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MOLECULAR PHYLOGENY OF THE GOLDENASTERS, SUBTRIBE CHRYSOPSIDINAE (ASTERACEAE: ASTEREAE), BASED ON NUCLEAR RIBOSOMAL AND CHLOROPLAST SEQUENCE DATA

by

Clayton M. Costa

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Clayton M. Costa, entitled Molecular phylogeny of the goldenasters, subtribe Chrysopsidinae (Asteraceae: Astereae), based on nuclear ribosomal and chloroplast sequence data, has been approved by the thesis committee as satisfactorily completing the thesis requirements for the degree Master of Science in Biology.

Chairperson, Thesis Committee Signature — Roland P. Roberts — 3rd July, 2014 Date

Committee Member Signature — David Hearn — 23 July 2014 Date

Committee Member Signature — Vicki Funk — 3 July 2014 Date

Dean of Graduate Studies — July 27, 2014 Date

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ABSTRACT

In this study, phylogenies were estimated based on molecular data from *ETS* and *ITS*, nuclear ribosomal DNA, and *ycf1* and *psbA-trnH*, chloroplast DNA for all known genera of the Chrysopsidinae. The resulting phylogenies were used as the basis for addressing questions related to subtribal and generic monophyly, the relationship of the Central and South American genera *Osbertia*, *Tomentaurum*, and *Noticastrum* to North American genera, biogeography, morphological trait evolution and convergence among morphological features commonly used to delimit generic and species boundaries. Additionally, modifications to commonly used DNA extraction techniques allowed us to access DNA from relatively old herbarium specimens. Results from analyses of individual and combined datasets support the monophyly of the Chrysopsidinae and allowed some resolution of both the relationships among the genera of the Chrysopsidinae and the relationships among sections of the genus *Heterotheca*.

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Chapter 1

Molecular phylogeny of the goldenasters, subtribe Chrysopsidinae (Asteraceae: Astereae), based on nuclear ribosomal and chloroplast sequence data

ABSTRACT

Subtribe Chrysopsidinae, also known as goldenasters, is a group of wildflowers distributed from North to South America and includes eight genera *sensu* Semple (*Bradburia*, *Chrysopsis*, *Croptilon*, *Heterotheca*, *Osbertia*, *Noticastrum*, *Pityopsis* and *Tomentaurum*). Historically, phylogenetic inference within the Chrysopsidinae has been based on morphological characters, resulting in multiple classifications put forward by various researchers. Previous studies based on chloroplast DNA restriction site data were inconclusive with regard to the resolution of relationships among all the genera and species in this subtribe. Furthermore, no single study has sampled all known genera and species of the Chrysopsidinae, so there was little understanding of the evolutionary relationships. This study estimates phylogenies based on molecular data from *ETS* and *ITS*, nuclear ribosomal DNA, and *ycf1* and *psbA-trnH*, chloroplast DNA for all known genera of the Chrysopsidinae. Results from analyses of individual and combined datasets support the monophyly of the Chrysopsidinae. We observe resolution of relationships among genera of the Chrysopsidinae, and some sectional resolution within the genus *Heterotheca*. All genera *sensu* Semple except *Tomentaurum*, are monophyletic lineages with strong support. However, like for other groups of tribe Astereae, the search continues for genetic markers that are variable enough to resolve relationships among species.

INTRODUCTION

The Asteraceae (Compositae) is the largest family of flowering plants with its species classified into 12 subfamilies (Funk et al. 2009). The family is considered to be relatively young geologically with the proposed split from its sister group in the early Eocene, approximately 50 million years ago (Barreda et al. 2010). Although the Asteraceae is considered a relatively young family, the group has rapidly evolved into a large number of species, approximately 30,000 distributed globally (Funk et al. 2005). This rapid evolution has resulted in species that are morphologically diverse but may be closely related genetically, or some that appear morphologically similar but genetically diverse (Anderson 1995).

The Astereae, a tribe within the Asteraceae, is the second largest tribe in subfamily Asteroideae (Nesom and Robinson 2007). The tribe comprises 222 genera and approximately 3,100 species (Brouillet et al. 2009). Tribe Astereae is distributed globally (Brouillet et al. 2009) and it is subdivided into 18 subtribes *sensu* Nesom (Nesom and Robinson 2007), 13 of which are primarily distributed in the Western Hemisphere. There is evidence for multiple dispersal events from among basal lineages of Astereae in Africa to other regions of the globe (Brouillet et al. 2009). Currently, North American lineages of Astereae show close relationships to South American subtribes, Hinterhuberinae and Podocominae (Brouillet et al. 2009).

Among the North American lineages is subtribe Chrysopsidinae *sensu* Nesom, commonly known as goldenasters. This subtribe has a distribution that spans North and South America. The Chrysopsidinae includes eight genera and approximately 70 known

species; however, assessment of evolutionary relationships among genera, among species within genera, and among species within sections are equivocal (Semple 1996).

The Chrysopsidinae *sensu* Semple (2006) includes eight genera: *Bradburia* Torr. & Gray, *Croptilon* Raf., *Chrysopsis* (Nutt.) Ell., *Heterotheca* Cass., *Noticastrum* DC., *Pityopsis* Nutt., *Osbertia* Greene, and *Tomentaurum* Nesom. The group was originally circumscribed based on morphological criteria by Bremer and Anderberg (1994) and later named by Nesom (1994). North American genera include *Bradburia*, *Chrysopsis*, *Croptilon*, *Heterotheca* and *Pityopsis*, with some species of *Croptilon*, *Heterotheca* and *Pityopsis* occurring in Central America. Unlike the other genera that are primarily distributed in North America, *Osbertia* and *Tomentaurum* are native to Mexico (Nesom, 2000) and *Noticastrum* is distributed across South America (Zardini 1985). The relationship of species of *Osbertia*, *Noticastrum* and *Tomentaurum* to the North American Chrysopsidinae is still unclear and requires examination using an independent dataset.

