae. The results here show numerous ascending and descending dysploidy events within clades suggesting that the karyotype in *Polygonatum* is labile. This first estimation of chromosome evolution focusing on the Polygonateae must be considered preliminary given the absence of several species with reported base numbers of $x = 13$ in *P. sect. Verticillata* (see Zhao et al. 2014). Furthermore this dataset includes several taxa, some of which are novel species, with unknown chromosome numbers. Unknown numbers in the dataset can cause erroneous inferences in ChromEvol. To fully appreciate the diversity and better estimate the chromosome evolution in *Polygonatum* additional species, their chromosome numbers, and a more fully resolved phylogeny are needed to increase the robustness of the model inference.

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Appendix E

Table 3.1. Chromosome numbers of *Heteropolygonatum* and *Polygonatum* species produced for this study following methods in Floden (2014).

Species	voucher	Herbarium	Chromosome #
<i>H. marmoratum</i> (H. Lév.) Floden	BO sn	(TENN)	2n = 2x = 32
<i>P. acuminatifolium</i> Kom.	MM sn	(TENN)	2n = 2x = 22
<i>P. angelicum</i> Floden	Floden 2844	(TENN)	2n = 2x = 30
<i>P. biflorum</i> (Walt.) Ell. FL	Floden sn	(TENN)	2n = 4x = 40
<i>P. brevistylum</i> Baker	FBP 2761		2n = 2x = 30
<i>P. cf. brevistylum</i>	**		2n = 2x = 30
<i>P. bulbosum</i> H. Lév.	Floden sn	(TENN)	2n = 4x = 60
<i>P. cathcartii</i> Baker	BSWJ2421	(TENN)	2n = 2x = 30
<i>P. cathcartii</i>	BSWJ2429	(TENN)	2n = 2x = 30
<i>P. cathcartii</i>	Floden 2735	(TENN)	2n = 2x = 30
<i>P. cf. nervulosum</i> Baker	FBP 2762	(TENN)	2n = 2x = 30
<i>P. cryptanthum</i> H.Lév. & Vaniot	Floden 2727	(TENN)	2n = 2x = 18
<i>P. cryptanthum</i>	USNA56584H	(TENN)	2n = 2x = 18
<i>P. cyrtanema</i> Hua	FO15 sn	(TENN)	2n = 2x = 22
<i>P. cyrtanema</i>	Floden 2448	(TENN)	2n = 2x = 22
<i>P. cyrtanema</i>	**		2n = 2x = 22
<i>P. cyrtanema</i>	USN60726J		2n = 2x = 18
<i>P. cyrtanema</i>	**		2n = 2x = 22
<i>P. cyrtanema</i>	DJH		2n = 2x = 22
<i>P. darisii</i> H.Lév.	Floden 2907	(TENN)	2n = 2x = 30
<i>P. govanianum</i> Royle	**		2n = 2x = 22
<i>P. huanum</i> H. Lév.	Floden 2461	(TENN)	2n = 2x = 30
<i>P. inflatum</i> Komarov	Cheju	(TENN)	2n = 2x = 22
<i>P. Jacquemontianum</i> Royle	BSWJ sn	(TENN)	2n = 2x = 28
<i>P. kansuense</i> Maxim. ex Batalin	Floden 2427	(TENN)	2n = 4x = 56
<i>P. kingianum</i> Collett & Hemsley	BO sn	(TENN)	2n = 4x = 60
<i>P. kingianum</i>	BSWJ6545	(TENN)	2n = 2x = 30
<i>P. kingianum</i>	CY sn	(TENN)	2n = 2x = 30
<i>P. lasianthum</i> v. <i>coreanum</i>	A1K301	(TENN)	2n = 2x = 20
<i>P. luteoverrucosum</i> Floden	FBP2764	(CAL)	2n = 2x = 30
<i>P. luteoverrucosum</i>	**		2n = 2x = 30
<i>P. macranthum</i> (Maxim.) Koidz.	CPC97.11.4	(TENN)	2n = 2x = 22
<i>P. odoratum</i> (Mill.) Druce	TCM12-273	(TENN)	2n = 2x = 20
<i>P. orientale</i> Desf.	TCM12-194	(TENN)	2n = 2x = 18
<i>P. orientale</i>	TCM Osmaniye	(TENN)	2n = 2x = 18
<i>P. prattii</i> Baker	BO sn	(TENN)	2n = 4x = 56
<i>P. prattii</i>	Floden 3065	(TENN)	2n = 4x = 56
<i>P. sibiricum</i> Redouté	PBG	(TENN)	2n = 4x = 48
<i>P. sibiricum</i>	GB sn	(TENN)	2n = 2x = 24
<i>P. singalilense</i>	Floden 2731	(TENN)	2n = 2x = 28
<i>P. sp.</i>	USNA	(TENN)	2n = 2x = 17,18
<i>P. sp.</i> Emei	**		2n = ?x = 100+

