

Phylogeny of the popcorn flowers: Use of genome skimming to evaluate monophyly and interrelationships in subtribe Amsinckiinae (Boraginaceae)

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Abstract Subtribe Amsinckiinae, currently containing 13 genera and approximately 287 species, is a species-rich group of the family Boraginaceae. Past studies assessing relationships had a limited sample size and generally weak support. Here we study phylogenetic relationships of Amsinckiinae using a large sample size and considerably more sequence data in order to evaluate the interrelationships of genera and clades within this group. Using high-throughput, genome skimming sequencing of 139 samples of Amsinckiinae and four outgroup taxa, maximum likelihood and Bayesian analyses of separate plastome, cistron, and mitochondrial datasets are presented. In almost all analyses the common ancestor of the Amsinckiinae gives rise to an *Andersonglossum* or to an *Andersonglossum*+*Adelinia* clade. Most genera, including *Amsinckia*, *Eremocarya*, *Greeneocharis*, *Harpagonella*, *Oreocarya*, and *Pectocarya*, are consistently monophyletic with strong support. *Plagiobothrys* is confirmed to be non-monophyletic, composed of three clades conforming to generic sections. *Cryptantha* is also non-monophyletic, with most species within a strongly supported *Cryptantha* s.str. clade, but some nesting within *Johnstonella* or our *Maritimae* clade, all with strong support. Although genome skimming verifies the monophyly of many genera and clades of Amsinckiinae, relationships among those clades and along the backbone of the trees remain uncertain, their elucidation possibly a factor of short branch lengths and likely requiring different types of molecular data. Our study may serve as a baseline for future work on the morphology, reproductive biology, and biogeography of the Amsinckiinae.

Keywords Amsinckiinae; Boraginaceae; Cryptanthinae; genome skimming; high-throughput sequencing; phylogenetics

Supplementary Material The Electronic Supplement (Figs. S1–S3) is available in the Supplementary Data section of the online version of this article (<http://www.ingentaconnect.com/content/iapt/tax>); DNA sequence alignments are available from TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S21212>)

■ INTRODUCTION

Boraginales are a group of flowering plants consisting of up to 2700 species of trees, shrubs, or (often) herbs, with a nearly worldwide distribution (Mabberley, 2008; Luebert & al., 2016). This plant order has been the focus of several broad phylogenetic studies (Långström & Chase, 2002; Nazaire & Hufford, 2012; Cohen, 2014; Weigend & al., 2014; Luebert & al., 2016). The Boraginales have been subject to differing circumscriptions over the years, having been treated as equivalent to a single, large family Boraginaceae s.l. (e.g., Mabberley, 2008; APG IV, 2016) with several subfamilies, or split into as many as eleven families (e.g., Weigend & al., 2014; Luebert & al., 2016), including the Cordiaceae, Ehretiaceae, Heliotropiaceae, Hydrophyllaceae, and a more narrowly circumscribed Boraginaceae s.str. We elect to treat the Boraginaceae in this strict

sense, as in Luebert & al. (2016) and Chacón & al. (2016), and our use of the name “Boraginaceae” is with this circumscription for the remainder of this article.

Boraginaceae contain 1600–1700 species (Chacón & al., 2016) and possess numerous diagnostic morphological characteristics, including a hirsute to hispid vestiture, a usually circinate scorpioid cyme inflorescence, mostly actinomorphic flowers, a strongly four-lobed ovary from a gynobasic style, and a fruit that is a schizocarp of nutlets, the last two characters apomorphic for the family (Luebert & al., 2016). The focus of this study is a group within Boraginaceae, recently delimited as subtribe Amsinckiinae (of subfamily Cynoglossoideae, tribe Cynoglosseae, after Chacón & al., 2016), many members of which have been referred to as the “popcorn flowers”. The Amsinckiinae is currently calculated to contain approximately 287 species and 330 minimally ranked taxa (see Table 1), a

diversity that will undoubtedly increase as the taxa become better studied. This is a relatively high richness within the Boraginaceae, up to 18% of the species in the family. Here, we aim to infer the phylogenetic relationships within the Amsinckiinae in order to assess the monophyly of genera and clades and to evaluate their interrelationships.

Past phylogenetic studies. — Prior to molecular phylogenetic studies, the members of the Amsinckiinae belonged primarily to tribe Eritricheae (see, e.g., Riedl, 1997), along with many other genera now included in other tribes and subtribes of the Boraginaceae. Långström & Chase (2002), in an early molecular study that included 38 taxa of the Boraginaceae (their Boraginoideae of Boraginaceae s.l.), using the single chloroplast marker *atpB*, argued for the recognition of four, monophyletic tribes within the group: Boragineae, Cynoglosseae, Lithospermeae, and a new tribe, Echiochileae. These researchers were the first to include a member of the Amsinckiinae (as recognized here) in such a study, in this case a single sample of the genus *Cryptantha* Lehman ex G. Don, *C. virgata* (Porter) Payson (= *Oreocarya virgata* (Porter) Greene), which placed within their tribe Cynoglosseae, inclusive of the formerly

recognized tribe Eritricheae (within which *Cryptantha* had traditionally been placed). Their small sample size prevented these authors from making more than cursory observations about affinities of *Cryptantha*. Nazaire & Hufford (2012), in a broad study of the Boraginaceae s.l., using 318 samples and DNA sequence data from two cistron regions and four chloroplast markers, recovered the same four tribes (within their Boraginoideae of Boraginaceae s.l.) as did Långström & Chase (2002). Within their Cynoglosseae, four species of *Cryptantha* and one species of *Plagiobothrys* constituted a clade sister to another clade of two species of *Amsinckia*, the two linked with mixed support (i.e., strongly supported in one analysis but weakly supported in another).

Hasenstab-Lehman & Simpson (2012) focused on the interrelationships of *Cryptantha* in a molecular phylogenetic analysis of 70 taxa using sequence data from ITS and the chloroplast marker *trnL_{UAA}*. Although they had limited outgroup sampling, the authors resolved a mixed to strongly supported large clade, which they called subtribe Cryptanthinae (= Amsinckiinae). (Note that the name “Cryptanthinae” was determined to be invalid by the rules of the *International Code of Nomenclature for algae, fungi, and plants*—McNeil & al., 2012; K. Gandhi, J. McNeill, J. Reveal, J. Strother, pers. comm.; see below.) What had been circumscribed by most taxonomists as the genus *Cryptantha* (see Simpson & Hasenstab, 2009) was determined to be polyphyletic. Species traditionally classified in *Cryptantha* were found to comprise 5–6 separate and strongly supported clades, warranting the authors to split *Cryptantha* s.l. into five genera: *Cryptantha* s.str. and the resurrected genera *Eremocarya*, *Greeneocharis*, *Johnstonella*, and *Oreocarya*. Moreover, *Cryptantha* s.str. was resolved as two clades, *Cryptantha* s.str. 1 and *Cryptantha* s.str. 2, which were either sister taxa (in their parsimony analysis) or well separated from one another (in their Bayesian and maximum likelihood analyses). Other clades with strong support in their analyses were the genera *Amsinckia* and *Pectocarya*. Finally, the genus *Plagiobothrys* was inferred to be non-monophyletic, composed of a main clade containing members of sect. *Allocarya* and sect. *Plagiobothrys* only (*Plagiobothrys* s.str.), a clade corresponding to *Plagiobothrys* sect. *Sonnea* (= genus *Sonnea*) and a clade corresponding to *Plagiobothrys* sect. *Amsinckiopsis* (this last group monophyletic in one analysis, diphyletic in another, both with weak support). The interrelationships of these clades/genera varied in their analyses. Although some interrelationships among genera remained constant (see below), the so-called “backbone” interrelationships of these genera and clades varied by analysis with generally weak support along nodes.

Subsequent molecular phylogenetic analyses of the Boraginaceae encompassing members of this plant complex include the studies of Weigend & al. (2013), Cohen (2014, 2015), Otero & al. (2014), and Chacón & al. (2016), all using various combinations of ITS and coding and/or intergenic chloroplast markers. These studies have almost entirely converged on the recognition of a strongly supported clade that includes *Cryptantha* and relatives, within tribe Cynoglosseae. This clade is now formally recognized (see Chacón & al., 2016) as subtribe Amsinckiinae Brand, the first available name for

Table 1. Consensus classification of genera of subtribe Amsinckiinae with current number of species and minimally ranked taxa (including varieties and subspecies) accepted by the authors.

Genus	Species	Minimum-ranked taxa
<i>Adelinia</i> J.I.Cohen 2015	1	1
<i>Amsinckia</i> Lehm. 1831, nom. cons.	15	17
<i>Andersonglossum</i> J.I.Cohen 2015	2	2
<i>Cryptantha</i> Lehm. ex G. Don 1837	103	115
<i>Dasynotus</i> I.M.Johnst. 1948	1	1
<i>Eremocarya</i> Greene 1887	2	3
<i>Greeneocharis</i> Gürke & Harms 1899	2	3
<i>Harpagonella</i> A.Gray 1876	2	2
<i>Johnstonella</i> Brand 1925	13	15
<i>Oncaglossum</i> Sutorý 2010	1	1
<i>Oreocarya</i> Greene 1887	63	72
<i>Pectocarya</i> DC. ex Meisn. 1840	13	14
<i>Plagiobothrys</i> Fisch. & C.A.Mey. 1836		
sect. <i>Amsinckiopsis</i> I.M.Johnst.	2	3
sect. <i>Allocarya</i> I.M.Johnst., sect. <i>Echidiocarya</i> I.M.Johnst., sect. <i>Plagiobothrys</i> I.M.Johnst.	65	79
sect. <i>Sonnea</i> I.M.Johnst. (= <i>Sonnea</i> Greene 1887)	2	2
Total	287	330

Consensus from the phylogenetic analyses of Hasenstab-Lehman & Simpson (2012), Weigend & al. (2013), Cohen (2014, 2015), Otero & al. (2014), and Chacón & al. (2016). Note that, for now, we are not including the monospecific *Nesocaryum* I.M.Johnst. (Johnston, 1927), which was accepted by Chacón & al. (2016), as a member of the subtribe.

this complex of plants that currently includes members of 13 genera: *Adelinia*, *Amsinckia*, *Andersonglossum*, *Cryptantha*, *Dasynotus*, *Eremocarya*, *Greeneocharis*, *Harpagonella*, *Johnstonella*, *Oncaglossum*, *Oreocarya*, *Pectocarya*, and *Plagiobothrys* (Table 1).

Although there are differences in the details of these studies, these likely a function of taxon and DNA sequence sampling, several common patterns have emerged. In those studies in which they were included, three North American species of *Cynoglossum* arise as part of a polytomy (Weigend & al., 2013) as a grade (Cohen, 2015; Chacón & al., 2016) from the common ancestor of what is now recognized as the Amsinckiinae. This novel placement of these North American species of *Cynoglossum* within the complex was first discovered by Weigend & al. (2013), with Cohen (2015) summarizing their comparative morphology and naming them as genera nova, *Adelinia* and *Andersonglossum*. Two other North American genera (both monospecific) were also discovered to be members of the Amsinckiinae. The genus *Oncaglossum*, which had also been earlier classified in the genus *Cynoglossum*, was shown to be part of the Amsinckiinae by Cohen (2014, 2015), in the only studies where the genus was included. *Oncaglossum* was resolved as sister either to all other members of the Amsinckiinae (Cohen, 2014) or to all other members of the subtribe except *Adelinia* and *Andersonglossum* (when the latter two genera were included; Cohen, 2015). The genus *Dasynotus* was established by Weigend & al. (2013), Cohen (2014, 2015), and Chacón & al. (2016) to belong in the Amsinckiinae clade. Weigend & al. (2013) resolved *Dasynotus* in a polytomy with all other Amsinckiinae examined except *Adelinia* and *Andersonglossum*. Cohen (2014) resolved *Dasynotus* as sister to three *Oreocarya* species or to a clade containing *Cryptantha*, *Greeneocharis*, *Pectocarya*, and *Plagiobothrys* species. However, Cohen (2015), using the same molecular markers but a reduced taxon sampling focusing on tribe Cynoglosseae, resolved it as sister to all other examined Amsinckiinae, minus *Adelinia*, *Andersonglossum*, and *Oncaglossum*. Finally, Chacón & al., 2016 resolved *Dasynotus* to be sister to all other examined Amsinckiinae except *Adelinia*, *Andersonglossum*, and a *Pectocarya*-*Harpagonella* clade. (The placement of the South African *Cynoglossum obtusicalyx* in a polytomy with all other examined Amsinckiinae in the plastid analysis of Otero & al., 2014 is likely erroneous and needs to be tested.)