Historically, three genera (*Chrysopsis*, *Heterotheca* and *Pityopsis*) were commonly referred to as goldenasters, with no formal subtribal classification. The composition and boundaries of these genera were based on morphological criteria, such as color of ray florets, series of pappus (modified calyx) and ridges on cypsela, which have been subjected to many interpretations resulting in various estimations or relationships. Several researchers (Shinners 1951; Semple et al. 1980; Bremer and Anderberg 1994) have attempted to resolve issues of composition and relationships associated with the goldenasters, resulting in the formal subtribal classification of Chrysopsidinae by Nesom (1994). This, however, was only a first step towards the

development of an understanding of the relationships among these taxa, but much remains to be done.

Heterotheca, the prairie goldenaster, was originally circumscribed as a small genus by Cassini (1817) in tribe Astereae. The genus retained its small size until Shinners (1951) proposed the merger of *Chrysopsis*, a much larger genus, with *Heterotheca* based on morphology, particularly features of the pappus of the achenes (fruits). As a result, *Heterotheca* is currently the largest in the subtribe, comprising 24 species. Species of *Heterotheca* are distributed primarily in North America with some extension into Central America. In the years following the expanded concept of this genus, dozens of names were added to the literature as new species were described. *Heterotheca sensu* Shinners was later supported by the morphological studies by Wagenknecht (1960) and cytological evidence of Harms (1965). Later, while Harms continued to reorganize *Heterotheca*, he expanded its boundaries to include *Pityopsis* (Harms 1969) and *Chrysopsis* (Harms 1974), resulting in five sections: section *Ammodia* (Nutt.) Harms, section *Chrysopsis* (Nutt.) Harms, section *Heterotheca*, section *Phyllotheca* (Nutt.) Harms, and section *Pityopsis* (Nutt.) Harms. However, using cytological data, Semple (1977) did not find evidence to support Shinners' (1951) or Harms' (1974) hypotheses. Semple (1977) proposed the recognition of *Chrysopsis* as a genus distinct from *Heterotheca*. Subsequently, evidence from morphological, anatomical, habit, and habitat characteristics was used by Semple and Chinnappa (1980a, 1980b) and Semple et al. (1980) to treat *Chrysopsis*, *Heterotheca* (sects. *Heterotheca*, *Phyllotheca*, and *Ammodia*) and *Pityopsis* as distinct genera.

Chrysopsis was the largest of the original goldenaster genera. Species of this genus are only found in the continental United States and are distributed from New York to Texas. Presently, the genus comprises 11 species, far less than the dozens assigned to it historically. Though *Chrysopsis* was sometimes merged with *Pityopsis* and *Heterotheca*, currently there is agreement that *Chrysopsis* is morphologically quite different from both *Pityopsis* and *Heterotheca*. However, there is still debate among researchers about which genus, *Pityopsis* or *Heterotheca*, might be the closest relative of *Chrysopsis.* Semple (1981) proposed that *Chrysopsis* was more closely related to the monotypic genus *Bradburia* than to *Pityopsis or Heterotheca. Bradburia* was previously treated as a close relative of *Heterotheca* (Correll and Johnston 1970), thus it might be argued that the closest relatives of *Chrysopsis* were among species of *Heterotheca*. Additional observations by Semple and Chinnappa (1984) supported a very close relationship between *Bradburia* and *Chrysopsis*. That hypothesis was challenged by Nesom (1991a) who proposed placement of species of *Bradburia* in a broadly defined *Chrysopsis.* Semple (1996) refuted this claim and treated *Bradburia* as distinct from both *Chrysopsis* and *Heterotheca*. In addition, he proposed reclassification of *Chrysopsis pilosa* (the closest known relative of *Bradburia hirtella*) to *Bradburia*. Nesom and Semple, in all subsequent publications, failed to reach agreement on the boundaries and composition of *Chrysopsis*.

Bradburia's status as a genus is currently contested. *Sensu* Semple, *Bradburia* includes species *B. pilosa* (*Chrysopsis pilosa sensu* Nesom) and *B. hirtella* (*C. texana sensu* Nesom). Nesom (1991a) argues that variations in morphology are insufficient to recognize these genera (*Chrysopsis* and *Bradburia*) as distinct. In that study, Nesom

found these two species to be very closely related, and argued that if *Chrysopsis pilosa* is maintained taxonomically, *Bradburia hirtella* should be recognized as *Chrysopsis*. In addition, he stated that the achene morphology of *C. pilosa* was similar to other species of *Chrysopsis*, which suggested that these taxa formed a monophyletic group. Semple (2006), however, proposed that the unique pappus traits, which include broader scales in *B. pilosa* and broad scaly bases of bristles in staminate disk florets justifies the distinction, previously supported by cytological findings of Semple and Chinnappa (1984). In the most recent subtribal circumscription (Nesom and Robinson 2007), Nesom did not recognize *Bradburia* as a distinct genus from *Chrysopsis*.