Table 3.1. Continued.

<i>Species</i>	<i>voucher</i>	Herbarium	Chromosome #
<i>P. sp.</i> Daocheng	**		2n = 4x = 60
<i>P. sp.</i>	BSWJ2417		2n = 4x = 60
<i>P. sp.</i> "Himalayan Giant"	Floden 2900	(TENN)	2n = 4x = 60
<i>P. sp. nov.</i>	**		2n = 2x = 30
<i>P. sp. nov.</i>	**		2n = 2x = 30
<i>P. stewartianum</i> Diels	CLD325	(TENN)	2n = 4x = 56
<i>P. trinerve</i> Hua	YW sn		2n = 4x = 56
<i>P. trinerve</i> Hua	**		2n = 2x = 28
<i>P. uncinatum</i> Diels	Floden 2914	(TENN)	2n = 2x = 30
<i>P. uncinatum</i>	B5	(TENN)	2n = 2x = 30
<i>P. vietnamicum</i> Abramova	Floden 2873	(TENN)	2n = 2x = 30
<i>P. zanolanscianense</i> Pamp.	**	(TENN)	2n = 2x = 30
<i>P. zanolanscianense</i>	Floden 2750	(TENN)	2n = 2x = 30

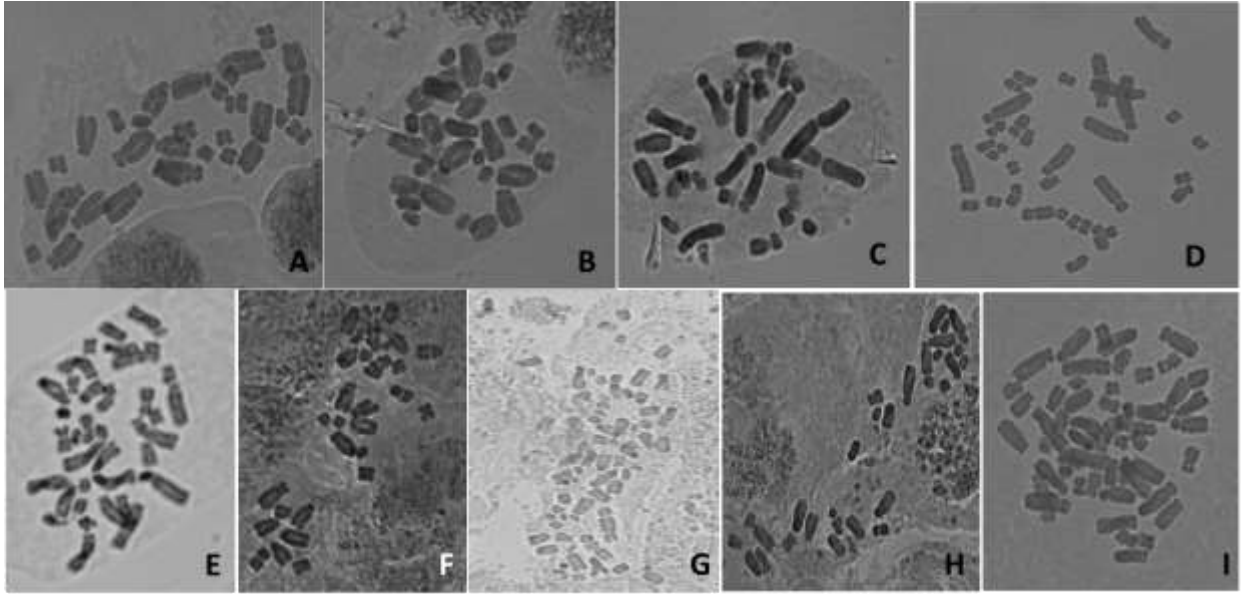
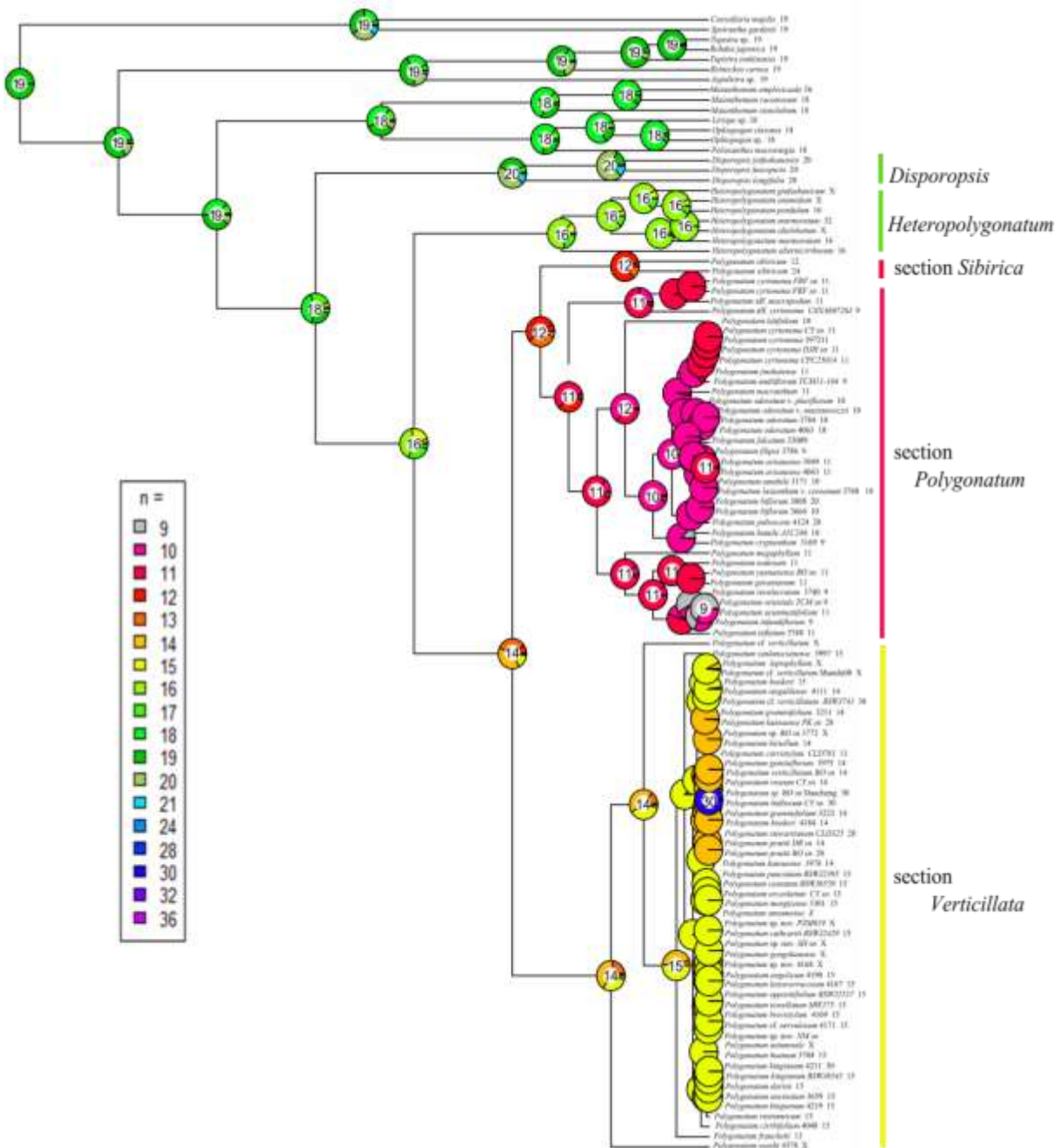


Figure 3.1. Squashes of chromosome counts of *Heteropolygonatum* and *Polygonatum* produced for this study. Top Row: A, *P. angelicum* $2n = 2x = 30$; B, *P. luteoverrucosum* $2n = 2x = 30$; C, *P. cf. brevistylum* $2n = 2x = 30$; D, *Heteropolygonatum marmoratum* $2n = 2x = 32$; Bottom Row: E, *P. cathcartii* $2n = 2x = 30$; F, *P. brevistylum* $2n = 2x = 30$; G, *P. biflorum* $2n = 4x = 40$; H, *P. cf. nervulosum* $2n = 2x = 30$; I, *P. sibiricum* $2n = 4x = 48$.