Phylogenetic relationships of the other genera of the Amsinckiinae have also varied in the aforementioned analyses. However, some general trends can be noted. The genera *Amsinckia* and *Pectocarya* are recovered as monophyletic in all analyses, and (where included) *Harpagonella* is always sister to *Pectocarya*. *Oreocarya* is monophyletic in the analyses of Hasenstab-Lehman & Simpson (2012), Weigend & al. (2013), Otero & al. (2014, in part), and Cohen (2015), and, when so, support values for the clade are high. *Eremocarya* is sister to *Oreocarya* in Hasenstab-Lehman & Simpson (2012) and in Otero & al. (2014, in part). The species of *Plagiobothrys* in sect. *Allocarya* and sect. *Plagiobothrys* consistently form a well-supported clade (Hasenstab-Lehman & Simpson, 2012; Weigend & al., 2013; Cohen, 2014, 2015; Otero & al., 2014;

Chacón & al., 2016), these being sister to *Greeneocharis* in some studies (Hasenstab-Lehman & Simpson, 2012; Otero & al., 2014, in part). *Eremocarya* and *Greeneocharis*, where included, are always monophyletic, but sample size of these two genera (each containing only two species) has often been limited to a single specimen. The genus *Johnstonella* is monophyletic in two studies (Hasenstab-Lehman & Simpson, 2012; Otero & al., 2014, in part). *Cryptantha* s.str. (minus the segregate genera *Eremocarya*, *Greeneocharis*, *Johnstonella*, and *Oreocarya*) remains consistently polyphyletic in all analyses, often with weak support. In these analyses *Cryptantha* s.str. is often (but not always) classified apart from *Eremocarya*, *Greeneocharis*, and *Oreocarya*, but species of *Johnstonella* are sometimes included within *Cryptantha* s.str. Both Hasenstab-Lehman & Simpson (2012) and the ITS analysis of Otero & al. (2014) recovered similar *Cryptantha* s.str. 1 and s.str. 2 clades. Finally, interrelationships of genera and major clades of the Amsinckiinae, especially along the backbone of the tree, generally vary significantly between analyses and often with poor support, the clades sometimes forming polytomies.

Members of the Amsinckiinae exhibit a great range of variation in nutlet morphology (size, shape, sculpturing patterns, gynobase attachment, heteromorphism, and dimorphism), plant duration (annuals, biennials, or perennials), and reproductive biology (cleistogamous versus chasmogamous, heterostyly versus homostyly; see Simpson & Hasenstab, 2009). In addition, many members of the group exhibit a classic American amphitropical distribution, occurring on either side of the American tropics (Raven, 1963; Wen & Ickert-Bond, 2009) (see Fig. 1). Thus, the Amsinckiinae constitutes a particularly interesting group of plants with respect to several facets of biology and biogeographic history.

Goals. — The overriding goal of this study is to infer, with greater confidence, the phylogeny of the Amsinckiinae. Using the high-throughput sequencing method of genome skimming, we compare different gene trees from analyses of most or all of the chloroplast (cpDNA) genome, most or all of the nuclear ribosomal cistron (nrDNA) genome (including ITS and ETS), plus a significant portion of the mitochondrial (mtDNA) genome. From these analyses, we assess the monophyly of named genera, infer the interrelationships of the major clades, and evaluate the need for future taxonomic changes in the group. We hope this study will lay the groundwork for additional work on the character evolution, divergence timing, and phylogeographic history of this interesting complex of plants, which we plan to address in future studies.

■ MATERIALS AND METHODS

Taxon sampling. — DNA was extracted from 143 silica-dried leaf samples collected concurrently with vouchered specimens or taken directly from previously collected herbarium specimens, generally within the last 20 years. Voucher specimens are housed at the following herbaria: ARIZ, CONC, DUKE, GH, JEPS, MERL, MO, NSW, RSA, SBBG, SD, SDSU, SGO, SI, UC, UCR, and UTC. Appendix 1 lists

voucher information and National Center for Biotechnology Information (NCBI) accessions. Sampling included four outgroup taxa, *Cynoglossum creticum* Mill., *Hackelia micrantha* (Eastw.) J.L.Gentry, *Microula tibetica* Benth., and *Myosotis laxa* Lehm. Amsinckiinae ingroup taxa ($n = 139$) encompassed 12 genera (all but the monotypic *Oncaglossum*), 123 species (42% of the subtribe), and 127 minimum-ranked taxa (38% of the subtribe) (Appendix 1). A map showing the distribution of all Amsinckiinae sampled in this study and general ranges of the subtribe is seen in Fig. 1.

DNA isolation and sequencing. — Genomic DNA extraction and sequencing follow Ripma & al. (2014). Briefly, genomic DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987; Friar, 2005). Whole genomic DNA was then sent to Global Biologics (Columbia, Missouri, U.S.A.) for library preparation. After

library preparation, three separate runs of high-throughput sequencing were performed. Run one utilized the Illumina HiSeq 2000 genetic analysis system (Illumina, San Diego, California, U.S.A.) at the University of Delaware Sequencing and Genotyping Center for 38 samples in one lane, with an average of 3,474,074 (1,332,453–7,593,640) reads. A second run of 53 samples was performed at the University of California at Riverside Genomics Core in a single lane, with an average of 2,926,555 (707,000–5,092,337) reads. A third run was performed on an Illumina HiSeq2500 at Global Biologics for 96 samples in a single lane, with an average of 1,998,447 (820,347–4,835,141) reads. All three sequencing runs produced single-end 100 bp reads with an average insert size of 250 bp. The last two runs were shared with other researchers to reduce costs. All reads are deposited at the Short Read Archive of the NCBI (<https://www.ncbi.nlm.nih.gov>; see Appendix 1).

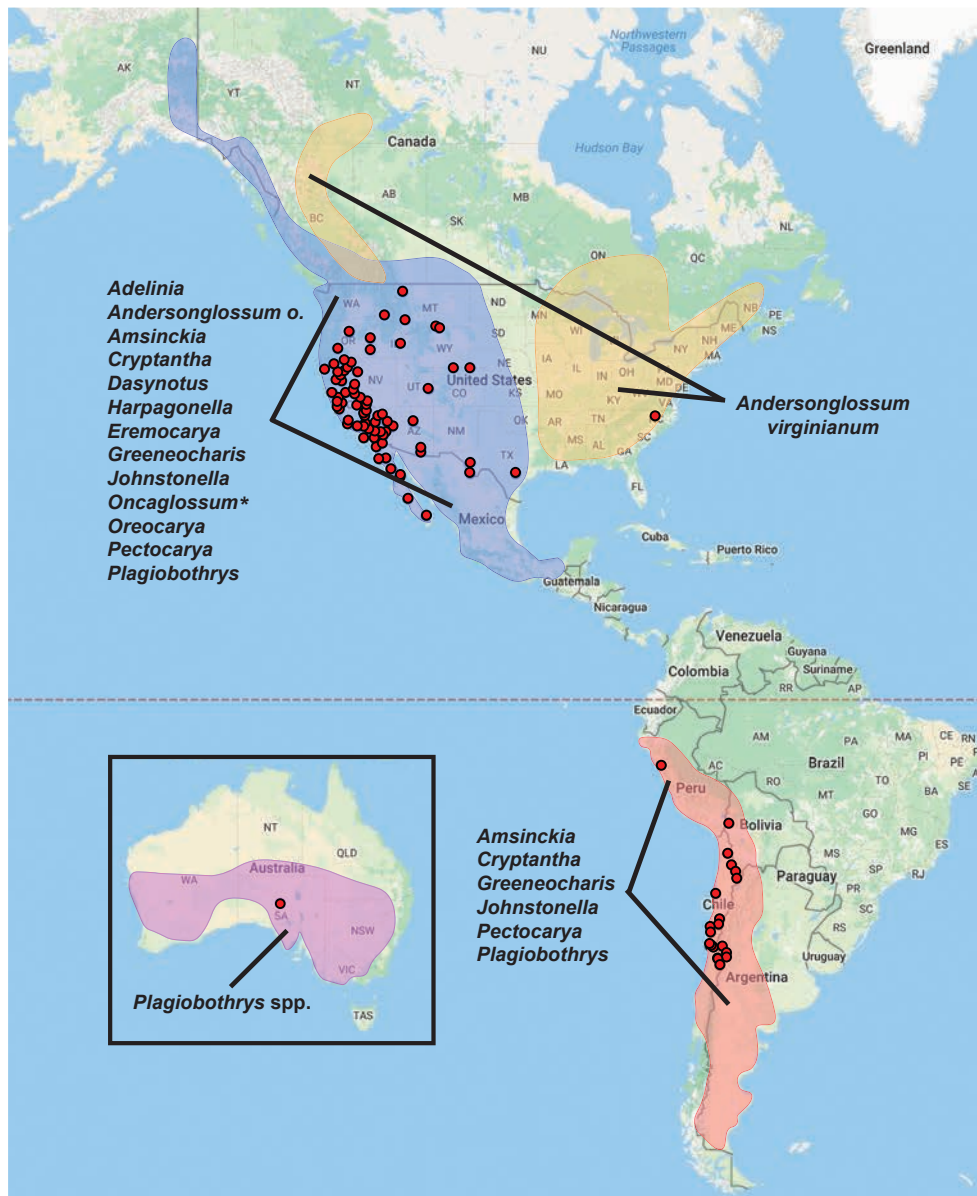


Fig. 1. Distribution map of the 139 sampled specimens (dots) and native ranges of genera (highlighted areas) in subtribe Amsinckiinae, members of which show an American amphitropical disjunct distribution. Only *Andersonglossum virginianum* (inclusive of *A. boreale* (Fernald) J.I.Cohen) is natively distributed in eastern U.S.A. (also occurring in western Canada), and only species of *Plagiobothrys* natively occur in Australia. **Oncaglossum* not included in our study. Distribution data, in part, from Global Biodiversity Information Facility (<http://www.gbif.org>).

DNA quality, assembly, and alignment. — Raw read quality control and filtering follows the methods in Ripma & al. (2014) using PRINSEQ (Schmieder & Edwards, 2011), with removal of reads being exact duplicates, having a mean quality Phred score below 30, or having more than one N. Both the 3' and 5' ends were trimmed to a Phred quality score of 30 using a window size of 1 (Straub & al., 2013). Any read less than 50 bp in length was removed, as well as any remaining sample barcodes. Post-quality control reads were imported into Geneious v.7.1.5 (Biomatters, Auckland, New Zealand, <http://www.geneious.com>) in FASTQ format, hereafter referred to as read pools.

For assembly of the plastome (cpDNA), we used a reference-guided assembly to an annotated 124,868 bp partial plastome sequence of *Pectocarya penicillata* (Hook. & Arn.) A.DC. (from Ripma & al., 2014), which was constructed using Geneious v.7.017 with default settings. Reference-guided assembly to the *P. penicillata* plastome was implemented in Geneious, with default settings and 25 iterations of the read pool from each sample. Consensus contigs for each sample were generated using a 75% similarity threshold, masking areas with less than 20 bp sequence depth with gaps, and retaining IUPAC ambiguity codes. Sequences were aligned using the MAFFT plugin v.1.3.3 in Geneious v.7.017 (Katoh & al., 2002) with default settings. Lastly, final alignments were examined for misaligned areas by eye, and adjusted accordingly. Total number of informative characters were calculated using GARLI v.2.0 (Zwickl, 2006), implemented in Geneious v.7.017 using default settings.

Ribosomal cistron (nrDNA) assembly followed methods detailed in Ripma & al. (2014), with some exceptions. The assembled cistron of *Cryptantha torreyana* (A.Gray) Greene var. *torreyana* from Ripma & al. (2014) was used for reference-guided assembly of the read pools in Geneious using 25 iterations, medium-low sensitivity, and default settings. The methods for generating nrDNA consensus sequences, sequencing editing, and alignment followed those employed for the plastome (above). Positions that contained an ambiguity code were removed from the alignment, as we were not able to determine whether these ambiguities were due to polymorphic states within the ITS/ETS regions or due to sequencing error.