Pityopsis, the grass-leafed goldenaster, was previously part of the large *Chrysopsis* group. However, as currently defined, *Pityopsis* comprises seven species that are distributed in North America and ranges from Texas to Canada. *Pityopsis* is quite distinct from other goldenasters, having grass-like leaves with parallel nerves. Harms (1969) proposed that *Pityopsis* be merged with *Heterotheca*, assigning it sectional status based on his morphological studies. Semple et al. (1980) re-elevated *Pityopsis* to generic rank, followed by additional revisions in Semple and Bower (1985), the status accepted since. This status is supported by phenotypic and habitat differences of *Pityopsis* (compared to other Chrysopsidinae), exemplified by fire-dependent flowering displayed by some species of the genus (Gowe and Brewer 2005).

Nesom (1991b) speculated that *Croptilon* was the sister taxon of *Pityopsis.* *Croptilon* was originally treated as *Haplopappus* sec. *Isopappus* (Hall 1928), *Croptilon* was first treated as a genus by Shinners (1951), a hypothesis disputed by Smith (1965, 1966). *Croptilon* was later described by Smith (1981), in agreement with Shinners

(1951), as comprising three species, two North American, and populations of one species, *Croptilon rigidifolium*, occurring in Mexico. Chloroplast DNA restriction site studies by Morgan (1990) placed *Croptilon* in the Chrysopsidinae.

Another group placed in *Haplopappus* and elevated to genus rank following *Haplopappus'* diminution was *Osbertia*. *Osbertia*, was elevated to the rank of genus by Turner and Sundberg (1986). At the time, they speculated a close relationship of this genus to *Heterotheca* and *Noticastrum*. Currently there are three known species of *Osbertia* restricted to Central America. Nesom (1994) included *Osbertia* in the Chrysopsidinae, a decision that was questioned by Semple (1996). Lane et al. (1996) supported Nesom's (1994) morphologically-based hypotheses of the composition of Chrysopsidinae, with the exclusion of *Osbertia*. Furthermore, Lane et al. (1996) inferred a close relationship between *Boltonia* L'Hér and the Chrysopsidinae, supported by chloroplast DNA restriction site data. However, that study did not include a large number of species that are representative of the genera in the Chrysopsidinae *sensu stricto*, nor did it include species of *Noticastrum* and *Tomentaurum* in the investigation.

Noticastrum, a South American genus, is widely distributed with approximately 20 species. Other than Zardini's (1985) treatment of this genus, there are no recent taxonomic treatments or studies addressing phylogenetic relationship of taxa in this genus. Nesom (1994) hypothesized a close relationship between *Heterotheca* and *Noticastrum*. The genus is characterized by white ray florets, atypical of the yellow rays commonly associated with most of the goldenasters. *Noticastrum* is the only genus in the Chrysopsidinae with a strictly South American distribution. Finally, Nesom (1994)

included the relatively recently described monotypic Mexican genus *Tomentaurum* in Chrysopsidinae.

There is consensus that all described genera of the Chrysopsidinae are related to *Heterotheca*, though which of these genera is the sister taxon of *Heterotheca* is yet unknown. Major confusion remains with regard to 1) generic composition of Chrysopsidinae, 2) the relationships among the genera within the Chrysopsidinae, and 3) the relationship of Chrysopsidinae to other subtribes in Astereae. Despite the lack of consensus regarding the composition of the Chrysopsidinae and the relationships of its constituent genera and species, there is no single study that has comprehensively sampled among genera and species of the subtribe for assessing evolutionary relationships. The use of morphological criteria in assessments of composition and relationships has resulted in conflicting hypotheses in this group. Evaluation of current hypotheses using molecular data may help to resolve some of the confusion discussed above for the Chrysopsidinae.

Most recent phylogenetic studies of subtribes within Astereae have been based in part on molecular data. However, many of these phylogenies lack resolution within and among genera (Suh and Simpson 1990; Morgan and Simpson 1992; Lane et al. 1996) because of their reliance on single genes. Despite the problems inherent in single-gene studies (e.g. lack of robust resolution among taxa), it has been demonstrated that DNA data are useful for diagnosing younger lineages and have been a successful tool for investigators (Markos and Baldwin 2001). Molecular studies spanning the 18S-26S region of ribosomal DNA (*r*DNA) have been successful in inferring phylogenies for investigations of genera in the Astereae (Markos and Baldwin 2002). Roberts and

Urbatsch (2003, 2004) inferred the phylogenies of *Ericameria*, *Chrysothamnus* and related genera in subtribes Hinterhuberinae and Solidagininae, close relatives of Chrysopsidinae, utilizing nuclear ribosomal DNA (*nr*DNA) data, spanning external transcribed spacer (*ETS*) and internal transcribed spacer (*ITS*). A broader investigation of tribe Astereae based on *ITS* data was reported by Brouillet et al. (2009). Their study, being limited to nuclear data, proposed a need for "better support and better resolution" based on additional molecular markers. In addition to *nr*DNA, chloroplast DNA (*cp*DNA) region *ycf1* and *psbA-trnH* have also been successfully used for phylogenetic analysis in the Asteraceae. In addition, chloroplast DNA region *ycf1* has been shown to be useful in phylogenetic analysis of Orchidaceae and Annonaceae by Neubig et al. (2009; 2010) and utility of this region was useful in tribe Astereae by Urbatsch (pers. comm.). The *cp*DNA region *psbA-trnH* was shown to be moderately informative among Asteraceae by Shaw et al. (2005).

A multi-gene approach is more likely to yield resolved phylogenies in related subtribes of Astereae by utilizing different rates of evolution among organelles and regions of DNA (Roberts 2002; Roberts and Urbatsch 2003, 2004; Urbatsch et al. 2003). Here we propose to analyze DNA sequence data for two chloroplast and two nuclear regions to address questions of boundaries and relationships of taxa of subtribe Chrysopsidinae. Specifically, the following questions are addressed; 1) Is Chrysopsidinae a monophyletic group? 2) Are genera within the Chrysopsidinae monophyletic? 3) What are the relationships of genera within the Chrysopsidinae? 4) Are the morphological features commonly used to delimit genera a result of unique or multiple evolutionary events?