Conclusions

The results of whole plastome phylogenetic analyses from NGS data, a comprehensive taxon sampling phylogeny with four loci, and a reduced subset of the previous dataset used in chromosomal evolution studies has greatly increased the knowledge of the Polygonateae.

Comparisons of plastome structure and phylogenetic analyses of the plastome strongly supported the reciprocal monophyly of *Disporopsis*, *Heteropolygonatum*, and *Polygonatum*. Furthermore, comparisons of chloroplasts genomes revealed extensive polyphyly in *P. verticillatum* and showed that the Himalayan plants are not closely related to the European plants. These data also supported the recognition of *P. govanianum*, indicating that it should not be referred to *P. multiflorum* from Europe and the Caucasus. Last, the analyses of the plastome provided a dataset that facilitated the selection of markers from relatively more divergent portions of the plastome to use in studies that assessed phylogenetic relationships in the tribe.

The second chapter provides the most comprehensive phylogenetic analyses of *Polygonatum* to date and provides the first species level phylogenies for *Disporopsis* and *Heteropolygonatum*. These data, with higher taxon sampling, corroborate extensive polyphyly in *P. verticillatum* and *P. cyrtonema*, and confirm the distinction of *P. govanianum*. Moreover *P. cathcartii* and *P. hookeri* were found to be polyphyletic. The series level classification that has been proposed in *P. sect. Verticillata* was mostly validated, and individual series were moderately to well-supported that seem to be natural groups based on data from morphology and cytology. Surprisingly, the relatively obscure species *P. wardii* was placed sister to *P. sect. Verticillata*. Most importantly, these data and the inclusion of several undescribed species revealed that the diversity in *Polygonatum* has been greatly underestimated.

The final phylogenetic study was the first estimation of chromosomal evolution in the Polygonateae and used the molecular phylogenetic tree analyzed with the software package ChromEvol. These results showed that descending and ascending dysploidy have occurred in the Polygonateae with losses estimated to predominate within *Polygonatum* though gains were estimated to have occurred in distal nodes; documenting the labile behavior of chromosome number in *Polygonatum*.

These studies provided the first comprehensive molecular phylogenetic study for the three genera of the Polygonateae. The molecular dataset and the methodology provided to sequence the universal barcode nuclear ITS, provide a foundation on which to build further studies with increased sampling to elucidate the polyphyly uncovered for several species groups and the intraspecific variation in some widely distributed species, e.g. *P. odoratum*. The molecular validation of several novel species shows the importance of continued work, especially field studies, in the Polygonateae. The initial estimation of chromosome evolution should be seen as preliminary given the absence of several rare *Polygonatum* species placed in *P. sect. Verticillata* based on their karyotype and morphology which have a base chromosome of $x = 13$. These species might fundamentally alter the structure of within the group and provide a more accurate estimation of chromosome evolution.

Vita

Aaron Floden was born in Whitefish, MT, to the parents of John and Anita Floden. He is the fourth of six children; Shane, Angliee, Lars, Cailin, and Errienne. He attended several elementary schools while his father was in the Airforce. After settling in Overland Park, KS, he attended Blue Valley Middle School and Blue Valley High School. After high school he worked as a chef at Da' Ginos in Malnisio di Gais in northern Italy. He began his bachelors at Kansas State University in Manhattan, KS as engineering major, and obtained his bachelors in Ecology and Evolutionary Biology in 2010 at the University of Tennessee, Knoxville. He accepted a graduate teaching assistantship at the University of Tennessee, Knoxville, in the Ecology and Evolutionary Biology Program in the Schilling Lab. Aaron graduated with a Doctor of Philosophy in 2017.