Mitochondrial (mtDNA) exons were obtained using a reference-guided assembly of each read pool to a GenBank *Nicotiana tabacum* L. mitochondrion (GenBank: BA000042), modified to include only one copy of each annotated repeat region. A consensus contig was saved using the same methods presented for the cpDNA plastome (above). A single contig from each sample was made into a custom database in Geneious, henceforth referred to as the mtDNA contig bin. Each *N. tabacum* exon was separated using the extract annotations feature in Geneious. To exclude chloroplast sequences from the mitochondrial assemblies, these exons were BLASTN searched against the cited plastome of *S. lycopersicum*, using an *E*-value of 1e-10, a *k*-mer length of 15, a scoring match-mismatch of 2-3, and a 5-2 open extend gap cost (this BLASTN search being more likely to find matches than the MegaBLAST search used elsewhere). Only *N. tabacum* exons with no match to chloroplast sequences were retained. Each retained exon was

MegaBLAST searched against the mtDNA contig bin using a query centric alignment output and the settings for LCNG gene searches. The result was an alignment of each *N. tabacum* exon and the corresponding sequence(s) from each sampled taxon. Only alignments with a single copy from each sample were retained, and several of these exons were partial. Exons were aligned using the MAFFT plugin (Katoh & al., 2002) with default settings. Sequences were edited with the same methods as those used for the cpDNA plastome (above).

Phylogenetic analyses. — Only sequence data represented in all samples for each genome were used in subsequent analyses. All analyses were run with the outgroups *Cynoglossum creticum*, *Hackelia micrantha*, *Microula tibetica*, and *Myosotis laxa* and rooted afterwards with *Hackelia micrantha*, following the results of Weigend & al. (2013) and Chacón & al. (2016). All partitioning schemes and models of molecular evolution were determined using PartitionFinder2 v.2.1.1 (Lanfear & al., 2016). Each genome was analyzed separately, with the cpDNA partitioned by codon position for the entire plastome, the nrDNA partitioned by gene and non-coding spacer regions (ETS, 18S, ITS1, 5.8S, ITS2, 26S), and the mtDNA partitioned by exons. Phylogenetic trees were inferred using maximum likelihood (ML) and Bayesian (BI) frameworks implemented on the CIPRES portal (Miller & al., 2010). For all three alignments, the GTR+I+G model of evolution was used for both ML and BI analyses. Searches for the tree topology with the highest likelihood score were conducted in RAxML v.8 (Stamatakis, 2014), with support assessed using 1000 rapid bootstrap (BS) replicates. Bayesian analyses were implemented in MrBayes v.3.2 (Ronquist & al., 2012), with the MCMC chain run for 10 million generations.

Resulting trees were viewed in FigTree (Rambaut, 2006–2014), with final graphics prepared with Adobe InDesign CC. Bootstrap support was considered strong if $\geq 70\%$ and posterior probabilities (PP) strong if ≥ 0.95 ; otherwise, either were considered to be weak in support. A combination of strong and weak support between the ML and BI analyses was indicated as “mixed”.

■ RESULTS

The total alignment lengths, number of parsimony-informative characters, and number of uninformative characters for the three datasets are seen in Table 2 and are available at TreeBase for download (<http://purl.org/phylo/treebase/phylovs/study/TB2:S21212>). The ML and BI analyses result

Table 2. Alignment lengths, number of parsimony-informative characters (PICs), and number of uninformative characters (UCs) for the three types of DNA used in this study.

DNA	Alignment length	PICs	UCs
cpDNA	107,155 bp	9,837	6,765
mtDNA	20,511 bp	4,567	2,154
nrDNA	5,532 bp	419	190

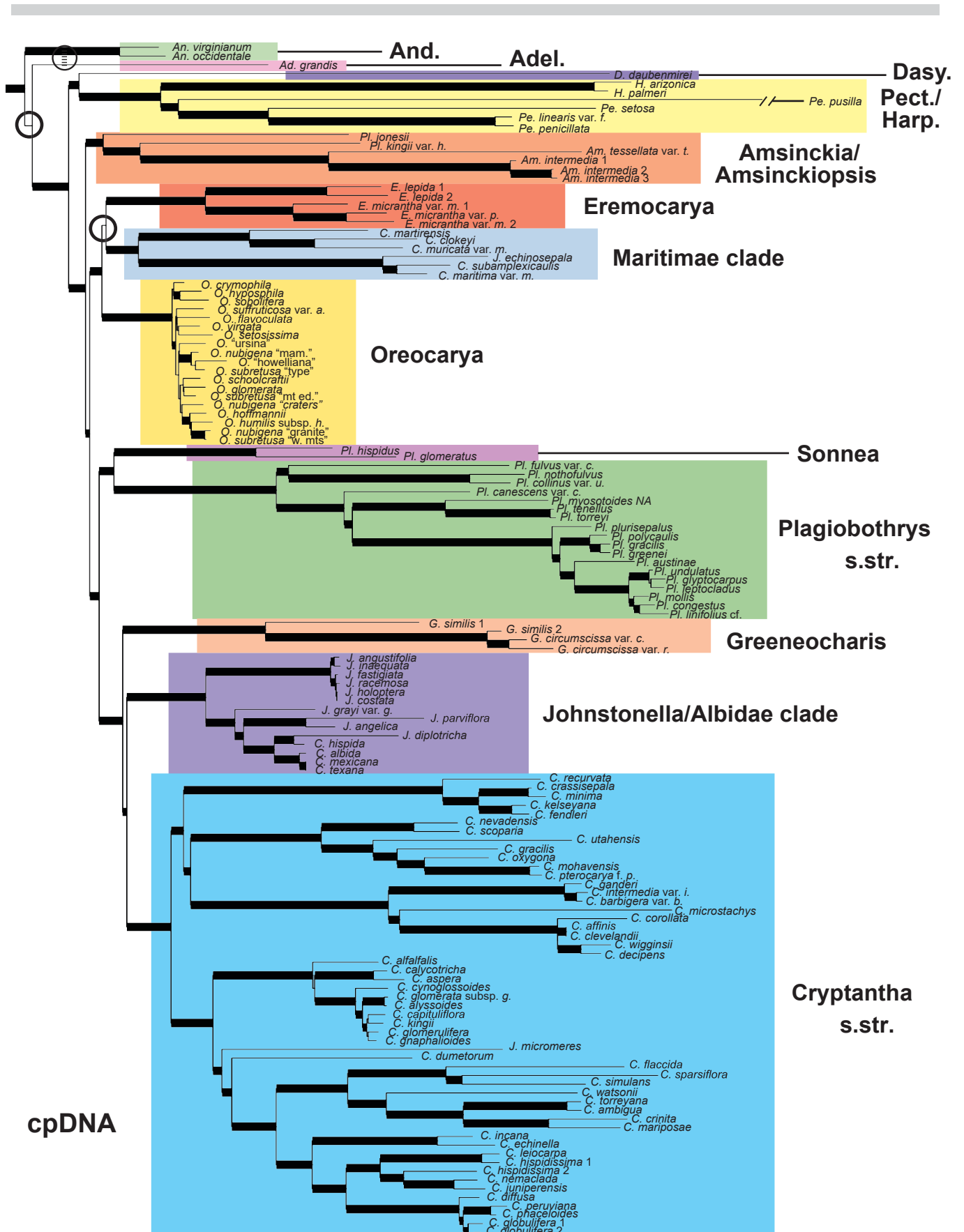
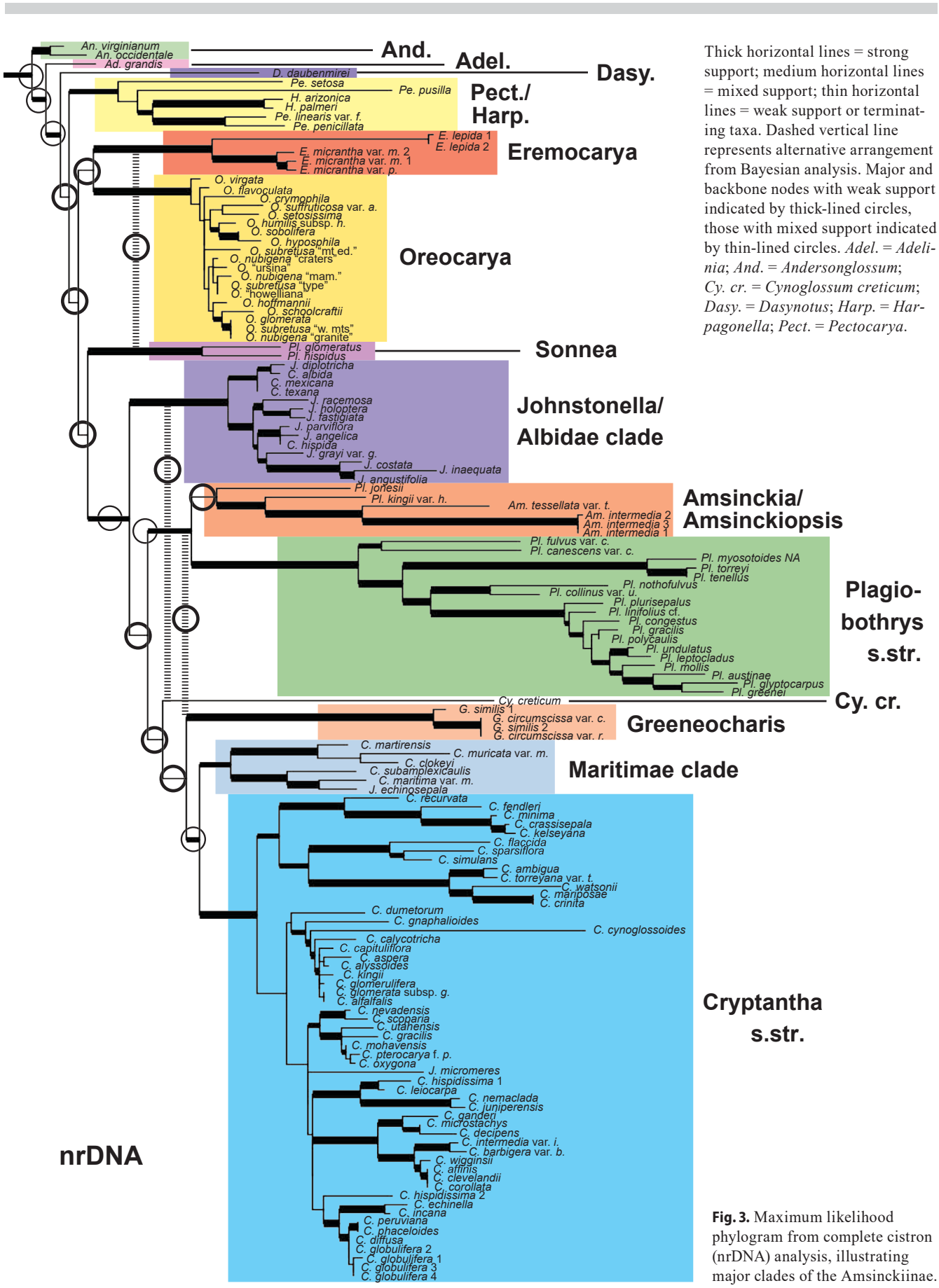


Fig. 2. Maximum likelihood phylogram from complete plastome (cpDNA) analysis, illustrating major clades of the Amsinckiinae. Graphics and abbreviations as in Fig. 3.



Thick horizontal lines = strong support; medium horizontal lines = mixed support; thin horizontal lines = weak support or terminating taxa. Dashed vertical line represents alternative arrangement from Bayesian analysis. Major and backbone nodes with weak support indicated by thick-lined circles, those with mixed support indicated by thin-lined circles. *Adel.* = *Adelina*; *And.* = *Andersonglossum*; *Cy. cr.* = *Cynoglossum creticum*; *Dasy.* = *Dasynotus*; *Harp.* = *Harpagonella*; *Pect.* = *Pectocarya*.

Fig. 3. Maximum likelihood phylogram from complete cistron (nrDNA) analysis, illustrating major clades of the Amsinckiinae.