MATERIALS AND METHODS

Sampling and DNA Extraction

Outgroup taxa included species from close as well as distantly related subtribes Conyzinae, Boltoniinae, Solidagininae, and Hinterhuberinae based on the relationships proposed by Brouillet et al. (2009). In addition, sequences were obtained from GenBank® for *Pseudognaphalium* and South American Hinterhuberinae. Ingroup taxa representing species in all eight genera of the Chrysopsidinae *sensu* Semple (2006) have been analyzed. Extensive taxon sampling within subtribe Chrysopsidinae included specimens that were field and herbarium collected and ranged from North to South America. A total of 252 individuals from 19 genera and 89 species were sampled (Table 1.1). With the exception of rare or threatened taxa, multiple specimens of each taxon were sampled with the specimens selected from different localities to maximize the possibility of variation. Although, the study was designed to exhaustive sample all known species in subtribe Chrysopsidinae, this investigation resorted to exemplar sampling due to limitations in resources, namely the availability of specimens from all genera studied. Identification of taxa was based primarily on the keys in Flora of North America (2006) but the keys in Weakley's Flora of the Southern and Mid-Atlantic States (2012) and Semple (1996) were also used.

In order to determine the impacts of outgroup selection on the resulting topology, each outgroup lineage was included one at a time during initial analyses of each dataset. This was followed by analyses with all of the outgroup lineages. Finally, (based on their availability in GenBank®) outgroup taxa from the distantly related genus *Pseudognaphalium* and were used in the analysis of *nr*DNA regions to determine the

basal structure of the phylogeny. All subsequent analyses of combined datasets included species from *Ericameria* as outgroup for rooting the phylogeny.

Tissues for DNA extraction (20-30mg) were collected from among the youngest leaves on each sampled stem. Primarily, herbarium specimens served as study material, however, freshly collected and dried field samples were also used in the study. Desiccated tissues were pulverized using a Mini-Beadbeater-8 (Bio Spec Products Inc., Bartlesville, OK). DNA was extracted from pulverized samples using Qiagen DNeasy® Plant Mini Kit and Miniprep CTAB extraction method modified from Doyle and Doyle (1990). These protocols were modified to improve quality and yields of DNA from specimens (Chapter 2). In order to determine the efficacy of the extraction protocols, several taxa were sampled multiple times using unmodified and modified protocols. DNA quality and concentration were verified using a NanoDrop Lite (NanoDrop Products, Wilmington, DE). Extracts were then stored in -20°C. Subsequently, working solutions were prepared through serial dilutions from the stock DNA for use as PCR template.

Polymerase Chain Reaction (PCR) and Sequencing:

Templates for sequencing were generated using 20µL PCR. Pilot studies provided the basis for selecting types of DNA polymerases used for this study. Premixed *Taq* DNA polymerase e2TAK (TaKaRa Bio Inc., Japan) and 5X e2TAK Buffer were used for most PCR. When premixed *Taq* DNA polymerase e2TAK failed to amplify products for recalcitrant templates, premixed *Tfl* DNA polymerase (Epicentre, Madison, WI) and 2X GN buffer were used. Two PCR cyclers were used in this study, Labnet MultiGene Thermal Cycler (Labnet International, Inc., Edison, NJ) and Bio-Rad MyCycler (Bio-Rad Laboratories, Hercules, CA).

Nuclear ribosomal DNA spanning the *ETS* region was isolated and amplified with primers 18S-2L and Ast 1, with nested primers 18S-ETS and Ast-8 when external primers failed to amplify (Table 1.2). *ITS* regions were isolated and amplified with primers ITS 4 and ITS 1 or ITS 20 if ITS 1 failed to amplify (Table 1.2). PCR protocols used for *ETS* and *ITS* were modified from Roberts (2002). The amplification profile included an initial denaturation cycle at 95°C for 5min, followed by 10 cycles of 95°C for 1min denaturation, 55°C for 1min annealing, 72°C for 1min extension, followed by 20 cycles of 95°C for 1min denaturation, 50°C for 1min annealing, 72°C for 1min extension. This profile ended with a 7-min extension at 72°C followed by storage at 4°C. For samples that failed to amplify with nested priming, PCR profiles were modified. In addition to the above PCR profile, we substituted another profile for *ITS* isolation with an initial denaturation cycle at 95°C for 5min, followed by 35 cycles of 95°C for 1min denaturation, 55°C for 1min annealing, 72°C for 1min extension, ending with one cycle at 72°C extension for 7min and storage at 4°C.

Chloroplast DNA *ycf1 3300-4280* region was amplified with primers ycf1-3300F and ycf1-4280R (Neubig et al. unpubl. data). Additionally, a modified primer ycf1TU-F was substituted when samples failed amplify. The reactions had a denaturation cycle at 98ºC for 2 min, followed by 30 cycles of 98ºC for 10sec denaturation, 55ºC for 15sec annealing, 72ºC for 1min extension, one cycle at 72°C for 10min extension with storage at 4°C. The second *cp*DNA region *psbA-trnH* was amplified with primers psbA-F and trnH-R (Shaw et al. 2005). Modified primers psbATU-Fdeg and psbATU-F were used as alternative forward primers when amplification failed. The reactions had a denaturation cycle at 80ºC for 2 min, followed by 25 cycles, 94ºC for 30sec denaturation, 56.5ºC for

30sec annealing, 72ºC for 1min extension, then one cycle 72°C for 10min extension with storage at 4°C

Following PCR, the presence of amplicons was verified using 1.5% agarose gel electrophoresis and/or Lonza FlashGel System (Lonza Group Ltd., Allendale, NJ). After verification, PCR products were purified using Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit and protocol (Macherey-Nagel Inc., Bethlehem, PA). DNA sequencing was outsourced to MacrogenUSA (Rockville, MD).