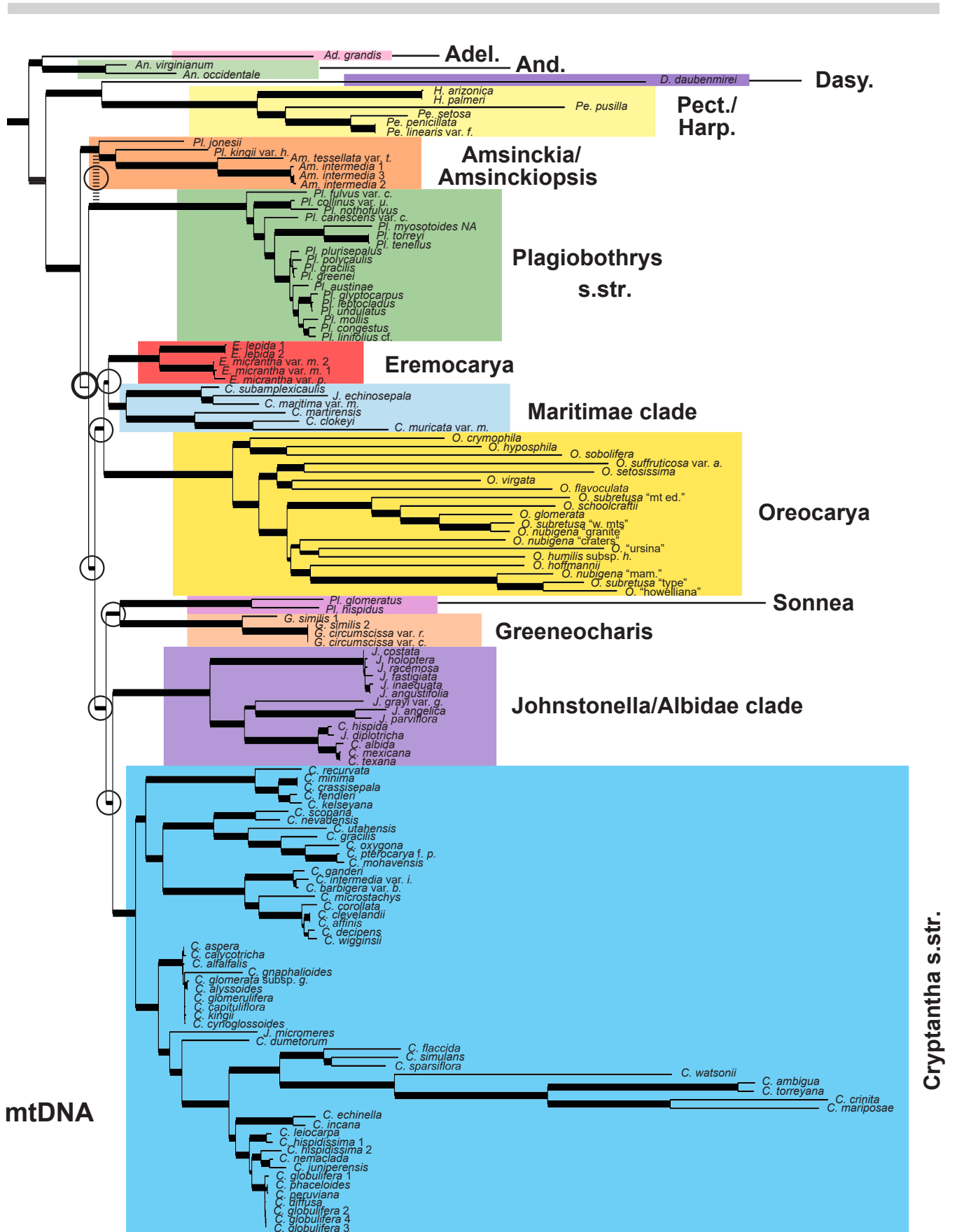


Fig. 4. Maximum likelihood phylogram from mitochondrial (mtDNA) analysis, illustrating major clades of the Amsinckiinae. Graphics and abbreviations as in Fig. 3.

in the same or almost the same topology for a given genome dataset (ML trees illustrated in Figs. 2–4). For all analyses, the *Andersonglossum* clade alone (cpDNA-ML, nrDNA) or an *Andersonglossum-Adelinia* clade (cpDNA-BI, mtDNA) is sister to all other examined members of the Amsinckiinae (Figs. 2–4). *Adelinia* is sister to all other subtribe members except *Andersonglossum* in the cpDNA-ML and nrDNA analyses (Figs. 2, 3).

In all analyses the examined species of *Harpagonella* and *Pectocarya* comprise a monophyletic group with strong support, termed here the *Pectocarya/Harpagonella* clade. In all cpDNA and mtDNA analyses, *Harpagonella* is sister to a monophyletic *Pectocarya* with strong support (Figs. 2, 4), but in the nrDNA analyses *Harpagonella* is sister to two of the four sampled species of *Pectocarya* with mixed support (Fig. 3). The monospecific *Dasynotus* is sister to the *Pectocarya/Harpagonella* clade with strong support in the cpDNA and mtDNA analyses, and in these same analyses this *Dasynotus-Pectocarya/Harpagonella* clade is sister to the rest of the Amsinckiinae (minus *Adelinia* and *Andersonglossum*) with strong support (Figs. 2, 4). However, in the nrDNA analyses *Dasynotus* is sister to all other Amsinckiinae (minus *Adelinia* and *Andersonglossum*) with mixed support (Fig. 3).

A clade containing all *Amsinckia* spp. plus *Plagiobothrys jonesii*, and *P. kingii* (the last two of *Plagiobothrys* sect. *Amsinckiopsis*), termed here the *Amsinckia/Amsinckiopsis* clade, is recovered in all analyses with strong (cpDNA, mtDNA) to weak (nrDNA) support. Within this clade *Plagiobothrys jonesii* is sister to *P. kingii*+*Amsinckia*, and *P. kingii* is sister to *Amsinckia*, with strong support in all analyses. This *Amsinckia/Amsinckiopsis* clade is sister to the remainder of the Amsinckiinae (minus *Adelinia*, *Andersonglossum*, *Dasynotus*, and the *Pectocarya/Harpagonella* clade) with strong support in the cpDNA and the mtDNA-ML analyses, whereas, in the mtDNA-BI analysis the *Amsinckia/Amsinckiopsis* clade is sister to *Plagiobothrys* s.str. with strong support (Figs. 2, 4). In the nrDNA analyses the *Amsinckia/Amsinckiopsis* clade is part of a clade with *Plagiobothrys* s.str. with mixed support (Fig. 3).

Johnstonella, as circumscribed by Hasenstab-Lehman & Simpson (2012), is not monophyletic in our study. However, in all analyses, 10 of the 12 included species of *Johnstonella* plus 4 species of *Cryptantha*—*C. albida* (Kunth) I.M.Johnst., *C. hispida* (Phil.) Reiche, *C. mexicana* I.M.Johnst., and *C. texana* Greene—together form a strongly supported monophyletic group, termed here the *Johnstonella/Albidae* clade (Figs. 2–4).

The remaining genera/clades—*Cryptantha* s.str., *Eremocarya*, *Greeneocharis*, *Maritimae* clade, *Oreocarya*, *Plagiobothrys* s.str. (sect. *Allocarya*, sect. *Echidiocarya*, and sect. *Plagiobothrys* only), and *Sonnea* (= *Plagiobothrys* sect. *Sonnea*)—are recovered in all analyses with strong support, except for what we term the *Maritimae* clade (see Discussion), recovered with mixed support in the nrDNA analyses (Figs. 2–4). The interrelationships of these seven genera/clades vary among analyses. However, some common patterns are evident. Three clades—*Eremocarya*, *Oreocarya*, and the *Maritimae* clade—together form a monophyletic group in the cpDNA and mtDNA analyses, with strong to mixed support (Figs. 2, 4).

Within this group, *Eremocarya* and the *Maritimae* clade are sister taxa with mixed to weak support, with *Oreocarya* sister to these two with strong to mixed support (Figs. 2, 4). In the nrDNA analyses *Eremocarya* and *Oreocarya* are sister taxa with mixed support, and the *Maritimae* clade is distantly related to these (Fig. 3).

Cryptantha s.str., the largest clade in our study, is sister to the *Johnstonella/Albidae* clade with strong to mixed support in the cpDNA and mtDNA analyses (Figs. 2, 4), but is sister to the *Maritimae* clade with mixed support in the nrDNA analyses (Fig. 3). *Plagiobothrys* s.str. is sister either to the *Amsinckia/Amsinckiopsis* clade with weak to strong support (nrDNA and mtDNA-BI analyses), to *Sonnea* with strong support (cpDNA), or to a group of seven major clades with weak support (mtDNA-ML analysis) (Figs. 2–4). The relationships of *Greeneocharis* and *Sonnea* differ among analyses. Finally, *Cynoglossum creticum*, which is sister to *Microula tibetica* among the outgroups in the cpDNA and mtDNA analyses (Figs. 2, 4), is nested within the Amsinckiinae in the nrDNA analyses, but with weak to mixed support (Fig. 3).

In each of the individual genome phylograms (cpDNA, nrDNA, and mtDNA; only ML illustrated) relative branch lengths along the backbone of the Amsinckiinae are generally short (Figs. 2–4). Relative branch lengths of *Oreocarya* taxa in the cpDNA (Fig. 2) and nrDNA (Fig. 3) analyses are quite short, whereas those in the mtDNA analysis (Fig. 4) are long, longer than for most other taxa. Also in the mtDNA-ML analysis, five species of *Cryptantha* s.str.—*C. ambigua* (A.Gray) Greene, *C. crinita* Greene, *C. mariposae* I.M.Johnst., *C. torreyana* (A.Gray) Greene, and *C. watsonii* (A.Gray) Greene—show particularly long branches relative to the rest of the genus (Fig. 4). These taxa do not show long branches in either the cpDNA or nrDNA analyses (Figs. 2, 3). A comparison of the three ML cladograms from the cpDNA, nrDNA, and mtDNA analyses illustrates the differences in the placement of major clades (Electr. Suppl.: Figs. S1–S3).

DISCUSSION

Phylogenetic relationships and taxonomic considerations.

— The analyses presented here of the “popcorn flowers”, subtribe Amsinckiinae (Boraginaceae), have a significantly increased sample size over past studies, and utilized high-throughput genome skimming sequencing, yielding much more data. For these analyses, we were able to obtain virtually all of the cpDNA (plastome), virtually all of the nrDNA (cistron), and many mtDNA markers. Nonetheless these analyses yielded trees of somewhat different topologies (Figs. 2–4). Bootstrap and posterior probability support values for genera or major clades are generally strong, but those for nodes along the “backbone” of the Amsinckiinae are mostly weak to mixed, except for the cpDNA (Fig. 2) analysis, in which backbone nodes are mostly strongly supported. (See summary cladograms for the analyses in Electr. Suppl.: Figs. S1–S3.) Thus, we feel that our studies are insufficient to confidently infer evolutionary relationships among many of the major clades of the Amsinckiinae.

The reasons for the conflicts among datasets could be for a variety of reasons. The only biparentally inherited marker, the nuclear cistron, is present within the organisms in thousands of copies, but may not have homogenized in all cases (Alvarez & Wendel, 2003). Sequences from the plastome and mitochondrion, though showing strongly supported relationships, are almost always uniparentally (usually maternally) inherited. Such organelle evolution may not necessarily correspond with species evolution. Moreover, the relatively short branch lengths along the backbone of the Amsinckiinae (Figs. 2–4) may be indicative of relatively rapid divergence of the major clades of the subtribe. This may explain both the varying results we obtained with different datasets and analyses (Figs. 2–4), and those across different studies (Hasenstab-Lehman & Simpson, 2012; Cohen, 2014; Otero & al., 2014; Weigend & al., 2014, Chacón & al., 2016). These relatively short branches along the major nodes of the backbone may confound future attempts to obtain a robust phylogeny of the subtribe (see Future Work).

Despite these conflicts among datasets, we have made significant progress in our understanding of the group. The Amsinckiinae as a whole is strongly supported as monophyletic in all but one (nrDNA) of the analyses, in which *Cynoglossum creticum* is nested within the subtribe, and its placement generally with low support values. Chacón & al. (2016) placed *C. creticum* within the more distantly related subtribe Cynoglosseae, one of the four subtribes of tribe Cynoglosseae, to which the Amsinckiinae belongs. Thus, we suspect that the placement of *C. creticum* in our nrDNA analysis is in error. Although we recognize that our outgroup sampling ($n = 4$) was limited, our study largely continues to support the recognition of subtribe Amsinckiinae, having the circumscription cited in Chacón & al. (2016), summarized in Table 1.