Sequence Editing and Alignment:

Sequence information, including chromatographs and raw sequence data, from Macrogen was edited with SEQUENCHER 4.7 (Gene Codes Corporation, Ann Arbor, Michigan). Preliminary alignment, to determine homology, was obtained with MUSCLE (Edgar 2004), CLUSTAL OMEGA (Sievers et al. 2011), and MAFFT (Katoh and Frith 2012). MAFFT was consistently the most robust alignment software (determined through qualitative visual analysis for misalignments) and was utilized as the primary alignment software for this study. Aligned sequences were further checked and modified manually using MESQUITE 2.75 (Maddison and Maddison 2011). Manual sequence adjustments were made to each individual dataset before combining into four concatenated datasets—*cp*DNA regions only, *nr*DNA regions only, *nr*DNA *ETS* with both *cp*DNA regions and all four DNA regions. Sequences of various lengths were produced for all regions due to the use of various primers. A conservative approach was taken by removing bases from the 5′ and 3′ ends of aligned sequence data, reducing the amount of missing data. Additionally, a large section ca. 150bp was excised from the middle of *psbA-trnH* aligned

sequence due to missing data for most taxa. Sequences for combined datasets were obtained by use of concatenation of edited individual datasets.

Model of Evolution and Phylogenetic Analysis:

The model of nucleotide evolution for each individual dataset was estimated with JMODELTEST (Posada 2008) using the Akaike information criterion. Bayesian phylogenetic analyses were conducted using MRBAYES (Ronquist et al. 2012). Based on the model of evolution, parameter values were defined *a priori* for analyses of the individual and combined datasets (Table 1.3). Concatenated data matrixes were analyzed producing four datasets of combined regions in addition to individual region data analyses. The *nr*DNA *ETS* and *ITS* datasets were combined into one matrix. Likewise, *cp*DNA *ycf1*and *psbA-trnH* regions were combined. Due to the similarity in composition of *ETS*, *ycf1*, and *psbA-trnH* datasets described below, they were combined. Similarly, all four regions (*nr*DNA and *cp*DNA) were combined into one dataset for Bayesian analysis. The concatenated dataset of *ETS*, *ycf1* and *psbA-trnH* consisted of a larger number of sequences for a greater number of ingroup species compared to the dataset with all four DNA regions. Unique identification numbers were assigned to each individual specimen. In both the three- and four-loci combined datasets, sequences were concatenated only when all targeted DNA regions were successfully amplified and sequenced. A mixed model approach was used for analysis of combined datasets using models selected by JMODELTEST (Table 1.3). As each dataset was concatenated, models were reevaluated due to variation in taxon composition. All independent and combined datasets were also analyzed using flat priors. Bayesian analysis was initiated with random starting trees and allowed to search for 2.0×10^6 generations. Two replicate Bayesian searches were

performed for each dataset where Markov Chains were sampled every 100 generations (a total of 40002 saved trees). Additionally, each dataset was independently analyzed three times.

Bayesian analysis requires Markov Chains to reach stationarity, resulting in trees that produce stable parameter estimates. Therefore, all sampled trees prior to stationarity were discarded as "burn-in" samples. The discarded samples also included some samples after stationarity was attained to minimize retention of any burn-in samples, since stationarity was reached within the first 1.0×10^5 generations in the analysis of both independent and combined datasets. Stationarity was determined using the overlay plots of summary parameters (an output of Bayesian analysis), and plotting of the log probability against generations in MICROSOFT EXCEL. Furthermore, all analyses were performed until the average standard deviation of the split frequencies was less than 0.01, another indicator of stationarity. For each Bayesian search, phylograms were plotted and compared for congruence in topology. Posterior probabilities were estimated by use of a 50% majority rule consensus tree. Lineages with posterior probability (PP) of 0.95 or higher were considered significantly supported. In addition to Bayesian analysis, maximum likelihood (ML) analysis was carried out on the concatenated dataset of all four DNA regions using the RAxML online server (Stamatakis 2008) with partitioned model analysis and defined outgroup. Bootstrap support (BS) values were calculated for RAxML analysis. Lineages with BS of 60 to 79 were considered moderately supported and BS values 80 or higher were considered strongly supported. The resulting ML trees were compared to Bayesian trees for congruence in topology. Phylogenetic trees were

edited for evaluation with FIGTREE v1.4.0 (Rambaut, Edinburgh, UK). Trees were further edited for presentation using INKSCAPE™ (Inkscape Project, Brooklyn, NY).

Morphological Character Evolution:

The history of morphological character states was traced using MESQUITE 2.75. These traits were subjected to rigorous analyses using stochastic character mapping and MK1 packaged in MESQUITE 2.75, following methods of analyses in Horn et al. (2012). Morphological characters and character states were selected from among those commonly used to delimit genera and species within the Chrysopsidinae (Table 1.4). Characters were scored from herbarium specimens from BALT, TEX/LL, US, DOV, LSU, MO and TENN (Table 1.1). These were supplemented by observations of living plants and secondarily from published descriptions of genera and species of Chrysopsidinae. Each terminal taxon had a single character state of binary or multistate characters. The tracing of character evolution facilitated the identification of synapomorphies supporting monophyletic groups.