Our analyses show generally strong support for the monophyly of most of the (non-monospecific) genera: *Amsinckia*, *Andersonglossum*, *Eremocarya*, *Greeneocharis*, *Harpagonella*, *Oreocarya*, and *Pectocarya*, although *Pectocarya* is paraphyletic in one analysis (nrDNA), this with weak support (below).

The phylogenetic positions of *Andersonglossum* and *Adelinia* generally correspond to or are compatible with the results of Weigend & al. (2013), Cohen (2015), and Chacón & al. (2016). One discrepancy is the recovery of a paraphyletic *Andersonglossum* by Chacón & al. (2016), who found *Andersonglossum virginianum* to be sister to *Adelinia grandis*. However, all of our analyses strongly support the two species of *Andersonglossum* as monophyletic (Figs. 2–4), agreeing with Weigend & al. (2013) and Cohen (2015). We were, however, limited to a single sample per species for both *Andersonglossum* and *Adelinia*. An increased sampling of these two genera, and of outgroup taxa, may be needed to verify their monophyly and interrelationships.

Harpagonella is sister to *Pectocarya* in all except our nrDNA analysis, in which the former is nested within the latter, but with weak support. This sister relationship of the two genera corresponds with that of Weigend & al. (2013), Otero & al., 2014, and Chacón & al. (2016). Our study confirms others in recognizing the close relationship between these two genera,

although studies with a greater sample size are needed to evaluate more detailed relationships in the complex.

In two of our analyses (Figs. 2, 4; see Electr. Suppl.: Figs. S1–S3), *Dasynotus* is sister to the *Pectocarya/Harpagonella* clade with strong support. This novel relationship was not obtained by Weigend & al. (2013), Cohen (2014, 2015), or Chacón & al. (2016), which have *Dasynotus* placed in polytomies or weakly supported relationships, but none of which are incompatible with our results based on support values. Aside from its relationships to *Dasynotus*, the general placement of the *Pectocarya/Harpagonella* clade in the subtribe generally agrees with or is compatible with that of several earlier studies (Hasenstab-Lehman & Simpson, 2012; Weigend & al., 2013; Chacón & al., 2016).

Aside from *Adelinia*, *Andersonglossum*, *Dasynotus*, and *Pectocarya/Harpagonella*, all but our nrDNA analyses recover a monophyletic grouping within the Amsinckiinae comprised of nine clades: *Amsinckia/Amsinckiopsis*, *Cryptantha* s.str., *Eremocarya*, *Greeneocharis*, *Johnstonella/Albidiae* clade, *Maritimae* clade, *Oreocarya*, *Plagiobothrys* s.str., and *Sonnea* (Figs. 2, 4). Relationships within this clade of nine groups vary among the different analyses, but some patterns are consistent.

Plagiobothrys s.l., as traditionally treated, is not monophyletic. However, three segregate clades are monophyletic with strong support: *Sonnea* (*Plagiobothrys* sect. *Sonnea*), the *Amsinckia/Amsinckiopsis* clade (including two species of *Plagiobothrys* sect. *Amsinckiopsis*), and our *Plagiobothrys* s.str. clade (including sect. *Allocarya*, sect. *Echidio-carya*, and sect. *Plagiobothrys* only). *Sonnea* is sister to either *Plagiobothrys* s.str. (with strong to mixed support), to *Greeneocharis* (with mixed support), or to a large component of the Amsinckiinae (with weak support; Figs. 2–4). In their study, Hasenstab-Lehman & Simpson (2012) sampled only *Plagiobothrys hispidus* of *Sonnea*, which consistently occurred separate from other *Plagiobothrys* species and was sister to their *Eremocarya*+*Oreocarya* clade. Other studies cited did not include samples of *Plagiobothrys* sect. *Sonnea*. Given its strongly supported monophyly, but ambiguous relationship to other clades in the subtribe, we propose that the species of *Plagiobothrys* sect. *Sonnea* (*P. glomeratus* A.Gray and *P. hispidus* (Greene) I.M.Johnst.) should be treated in genus *Sonnea*, as *S. glomerata* (A. Gray) Greene and *S. hispidus* (A.Gray) Greene.

Within the *Amsinckia/Amsinckiopsis* clade, *Plagiobothrys jonesii* and *P. kingii* (both of sect. *Amsinckiopsis*) consistently form a grade with the monophyletic genus *Amsinckia* in all of our analyses, generally with strong support. The morphological resemblance of these two *Plagiobothrys* species to *Amsinckia* has been recognized for some time (Johnston, 1923), reflected in the sectional name, *Amsinckiopsis*. This close relationship between *Plagiobothrys* sect. *Amsinckiopsis* and the genus *Amsinckia* was first confirmed phylogenetically in one of the analyses of Hasenstab-Lehman & Simpson (2012), although in that study the two *Amsinckiopsis* species formed a clade (rather than a grade) sister to *Amsinckia*. A similar pattern to our study was recovered by Williams (2015), who is revising the classification of *Plagiobothrys*, including

sect. *Amsinckiopsis* (work in prep.), which will likely entail generic transfers of these two species.

The placement of our *Plagiobothrys* s.str. clade also varied by analysis, being sister either to *Sonnea* (with strong support), to the *Amsinckia/Amsinckiopsis* clade (with weak to mixed support), or to a large component of the Amsinckiinae (with weak support). Given the strongly supported monophyly of *Plagiobothrys* s.str., but its variable placement in the Amsinckiinae, we propose that the genus *Plagiobothrys* be recognized only as inclusive of sect. *Allocarya*, sect. *Echidiocarya*, and sect. *Plagiobothrys*.

What we term the *Maritimae* clade, based on *Cryptantha* ser. *Maritimae* of Johnston (1925) (but inclusive of only *Cryptantha maritima* of that classification), is composed of *Cryptantha clokeyi* I.M.Johnst., *C. maritima* (Greene) Greene, *C. martirensis* M.G.Simpson & Rebman, *C. muricata* (Hook. & Arn.) A.Nelson & J.F.Macbr., *C. subamplexicaulis* (Phil.) Reiche, and *Johnstonella echinosepala* (J.F.Macbr.) Hasenstab & M.G.Simpson. This *Maritimae* clade is roughly equivalent to the “*Cryptantha* s.str. 2” group of Hasenstab-Lehman & Simpson (2012), which included two (North and South American) samples of *C. maritima*, plus the South American species *C. chaetocalyx* (Phil.) I.M.Johnst. and *C. granulosa* (Ruiz & Pav.) I.M.Johnst. Unfortunately, we were unable to obtain quality sequences of the last two species. However, Otero & al. (2014) sequenced both *C. maritima* and *C. granulosa* and found them to be sister taxa, agreeing with Hasenstab-Lehman & Simpson (2012) that at least the latter taxon should be placed in the *Maritimae* clade. However, the inclusion in our *Maritimae* clade of the South American *C. subamplexicaulis*, the North American *Johnstonella echinosepala*, and three species corresponding to Johnston’s 1925 North American ser. *Muricatae* (*C. muricata*, *C. clokeyi*, and *C. martirensis*) is novel. No obvious morphological features unite all of the taxa of the *Maritimae* clade, and the placement of *Johnstonella echinosepala* outside the *Johnstonella/Albidae* clade was unexpected. (The identification of this specimen was confirmed by us in review; see Appendix 1 for voucher information.) Despite this, our *Maritimae* clade is strongly supported in all of our analyses and is well-separated from *Cryptantha* s.str. in all analyses (with strong support) except in the nrDNA analysis, in which it is sister to *Cryptantha* s.str. (but with mixed support). More samples are needed to establish the validity and full membership of our *Maritimae* clade, but it seems to represent an intriguing offshoot within the Amsinckiinae. Given the strong molecular evidence for its phylogenetic distinctiveness, the group may require elevation to the rank of genus in the future.

A clade consisting of *Eremocarya*, *Oreocarya*, and the *Maritimae* clade, with *Oreocarya* sister to the other two, was recovered with strong support in most of our analyses. This grouping was not detected in previously published studies. However, an *Eremocarya+Oreocarya* clade was detected by Hasenstab-Lehman & Simpson (2012), who recovered this relationship in both of their analyses (one with strong support), and was also recovered in the ITS tree of Otero & al. (2015). No morphological apomorphy is evident for either of these groupings, but this complex appears to represent a natural group.

In the genus *Oreocarya*, the relatively short branch lengths for taxa in the cpDNA (Fig. 2) and nrDNA (Fig. 3) analyses might be expected for this clade of perennials, which have a longer generation time correlated with relatively slow sequence divergence (Andreasen & Baldwin, 2001; Cenci & al., 2013). However, in our mtDNA analysis (Fig. 4), the branch lengths of *Oreocarya* are actually longer than most other taxa, an unexpected result. Why the mitochondrial data would show this reverse trend is a mystery, one we hope to elucidate in the future.

The *Johnstonella/Albidae* clade largely corresponds to the *Johnstonella* clade of Hasenstab-Lehman & Simpson (2012) but with the addition of the North American *Cryptantha albida* and *C. mexicana* (both of ser. *Albidae* of Johnston, 1925, 1961), the North American *C. texana* (ser. *Texanae* of Johnston, 1925), and the South American *C. hispida* (ser. *Phaceloides* of Johnston, 1927). These four taxa were not included in the study by Hasenstab-Lehman & Simpson (2012). The placement within *Johnstonella* of the two species of *Cryptantha* ser. *Albidae*, *C. albida* and *C. mexicana*, is not surprising based on nutlet morphology. Members of the *Albidae* group have white nutlet tubercles typical of *Johnstonella* and resemble the shape of certain *Johnstonella* species (e.g., *J. angustifolia*; see Hasenstab-Lehman & Simpson, 2012). The discovery of the South American *Cryptantha hispida* (of Johnston’s 1927 ser. *Phaceloides*) nested within the *Johnstonella/Albidae* clade is also supported by morphology, as the ovate, acutely margined nutlets of this taxon are similar to several other *Johnstonella* species, differing only in having a smooth sculpturing (see Simpson & al., 2014). However, the fourth *Cryptantha* species nesting within *Johnstonella*, *C. texana* of Johnston’s (1925) ser. *Texanae*, seems aberrant within *Johnstonella*. The nutlet and calyx morphology of *C. texana* do not resemble members of other *Johnstonella* species. Other members of Johnston’s (1925) ser. *Texanae* include *C. crassiseppala* (Torr. & A.Gray) Greene and *C. kelseyana* Greene, both of which nested firmly within *Cryptantha* s.str. in our analyses. We believe that this discrepancy between phylogeny and morphology warrants additional sampling of *C. texana* in future analyses before nomenclatural changes are considered.

The *Johnstonella/Albidae* clade is sister to *Cryptantha* s.str. in two (cpDNA, mtDNA) of our gene analyses, with mixed to strong support. However, the stem lineage of this sister-taxon grouping is very short (see Figs. 2, 4). The *Johnstonella/Albidae* clade is sister to clades other than to *Cryptantha* s.str. in the nrDNA analyses, with mixed to weak support. If the *Johnstonella/Albidae* clade were unequivocally sister to *Cryptantha* s.str., one choice of classification would be to combine the two groups, transferring all *Johnstonella* species back to the genus *Cryptantha*. However, given that the relationship of the *Johnstonella/Albidae* clade relative to *Cryptantha* s.str. is still ambiguous, and given the short branch length linking them in analyses in which they are sister taxa, a second choice is to retain *Johnstonella* and to transfer those *Cryptantha* species nested within the *Johnstonella/Albidae* clade to the genus *Johnstonella*. We are convinced that the *Johnstonella/Albidae* clade as presented here is a monophyletic group (with the possible exception of *C. texana*) and should be recognized as *Johnstonella*, either at the generic or subgeneric rank.

As mentioned earlier, in all analyses, two previously classified *Johnstonella* species occurred outside the *Johnstonella/Albidae* clade, namely *J. echinosepala*, placing in the *Maritimae* clade, and *J. micromeres*, occurring in the *Cryptantha* s.str. clade. We hope to obtain additional samples of these two species in order to test these phylogenetic relationships before considering formal changes in classification.