RESULTS

The variation in taxon composition, seen in the individual datasets, was linked to the success or failure of amplification and sequencing each DNA region. There was also variation in sequence length among individuals of the same taxon for each DNA region. The *ETS* region of *nr*DNA yielded sequences between 354bp and 552bp in size and a total of 164 individuals representing 79 species were sequenced for this region. The resulting aligned dataset contained 565 characters. The *ITS* region of *nr*DNA yielded sequences between 600bp and 866bp in size and a total of 96 individuals representing 60 species were sequenced for this region, with an edited aligned data matrix of 739

characters. The *psbA-trnH* region of *cp*DNA yielded sequences between 270bp and 413bp in size and a total of 126 individuals representing 72 species were sequenced for this region, with an edited aligned data matrix of 359 characters. The *ycf1 3300-4200* region of *cp*DNA yielded sequences between 723bp and 963bp in size and a total of 131 individuals representing 65 species were sequenced for this region, with an edited aligned data matrix of 952 characters. The combined nuclear dataset included a total of 90 sequences representing 61 species spanning *ETS* and *ITS*, yielding a matrix of 1249 characters. The combined chloroplast dataset included a total of 106 sequences representing 63 species spanning *psbA-trnH* and *ycf1 3300-4280*, a matrix of 1225 characters. *ETS, ycf1 3300-4280* and *psbA-trnH* aligned data matrix included 99 sequences representing 61 species with 1770 characters. A data matrix with concatenated data of all four regions included 76 sequences representing 52 species, and 2444 characters.

The aligned sequences for individual and combined datasets show several indels and substitutions as informative in that they mostly characterized monophyletic groups. Sequences resulting from *nr*DNA showed the largest variability among individuals with the *ETS* region being the most informative (43% variable). When compared, trees resulting from Bayesian analyses with model parameters defined *a priori* and trees resulting from analyses with flat *priors* showed no difference in topology, hence trees presented here are those derived with *a priori* parameters.

Phylogenies estimated with nuclear data, both individual and combined, showed resolution of relationships among subtribes and some genera. Resolution among species, however, was not observed in many cases, or terminal branches had very little support

(Figs. 1.1-1.3). With 90 individuals in the combined nuclear dataset, there is a grade within the Chrysopsidinae lineage (Fig. 1.3). The lineage is well supported (PP=1.0) and within the clade, *Osbertia* occurs at the base. There is also support for distinct lineages of *Chrysopsis*, *Pityopsis*, *Noticastrum*, *Croptilon* and *Heterotheca*. *Bradburia* is located at the base of the *Chrysopsis* clade (PP=0.78).

Phylogenies estimated with chloroplast data, both individual and combined, showed varying degrees of resolution. The resulting phylogeny of the *psbA-trnH* dataset showed the least amount of resolution among species, with many polytomies (Fig. 1.4). Additionally, the number of variable sites was quite low (13%), with indels serving as the most informative characters. A few lineages did have some support, namely *Heterotheca* section *Ammodia* and *Croptilon* (Fig. 1.4). The phylogeny estimated with *ycf1 3300-4280* was slightly more informative but there was little resolution among species (Fig. 1.5). With 106 individuals in the combined chloroplast dataset, there is support for a monophyletic Chrysopsidinae lineage (PP=0.98), and strong support for lineages of *Bradburia*, *Croptilon*, *Noticastrum* and *Osbertia* (Fig. 1.6). Species of *Chrysopsis* formed a weakly supported clade near the base of the Chrysopsidinae lineage, and one species, *Chrysopsis highlandsensis*, was nested within *Pityopsis*. Within the *Heterotheca* lineage (PP=1.0), *Tomentaurum* was observed at the base. Additionally, *Croptilon* is nested within this lineage. *Heterotheca* section *Ammodia* has strong support within this lineage (PP=1.0), however, there is no resolution among the other sections and species of *Heterotheca*.

Phylogenies were also constructed with three concatenated regions, *ETS*, *ycf1 3300-4280* and *psbA-trnH*, since a large number of sequences were available for these

genes representing a larger number of species (Fig. 1.7). The mixed model analysis with this dataset supports a monophyletic Chrysopsidinae (PP=1.0). Within the Chrysopsidinae, there is support for monophyly of *Bradburia*, *Chrysopsis*, *Croptilon*, *Noticastrum*, *Osbertia* and *Pityopsis*. *Bradburia*, *Chrysopsis* and *Osbertia* are located at the base of the Chrysopsidinae lineage, but the relationship of *Bradburia* and *Osbertia* is weakly supported. The *Heterotheca* lineage has weak support (PP=0.93), and includes *Tomentaurum*. There is resolution among two of the three sections of *Heterotheca*, sections *Ammodia* (at the base of the *Heterotheca* lineage) and *Heterotheca* (nested within the unresolved section *Phyllotheca*). In this analysis, subtribe Boltoniinae is sister to the Chrysopsidinae. Of note, a lineage of subtribe Conyzinae occurs unresolved with two clades of Solidagininae. Subtribe Hinterhuberinae occurs at the base of the phylogeny.

The phylogeny with the best resolution was estimated through analysis of the combined dataset with *ETS, ITS, psbA-trnH* and *ycf1 3300-4280*. This dataset was subjected to Bayesian and Maximum Likelihood analyses. The resulting phylogenies showed little to no difference in topology, particularly in regards to strongly supported lineages. Phylogenies from combined data support Chrysopsidinae as monophyletic, PP=1.0, BS=100 (Fig. 1.8). Since, *Ericameria* was selected as outgroup for the analysis of this dataset, subtribe Hinterhuberinae appears at the base of the phylogeny. Two distinct clades of Solidagininae also appear near the base of the phylogeny. One lineage of Solidagininae (PP=1.0, BS=100) includes *Columbiadoria* as the sister taxon of two species of *Chrysothamnus*. The other lineage of Solidagininae with *Euthamia* and *Bigelowia* (PP=1.0, BS=100), appears to be associated with *Erigeron*, a genus in subtribe

Conyzinae. There is some support for the hypothesized sister relationship of subtribe Boltoniinae to the Chrysopsidinae (PP=0.93, BS=86). *Osbertia* (3 of 3 known species examined) is the sister group to the rest of the Chrysopsidinae, PP=1.0. There is resolution among other genera of the Chrysopsidinae, including *Bradburia* (1 of 2 species examined), *Chrysopsis* (4 of 11 species examined), *Pityopsis* (5 of 7 species examined), *Noticastrum* (3 of 20 species examined), *Croptilon* (3 of 3 species examined) and *Heterotheca* (20 of 24 species examined). *Tomentaurum niveum* is unresolved in a lineage consisting of species of *Heterotheca*. There are also strongly supported lineages of *Heterotheca* section *Heterotheca* and section *Ammodia*, but section *Phyllotheca* is unresolved. *Heterotheca* section *Heterotheca* was nested within section *Phyllotheca.*

Morphological characters used for delimiting genera and species of this subtribe were traced on Figure 1.9 (Bayesian analysis of all four DNA regions). These characters included capitulescence, presence of ray florets, color of ray florets, venation patterns of leaves, glands on phyllaries, pappus series, presence of scaled pappus and ribs on cypsela. These traits were scored on the Bayesian phylogram based on estimates by MESQUITE 2.75. Among the eight characters studied, varying levels of changes were observed.

DISCUSSION

Monophyly of subtribe Chrysopsidinae

There was strong support for the lineage of Chrysopsidinae (Fig. 1.8, PP=1.0, BS=100). All species representing genera of Chrysopsidinae *sensu* Semple that were included in this study appeared within this lineage. Although subtribe Chrysopsidinae has

been generally accepted and reported as a monophyletic group (Nesom 1991b, 2000; Semple 2006; Nesom and Robinson 2007) prior to this study, the hypothesis of relationships has not been tested using molecular data. This study provided further evidence supporting Nesom and Robinson's (2007) most recent classification for the group.

Most lineages of Chrysopsidinae, with the exception of *Chrysopsis* (confined to United States), *Noticastrum* (confined to South America) and *Osbertia* (confined to Central America), are composed of both Central and North American species. This pattern, while not unique, supports the general trend of New World Astereae biogeography, radiation northwards from South America.

Relationships within Chrysopsidinae

All known species of *Osbertia* were included in this study and formed a monophyletic lineage (PP=1.0, BS=100). *Osbertia stolonifera* and *O. chihuahuana* were sister taxa of *O. bartlettii* at the base of this clade. While there is confusion in the study by Lane et al. (1996), our data showed that Nesom's (1991b, 1991c) inclusion of *Osbertia* in Chrysopsidinae is justified. Similar support was seen with a larger number of individuals and species in the analysis of three gene regions (Fig. 1.7). In their study of the systematics of *Osbertia*, Turner et al. (1986), hypothesizes *Osbertia* is a close relative of *Noticastrum*, *Chrysopsis*, and *Heterotheca*. They also describe morphological similarities among species of *Osbertia* with those of *Erigeron*, contending *Osbertia* as a "remnant of the ancestral stock of Astereae." *Osbertia* was certainly a sister group to the rest of the Chrysopsidinae but our results did not support a basal position in the tribe Astereae. *Osbertia* is native to Mexico and sympatric with some species of *Heterotheca*.

Also observed was an association between *Osbertia* and *Bradburia* in analyses excluding *ITS* sequence data (Fig. 1.7). Nesom's (2000) hypothesis of *Osbertia*'s close relation with *Chrysopsis* was supported by the analysis of all four DNA regions (Fig. 1.8).

Chrysopsis, a lineage with strong support (PP=1.0, BS=99), included *C. mariana*, *C. highlandsensis*, *C. gossypina* and *C. linearifolia*. The results of this investigation supported Semple's (2006) hypothesis about the composition of *Chrysopsis*. Most species of *Chrysopsis* are distributed in the southeastern United States and all but *C. gossypina* and *C. mariana* are restricted to Florida. Semple's (1981) revision of *Chrysopsis* describes much of the confusion associated with the taxonomy of this genus and its elevation from a section within *Heterotheca* to generic rank. He also proposes that the ancestor of this genus most likely originated in the Mexico-Texas region, and that geographic isolation and adaptation to various habitats were key factors in its speciation. In this study, there was insufficient resolution of relationships among species of *Chrysopsis*. The known species of *Chrysopsis* have been reported to hybridize despite the distinct morphology among taxa (Semple 1981), indicating these taxa may be genetically very close. There was evidence of reticulation based on *cp*DNA data with *Chrysopsis* and *Pityopsis* (Fig. 1.6), suggesting shared maternal ancestry among these genera. Semple (1981) indicated *C. pilosa* as sister to *C. mariana*, a hypothesis later revised when *C. pilosa* was classified as *Bradburia pilosa* the sister species of *B. hirtella*. There was insufficient support for this hypothesized sister relationship of *C. mariana* to *B. pilosa*.