The *Cryptantha* s.str. clade corresponds largely to “*Cryptantha* s.str. 1” of Hasenstab-Lehman & Simpson (2012). Other analyses, e.g., Weigend & al. (2013), Cohen (2014, 2015), Chacón & al. (2016), recovered a non-monophyletic *Cryptantha*, with quite variable and weakly supported relationships. However, our analyses firmly place the bulk of this genus (50 of the 59 species sequenced in our study) within the strongly supported *Cryptantha* s.str. clade. As noted earlier, however, four *Cryptantha* species place within the *Johnstonella/Albidae* clade and five within the *Maritimae* clade. Even if these nine species were ultimately transferred to other genera, *Cryptantha* still would be the largest genus of the Amsinckiinae. In the mtDNA analysis, the five species of *Cryptantha* (*C. ambigua*, *C. crinita*, *C. mariposae*, *C. torreyana*, *C. watsonii*) that have particularly long branches relative to the rest of the genus (Fig. 4) show no remarkable rate increases in either the cpDNA or nrDNA analyses. We are puzzled as to an explanation for this pattern in the mitochondrial analyses. Mitochondrial DNA in plants is known to have a high degree of plasticity in terms of genomic rearrangements, insertion or transfer of DNA to the chloroplast or nucleus, disruption of intron/exon gene continuity, and evolutionary changes in gene expression (Knoop, 2004) and is generally less useful in phylogenetic studies. We will be interested to see if this pattern is maintained in future analyses with greater taxon sampling.

Future work. — Our first recourse in future work will be to acquire sequence data for additional samples in the Amsinckiinae. These should ideally include *Oncaglossum* and more samples of *Adelinia*, *Andersonglossum*, and *Dasynotus*, to better evaluate their monophyly and placement in the subtribe. We hope to acquire sequence data for additional species not yet sampled, particularly the larger genera *Cryptantha* (59 of ca. 103 spp. = ca. 57% sampled in this study) and *Plagiobothrys* (22 of ca. 69 spp. (including members of *Sonnea* sect. *Amsinckiopsis*) = ca. 32% sampled in this study). We wish to acquire several additional samples of taxa that had an unexpected placement in our analyses, particularly *Cryptantha texana*, *Johnstonella echinosepala*, and *J. micromeres*. The latter in particular merits a population-level study or more nuclear data to understand its evolutionary history, as different samples of this species have placed in different areas of the subtribe with traditional Sanger sequence data (Hasenstab, unpub. data).

Following the acquisition of sequences of some additional taxa, future studies in subtribe Amsinckiinae will include broad surveys of character evolution, divergence timing, and phylogeographic history. Of particular interest in character assessment are the evolutionary transitions in cleistogamy, heterostyly, plant duration, ploidy levels, and details of nutlet morphology such as sculpturing, number per fruit, heteromorphism, and dimorphism (after Williams & al., 2013). The Amsinckiinae

contains a number of taxa/clades with American amphitropical disjunct distributions (Williams & al., 2016). The number and timing of putative dispersal events and their correlation with current vegetation types, geologic and climatic history, and possible selective pressures leading to the above morphological features are of great biological interest and a work in progress.

The techniques we used in this study may continue to be useful in studies of some genera or clades of the Amsinckiinae, especially those with relatively longer branch lengths. However, one of the conclusions from our work is that, despite the acquisition of virtually the entire plastome, the entire cistron, and numerous mitochondrial markers, we have still been unable to obtain strong support for the interrelationships of many genera and major clades, especially along the backbone of the tree. This may be a function of the fact that divergences of major clades in the Amsinckiinae occurred rapidly. Thus, these lineages diverged not only relatively long ago, but also over a brief period of time, confounding our efforts to obtain an accurate phylogeny. Different molecular techniques will likely be needed for refining the backbone relationships of the subtribe. Targeted sequences of numerous nuclear genes using methods such as Hyb-Seq (Weitemier & al., 2014) would enable a more rigorous application of species coalescent methods. In addition, there are many research problems that could be addressed on various species complexes within the subtribe. These latter studies may be tractable using methods such as RAD-Seq (Eaton & Ree, 2013; Eaton, 2014) for elucidating relationships within a recently evolved group. Still, we hope that the present investigation has enhanced our understanding of phylogenetic relationships in Amsinckiinae and can serve as a framework for additional studies in the future.

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Appendix 1. Taxa included in the phylogenetic analyses with their corresponding country of origin, collector/collection number, herbarium accessions, and NCBI Short Read Archive accession numbers.

OUTGROUP TAXA: *Cynoglossum creticum* Mill., Chile, Hasenstab s.n. (SDSU 21418), SRR5713409; *Hackelia micrantha* (Eastw.) J.L.Gentry, U.S.A., *Guilliams* 2606 (SBBG 132390), SRR5713385; *Microula tibetica* Benth., China, Boufford 31295 (GH 00466293), SRR5713384; *Myosotis laxa* Lehm., U.S.A., *Guilliams* 1246 (SBBG 132391), SRR5713383. **INGROUP TAXA:** *Adelinia grandis* (Douglas ex Lehm.) J.I.Cohen, U.S.A., *Simpson* 3007 (SDSU 19197), SRR5713435; *Amsinckia intermedia* 1 Fisch. & C.A.Mey., U.S.A., *Guilliams* 1466 (SBBG 132389), SRR5713381; *Amsinckia intermedia* 2 Fisch. & C.A.Mey., U.S.A., *Mabry* 65 (SDSU 20756), SRR5713430; *Amsinckia intermedia* 3 Fisch. & C.A. Mey., U.S.A., *Guilliams* 1447 (SBBG 132388), SRR5713433; *Amsinckia tessellata* A.Gray var. *t.*, U.S.A., *Mabry* 29 (SDSU 20350), SRR5713421; *Andersonglossum occidentale* (A.Gray) J.I.Cohen, U.S.A., *Bell* 3822 (RSA 793153), SRR5713450; *Andersonglossum virginianum* (L.) J.I.Cohen, U.S.A., *Wilbur* 73075 (DUKE 379623), SRR5713449; *Cryptantha affinis* (A.Gray) Greene, U.S.A., *Rebman* 18116 (SD 199070), SRR5713395; *Cryptantha albida* (Kunth) I.M.Johnst., U.S.A., *Kelley* 1426 (SDSU 20612), SRR5713394; *Cryptantha alfalfalis* (Phil.) I.M.Johnst., Chile, *Arroyo* 995313 (CONC 163659), SRR5713397; *Cryptantha alyssoides* (DC.) Reiche, Chile, *Teiller* 5210 (CONC 156553), SRR5713396; *Cryptantha ambigua* (A.Gray) Greene, U.S.A., *Benet-Pierce* 524 (SDSU 20524), SRR5713391; *Cryptantha aspera* (Phil.) J.Grau, Chile, *Munoz & al.* 2634 (MO 4317599), SRR5713390; *Cryptantha barbiger* (A.Gray) Greene var. *b.*, U.S.A., *Mabry* 27 (SDSU 20349), SRR5713393; *Cryptantha calycotricha* I.M.Johnst., Chile, *Luebert* 3023 (CONC 150898), SRR5713392; *Cryptantha capituliflora* (Clos) Reiche, Chile, *Arroyo* 991122 (CONC 166914), SRR5713389; *Cryptantha clevelandii* Greene, U.S.A., *Simpson* 3733 (SDSU 20782), SRR5713388; *Cryptantha clokeyi* I.M.Johnst., U.S.A., *Andre* 4153 (UCR 164170), SRR5713360; *Cryptantha corollata* (I.M.Johnst.) I.M.Johnst., U.S.A., *Mabry* 83 (SDSU 20775), SRR5713361; *Cryptantha crassisepala* (Torr. & A.Gray) Greene, U.S.A., *Kelley* 1997 (SDSU 20623), SRR5713362; *Cryptantha crinita* Greene, U.S.A., *Lepley* s.n. (SDSU 20823), SRR5713363; *Cryptantha cynoglossoides* (Phil.) I.M.Johnst., Argentina, *Kiesling* 8083 (SI 87776), SRR5713364; *Cryptantha decipens* (M.E.Jones) A.Heller, U.S.A., *Simpson* 3661 (SDSU 20014), SRR5713365; *Cryptantha diffusa* (Phil.) I.M.Johnst., Argentina, *Mendez* 9862 (MERL 56799), SRR5713366; *Cryptantha dumetorum* (Greene ex A.Gray) Greene, U.S.A., *Hasenstab* 57 (SDSU 18694), SRR5713367; *Cryptantha echinella* Greene, U.S.A., *Simpson* 3319 (SDSU 19611), SRR5713335; *Cryptantha fendleri* (A.Gray) Greene, U.S.A., *Ripma* 372 (SDSU 20114), SRR5713334; *Cryptantha flaccida* (Douglas ex Lehm.) Greene, U.S.A., *Simpson* 3619 (SDSU 19846), SRR5713333; *Cryptantha ganderi* I.M.Johnst., U.S.A., *Hasenstab* 40 (SDSU 20345), SRR5713332; *Cryptantha globulifera* 1 (Clos) Reiche, Chile, *Teillier* 3845 (SGO 147985), SRR5713328; *Cryptantha globulifera* 2 (Clos) Reiche, Chile, *Arroyo* 995294 (SGO 146942), SRR5713329; *Cryptantha globulifera* 3 (Clos) Reiche, Chile, *Arroyo* 993602 (CONC 163475), SRR5713331; *Cryptantha globulifera* 4 (Clos) Reiche, Chile, *Arroyo* 993602 (SGO 147688), SRR5713330; *Cryptantha glomerata* Lehmann ex G. Don subsp. *g.*, Chile, *Arroyo* 995177 (SGO 146941), SRR5713337; *Cryptantha glomerulifera* (Phil.) I.M.Johnst., Chile, *Teiller* 5579 (CONC 166867), SRR5713336; *Cryptantha gnaphalioides* (Phil.) Reiche, Chile, *Eggl* 2983 (SGO 146002), SRR5713453; *Cryptantha gracilis* Osterh., U.S.A., *Andre* 12644 (UCR 217631), SRR5713454; *Cryptantha hispida* (Phil.) Reiche, Chile, *Teillier* 4754 (CONC 150914), SRR5713451; *Cryptantha hispidissima* 1 Greene, U.S.A., *Helmkamp* 8471 (RSA 710334), SRR5713358; *Cryptantha hispidissima* 2 Greene, U.S.A., *Hasenstab* 30 (SDSU 18342), SRR5713359; *Cryptantha incana* Greene, U.S.A., *Myers* 1032 (UCR 227031), SRR5713452; *Cryptantha intermedia* (A.Gray) Greene var. *i.*, U.S.A., *Simpson* 3686 (SDSU 20037), SRR5713457; *Cryptantha junipereneis* R.B.Kelley & M.G.Simpson, U.S.A., *Mabry* 75 (SDSU 20766), SRR5713448; *Cryptantha kelseyana* Greene, U.S.A., *Kelley* 2254 (SDSU 20630), SRR5713458; *Cryptantha kingii* (Phil.) Reiche, Chile, *Muñoz* 2580 (SGO 123832), SRR5713455; *Cryptantha leiocarpa* (Fisch. & C.A.Mey.) Greene, U.S.A., *Mabry* 68 (SDSU 20759), SRR5713456; *Cryptantha mariposa* I.M.Johnst., U.S.A., *Helmkamp* 15796 (SDSU 20826), SRR5713459; *Cryptantha maritima* (Greene) Greene var. *m.*, U.S.A., *Simpson* 3665 (SDSU 20050), SRR5713460; *Cryptantha martirensis* M.G.Simpson & Rebman, Mexico, *Rebman* 15973 (SDSU 18625), SRR5713440; *Cryptantha mexicana* (Brandegee) I.M.Johnst., U.S.A., *Kelley* 1230 (SDSU 20610), SRR5713439; *Cryptantha microstachys* (Greene ex A.Gray) Greene, U.S.A., *Rebman* 21420B (SD 216851), SRR5713442; *Cryptantha minima* Rydb., U.S.A., *Kelley* 2248 (SDSU 20629), SRR5713441; *Cryptantha mohavensis* (Greene) Greene, U.S.A., *Ripma* 348 (SDSU 20877), SRR5713444; *Cryptantha muricata* (Hook. & Arn.) A.Nelson & J.F.Macbr. var. *m.*, U.S.A., *Simpson* 3818 (SDSU 20749), SRR5713443; *Cryptantha nemaclada* Greene, U.S.A., *Mabry* 82 (SDSU 20774), SRR5713446; *Cryptantha nevadensis* A.Nelson & P.B.Kenn., U.S.A., *Barth* 913 (SDSU 20393), SRR5713445; *Cryptantha oxygona* (A.Gray) Greene, U.S.A., *Honer* 811 (RSA 685321), SRR5713447; *Cryptantha peruviana* I.M.Johnst., Chile, *Teillier* 4100 (SGO 140959), SRR5713426; *Cryptantha phaceloides* (Clos) Reiche, Chile, *Ackerman* 211 (SGO 146206), SRR5713427; *Cryptantha pterocarya* (Torr.) Greene f. *p.*, U.S.A., *Mabry* 33 (SDSU 20355), SRR5713428; *Cryptantha recurvata* Coville, U.S.A., *Sanders* 39404 (UCR 225245), SRR5713429; *Cryptantha scoparia* A.Nelson, U.S.A., *Andre* 10360 (UCR 211150), SRR5713422; *Cryptantha simulans* Greene, U.S.A., *Hains* 258 (SDSU 20390), SRR5713423; *Cryptantha sparsiflora* (Greene) Greene, U.S.A., *Sanders* 34146 (UCR 184326), SRR5713424; *Cryptantha subamplexicaulis* (Phil.) Reiche, Chile, *Teillier* 2620 (SGO 129437), SRR5713425; *Cryptantha texana* (A.DC.) Greene, U.S.A., *Kelley* 1415 (SDSU 20611), SRR5713431; *Cryptantha torreyana* (A.Gray) Greene var. *t.*, U.S.A., *Ripma* 377 (SDSU 20124), SRR5713432; *Cryptantha utahensis* (A.Gray) Greene, U.S.A., *Mabry* 28 (SDSU 20348), SRR5713416; *Cryptantha watsonii* (A.Gray) Greene, U.S.A., *Andre* 15116 (UCR 226737), SRR5713415; *Cryptantha wigginsii* I.M.Johnst., U.S.A., *Clonessy* s.n. (SDSU 20082), SRR5713414; *Dasynotus daubenmirei* I.M.Johnst., U.S.A., *Kelley* 1951 (SDSU 20343), SRR5713413; *Eremocarya lepida* 1 (A.Gray) Greene, U.S.A., *Simpson* 3851 (SDSU 21209), SRR5713419; *Eremocarya lepida* 2 (A.Gray) Greene, U.S.A., *Simpson* 3184 (SDSU 19533), SRR5713420; *Eremocarya micrantha* (Torrey) Greene var. *m. 1.*, U.S.A., *Guilliams* 602 (SDSU

Appendix 1. Continued.

18956), SRR5713418; *Eremocarya micrantha* (Torrey) Greene var. *m. 2*, U.S.A., *Hendrickson 2640* (SD 203297), SRR5713417; *Eremocarya micrantha* var. *pseudolepida* M.G.Simpson, L.M.Simpson & Rebman, Mexico, *Simpson 3847* (SDSU 21205), SRR5713412; *Greeneocharis circumscissa* (Hook. & Arn.) Rydb. var. *c.*, U.S.A., *Simpson 3875* (SDSU 21417), SRR5713411; *Greeneocharis circumscissa* var. *rosulata* (J.T.Howell) Hasenstab & M.G.Simpson, U.S.A., *Kelley 1625* (SDSU 20663), SRR5713374; *Greeneocharis similis* (K.Mathew & P.H.Raven) Hasenstab & M.G.Simpson, U.S.A., *Kelley 1015* (SDSU 20605), SRR5713375; *Greeneocharis similis* (K.Mathew & P.H.Raven) Hasenstab & M.G.Simpson, U.S.A., *Kelley 1015* (SDSU 20605), SRR5713410; *Harpagonella arizonica* (I.M.Johnst.) Williams & B.G.Baldwin, U.S.A., *Tedford 599* (ARIZ 388168), SRR5713372; *Harpagonella palmeri* A.Gray, U.S.A., *Guilliams 1414* (SBBG 132412), SRR5713373; *Johnstonella angelica* (I.M.Johnst.) Hasenstab & M.G.Simpson, Mexico, *Rebman 18550* (SDSU 19425), SRR5713370; *Johnstonella angustifolia* (Torr.) Hasenstab & M.G.Simpson, U.S.A., *Boyd 11841* (RSA 731212), SRR5713371; *Johnstonella costata* (Brandege) Hasenstab & M.G.Simpson, U.S.A., *Guilliams 538* (SDSU 18964), SRR5713368; *Johnstonella diplotricha* (Phil.) Hasenstab & M.G.Simpson, Argentina, *Mabry 89* (SDSU 21232), SRR5713369; *Johnstonella echinosepala* (J.F.Macbride) Hasenstab & M.G.Simpson, Mexico, *Rebman 25402* (SD 228804), SRR5713376; *Johnstonella fastigiata* (I.M.Johnst.) Hasenstab & M.G.Simpson, Mexico, *West 99-23* (SDSU 15588), SRR5713377; *Johnstonella grayi* (Vasey & Rose) Hasenstab & M.G.Simpson var. *g.*, Mexico, *Fritsch 1264* (RSA 544081), SRR5713343; *Johnstonella holoptera* (A.Gray) Hasenstab & M.G.Simpson, U.S.A., *Simpson 811198H* (SDSU 13036), SRR5713342; *Johnstonella inaequata* (I.M.Johnst.) Brand, U.S.A., *Andre 8132* (RSA 732141), SRR5713345; *Johnstonella micromeres* (A.Gray) Hasenstab & M.G.Simpson, U.S.A., *Mabry 71* (SDSU 20762), SRR5713344; *Johnstonella parviflora* (Phil.) Hasenstab & M.G.Simpson, Chile, *Teillier 4616* (CONC 150821), SRR5713339; *Johnstonella racemosa* Brand, U.S.A., *Hasenstab 68* (SDSU 18710), SRR5713338; *Oreocarya crymophila* (I.M.Johnst.) Jeps. & Hoover, U.S.A., *Ripma 390* (SDSU 20116), SRR5713341; *Oreocarya flavoculata* A.Nelson, U.S.A., *Ripma 307* (SDSU 20030), SRR5713340; *Oreocarya glomerata* (Pursh) Greene, U.S.A., *Ripma 379* (SDSU 20113), SRR5713347; *Oreocarya hoffmannii* (I.M.Johnst.) Abrams, U.S.A., *Ripma 306* (SDSU 20036), SRR5713346; *Oreocarya "howelliana"* (R.B. Kelley, in prep.), U.S.A., *Ripma 312* (SDSU 20004), SRR5713324; *Oreocarya humilis* (A.Gray) Greene subsp. *h.*, U.S.A., *Ripma 303* (SDSU 20029), SRR5713325; *Oreocarya hyposphila* (I.M.Johnst.) Hasenstab & M.G.Simpson, U.S.A., *Ripma 374* (SDSU 20086), SRR5713326; *Oreocarya nubigena "craters"* Greene, U.S.A., *Ripma 399* (SDSU 20094), SRR5713327; *Oreocarya nubigena "granite"* Greene, U.S.A., *Ripma 363* (SDSU 20079), SRR5713320; *Oreocarya nubigena "mammoth"* Greene, U.S.A., *Ripma 301* (SDSU 20055), SRR5713321; *Oreocarya schoolcraftii* (Tiehm) R.B.Kelley, U.S.A., *Ripma 370* (SDSU 20123), SRR5713322; *Oreocarya setosissima* (A.Gray) Greene, U.S.A., *Kelley 1466* (SDSU 20242), SRR5713323; *Oreocarya sobolifera* (Payson) R.B.Kelley, U.S.A., *Kelley 1173* (SDSU 20211), SRR5713318; *Oreocarya subretusa "mt eddy"* (I.M.Johnst.) Abrams, U.S.A., *Kelley 928* (SDSU 20232), SRR5713319; *Oreocarya subretusa "type"* (I.M.Johnst.) Abrams, U.S.A., *Ripma 384* (SDSU 20107), SRR5713353; *Oreocarya subretusa "warner mts"* (I.M.Johnst.) Abrams, U.S.A., *Ripma 389* (SDSU 20110), SRR5713352; *Oreocarya suffruticosa* var. *abortiva* (Greene) J.F.Macbr., U.S.A., *Ripma 308* (SDSU 20024), SRR5713351; *Oreocarya "ursina"* (R.B. Kelley, in prep.), U.S.A., *Ripma 395* (SDSU 20098), SRR5713350; *Oreocarya virgata* (Porter) Greene, U.S.A., *Ripma 371* (SDSU 20117), SRR5713357; *Pectocarya linearis* var. *ferocula* I.M.Johnst., U.S.A., *Kelley 1962* (SBBG 132392), SRR5713356; *Pectocarya penicillata* A.D.C., U.S.A., *Kelley 1967* (SBBG 132393), SRR5713355; *Pectocarya pusilla* A.Gray, U.S.A., *Guilliams 995* (SBBG 132394), SRR5713354; *Pectocarya setosa* A.Gray, U.S.A., *Gowen 306* (JEPS 108272), SRR5713349; *Plagiobothrys austinae* (Greene) I.M.Johnst., U.S.A., *Guilliams 1010B* (SBBG 132398), SRR5713348; *Plagiobothrys canescens* Benth. var. *c.*, U.S.A., *Guilliams 929* (SBBG 132399), SRR5713437; *Plagiobothrys collinus* var. *ursinus* (A.Gray) Higgins, U.S.A., *Guilliams 1067* (SBBG 132400), SRR5713438; *Plagiobothrys congestus* (Wedd.) I.M.Johnst., Bolivia, *Villavicencio 323* (MO 5203201), SRR5713382; *Plagiobothrys fulvus* var. *campestris* (Greene) I.M.Johnst., U.S.A., *Guilliams 1105* (SBBG 132401), SRR5713436; *Plagiobothrys glomeratus* A.Gray, U.S.A., *Tiehm 12542* (UTC 230182), SRR5713380; *Plagiobothrys glyptocarpus* (Piper) I.M.Johnst., U.S.A., *Guilliams 1142* (SBBG 132402), SRR5713434; *Plagiobothrys gracilis* (Ruiz & Pav.) I.M.Johnst., Chile, *Guilliams 1688* (SBBG 132396), SRR5713378; *Plagiobothrys greenei* (A.Gray) I.M.Johnst., U.S.A., *Forrestal 4-15-09* (SBBG 132403), SRR5713379; *Plagiobothrys hispidus* A.Gray, U.S.A., *Oswald & Ahart 5655* (JEPS 87508), SRR5713386; *Plagiobothrys jonesii* A.Gray, U.S.A., *André & Clifton 10750* (UCR 215416), SRR5713387; *Plagiobothrys kingii* var. *harknessii* (Greene) Jeps., U.S.A., *Taylor 15044* (UC 1876874), SRR5713403; *Plagiobothrys leptocladus* (Greene) I.M.Johnst., Mexico, *Guilliams 1772* (SBBG 132404), SRR5713402; *Plagiobothrys linifolius* cf. (Willd. ex Lehm.) I.M.Johnst., Peru, *Weigend & Schwarzer 8073* (MO 6145366), SRR5713405; *Plagiobothrys mollis* (A.Gray) I.M.Johnst., U.S.A., *Guilliams 1347* (SBBG 132404), SRR5713404; *Plagiobothrys myosotoides* NA (Lehm.) Brand, U.S.A., *Gowen 1029* (SBBG 132405), SRR5713399; *Plagiobothrys nothofulvus* (A.Gray) A.Gray, U.S.A., *Guilliams 1481* (SBBG 132406), SRR5713398; *Plagiobothrys plurisepalus* I.M.Johnst., Australia, *Weber 5653* (NSW 647734), SRR5713401; *Plagiobothrys polycaulis* (Phil.) I.M.Johnst., Chile, *Guilliams 1687* (SBBG 132395), SRR5713400; *Plagiobothrys tenellus* (C.A.Mey. ex Ledeb.) A.Gray, U.S.A., *Guilliams 1183* (SBBG 132407), SRR5713407; *Plagiobothrys torreyi* (A.Gray) A.Gray, U.S.A., *Guilliams 1888* (SBBG 132408), SRR5713406; *Plagiobothrys undulatus* (Piper) I.M.Johnst., U.S.A., *Guilliams 1138* (SBBG 132409), SRR5713408.

TAXON

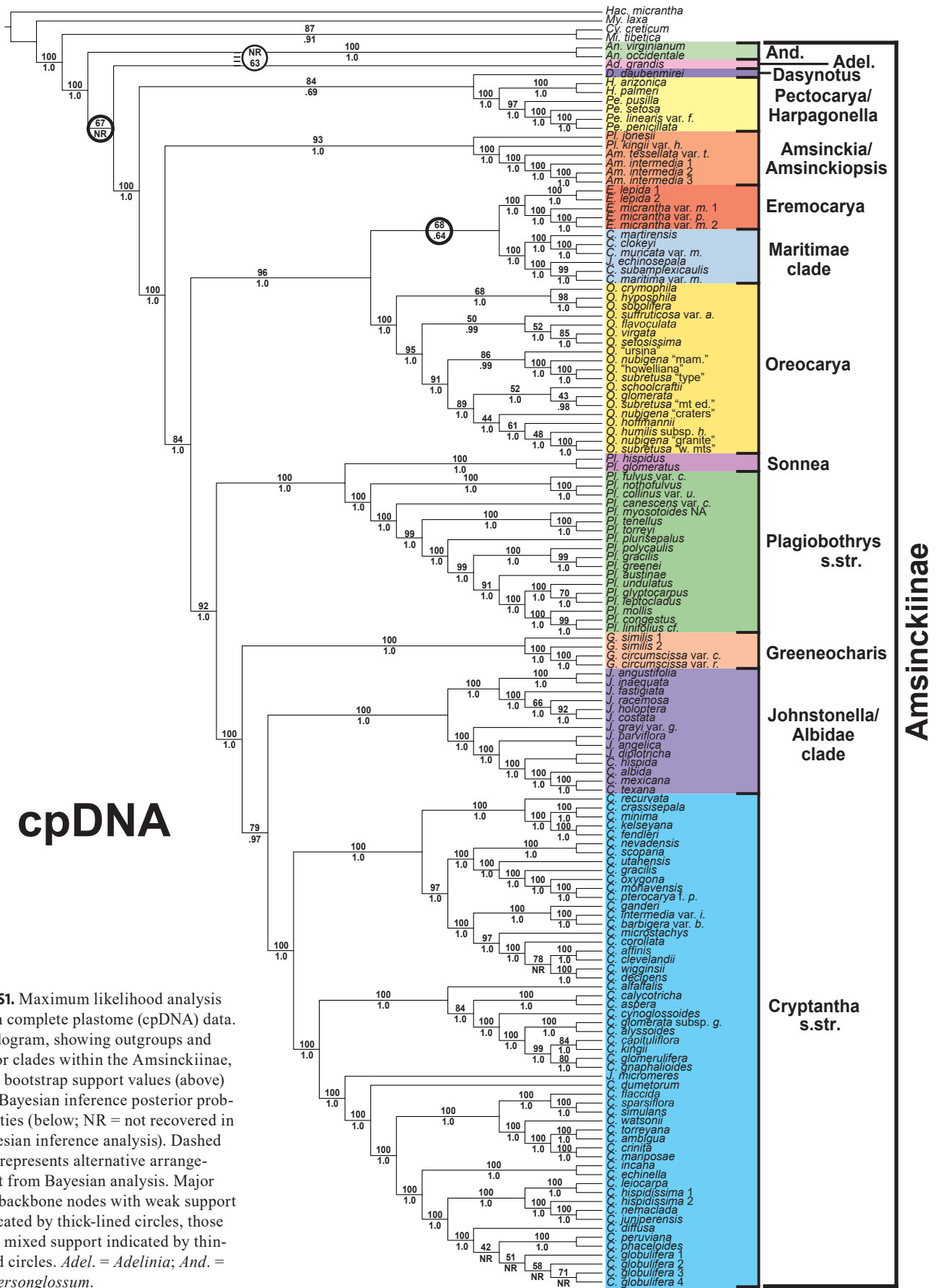
International Journal of Taxonomy, Phylogeny and Evolution

Electronic Supplement to

**Phylogeny of the popcorn flowers: Use of
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(Boraginaceae)**

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Makenzie E. Mabry & Lee Ripma**

***Taxon* 66: 1406–1420**



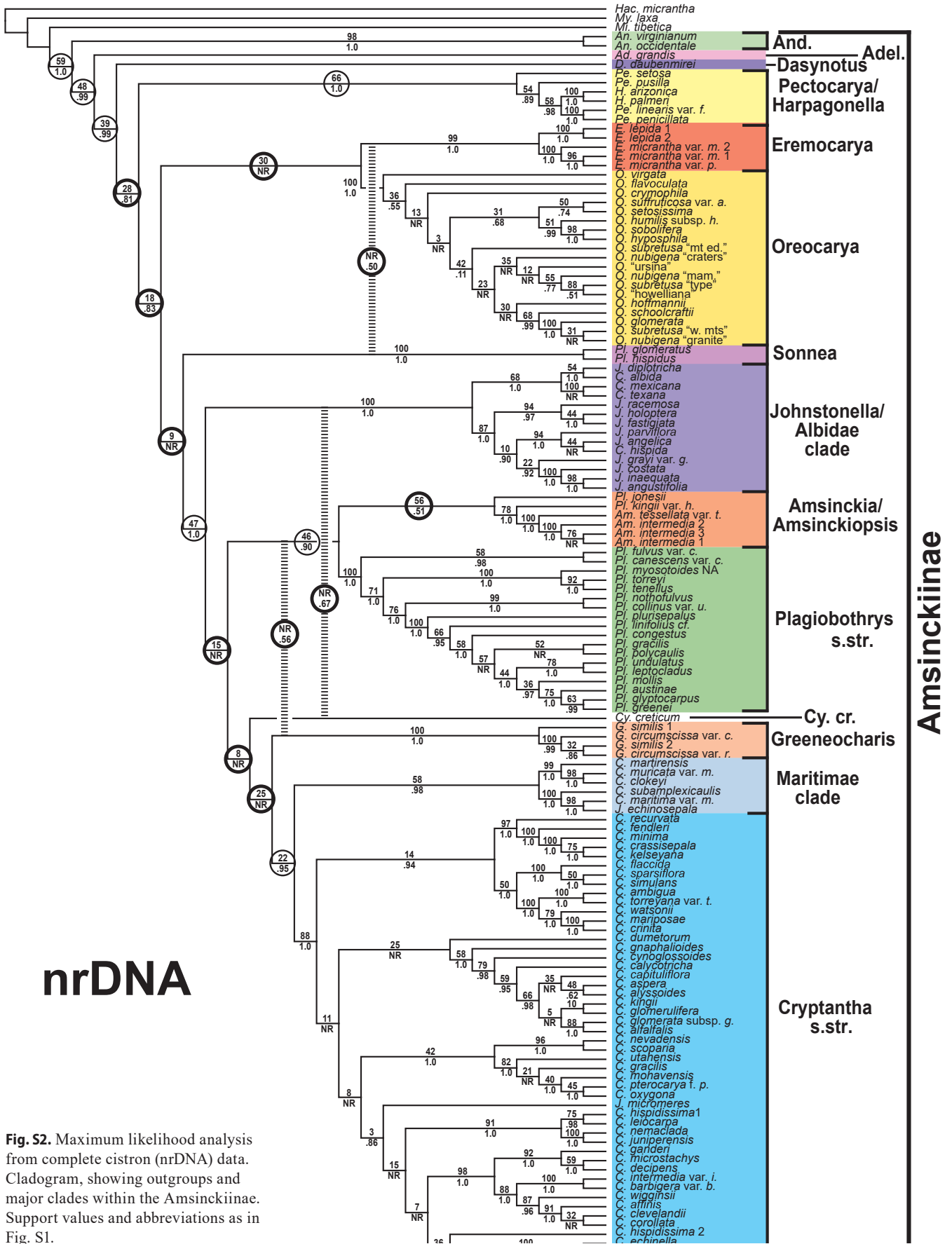


Fig. S2. Maximum likelihood analysis from complete cistron (nrDNA) data. Cladogram, showing outgroups and major clades within the Amsinckiinae. Support values and abbreviations as in Fig. S1.

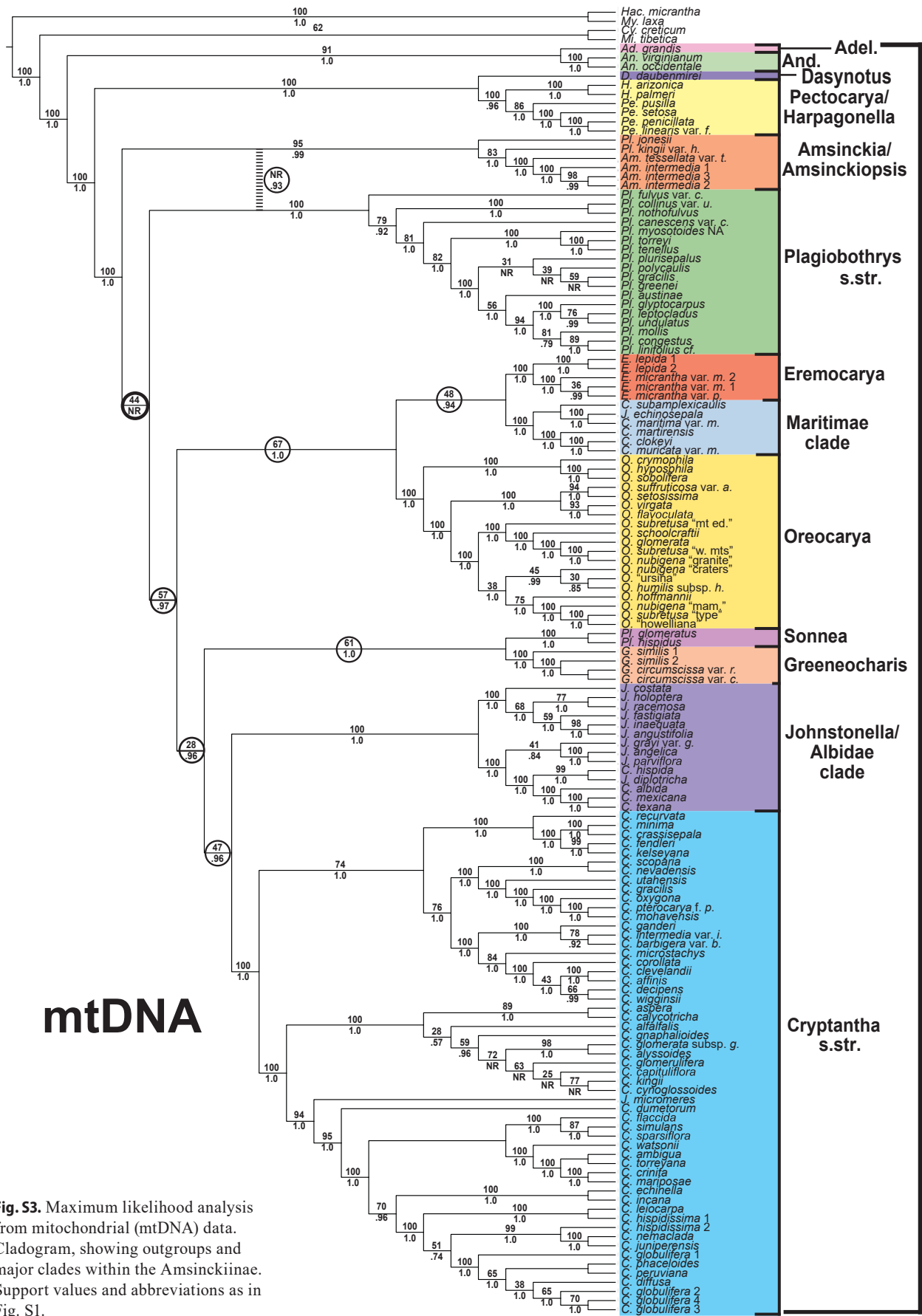


Fig. S3. Maximum likelihood analysis from mitochondrial (mtDNA) data. Cladogram, showing outgroups and major clades within the Amsinckiinae. Support values and abbreviations as in Fig. S1.