Nesom's (1991a, b) merger of *Bradburia* with *Chrysopsis* could not be supported by our findings. *Bradburia*'s relationship with other genera of the Chrysopsidinae appeared ambiguous (Fig. 1.8). Our data suggested that Semple's (1996) hypothesis of

generic rank for *Bradburia* may be supported. While unable to include all known species of *Bradburia* in the combined analysis of all four DNA regions, the analysis of *nr*DNA *ETS* data, which included both known species of *Bradburia*, supported a monophyletic *Bradburia* lineage (Fig. 1.1; PP=1.0). Furthermore, *Bradburia* was closely aligned with *Osbertia* based on phylogenetic analysis the two *cp*DNA regions combined with *nr*DNA *ETS* (Fig. 1.7). In the analyses of all four DNA regions, *B. pilosa* showed weak association with the *Pityopsis* lineage, however, it can be argued that *Chrysopsis* was just as close a relative. While Semple (2006) speculated that *Bradburia* may have derived from either *Chrysopsis* or *Croptilon*, this close association with *Osbertia* (Fig. 1.7) and *Chrysopsis* (Fig. 1.8) might be indicative of shared ancestry. *Bradburia* is native to the southeastern United States (Texas to Tennessee), but does not occur in Florida. However, it is quite possible that the last common ancestor of *Osbertia*, *Bradburia* and *Chrysopsis* radiated from Mexico northwest towards Florida resulting in these three genera.

In most previous studies, *Pityopsis* was generally accepted as a close relative of *Heterotheca*. More recently, Nesom (1991a) and Semple (1996) proposed it is sister to *Croptilon*. This latter hypothesis was not supported in the current study. The *Pityopsis* lineage (PP=1.0, BS=100) had two distinct clades with strong support – *P. pinifolia* and *P. ruthii* forming a lineage sister to *P. aspera*, *P. oligantha* and *P. graminifolia* (Fig. 1.8). This was consistent with the hypothesis of clade division within *Pityopsis* in Toah's (2008) thesis, where *P. falcata*, *P. pinifolia* and *P. ruthii* form the Ruthii clade, and the remaining species of *Pityopsis* form the Flexuosa clade. The combined molecular analysis placed *P. graminifolia* and *P. oligantha* as sisters taxa and *P. aspera* at the base of the Flexuosa clade (Fig. 1.8). *Pityopsis* are found in the eastern United States and

Central America, many species are abundant throughout the region of distribution. However, *P. ruthii* is rare and found only in Tennessee. This lineage, as previously discussed, was more closely aligned with *Bradburia* and *Noticastrum*.

Noticastrum is the only genus among the Chrysopsidinae native to South America. Little work has been done on this genus since Zardini's (1985) contributions. Study of specimens of this genus proved to be quite difficult due to limited availability of recently collected specimens in the United States. Of the three species examined in the combined data analysis, there was some resolution of relationships among species, though with low support (Fig. 1.8). A larger number of individuals were sampled for *ETS* sequences representing eight species, and analysis of *ETS* data showed some resolution among species (Fig. 1.1). The genus was nested between genera that occur in Central and North America only (Fig. 1.8). The inclusion of *Noticastrum* within the Chrysopsidinae based on morphological criteria was further supported by the molecular data presented here. Furthermore, the biogeographic relationship of *Noticastrum* as compared to other genera of Chrysopsidinae was consistent with the observations for tribe Astereae (Brouillet et al. 2009) – a movement of South American Astereae to North America then recolonization in South America.

The *Croptilon* and *Heterotheca* clades were sister lineages. Thus, Nesom's (1991) hypothesis of *Croptilon's* relationship with *Pityopsis* was not supported by this study. With all three known species included in this study, relationships among species were not well supported (Fig. 1.8). The findings of this study suggest a close relationship of *C. divaricatum* and *C. hookerianum*, however, the support for this relationship is weak (PP=0.52, BS=95). Analyses including more individuals of each species have revealed

little to help resolve relationships among species of *Croptilon* (Fig. 1.1, 1.4). *Croptilon rigidifolium* is native to Mexico and Texas while the other species are found in southeastern United States only.

The *Heterotheca* lineage was strongly supported (PP=1.0, BS=97). This genus has been extensively studied by many and there have been varying opinions related to the relationships among its species (Semple 1996 and citations within). This study, unfortunately, was unable to find genes that were sufficiently variable to resolve species relationships. There were, however, many revelations based on these analyses (Fig. 1.8). *Tomentaurum niveum* occurred within the *Heterotheca* lineage unresolved with *H. viscida*, both of which are found in Chihuahua, Mexico. *Heterotheca viscida* is also found in several southern states in the United States. Turner (1987) placed *Tomentaurum* in *Heterotheca*, a decision challenged by Nesom (1991d). Nesom's (1991d) classification of the genus is based on morphological differences in pubescence, habit, leaf morphology and capitula. Many of the "unique" characters found in *Tomentaurum*, though not present as a suite in any single species of *Heterotheca*, were found individually among species of *Heterotheca*. Based on molecular data, if *Tomentaurum* maintains generic rank, it might be argued that *H. viscida* must also be elevated to this rank. Simple (1996) stated that if *Tomentaurum* (formerly *H. vandevenderorum*) is retained in *Heterotheca* "an entirely new way to delimit the genus [*Heterotheca*]" is necessary. As a result of findings in this study, the evidence for elevation of *H. viscida* to genus rank was unequivocal.

Species of *Heterotheca* are usually divided among three sections. Our study provided some support for this sectional classification. Section *Ammodia*, consisting of varieties of *H. oregona*, was strongly supported (PP=1.0, BS=100). Section *Heterotheca*

also had strong support (PP=1.0, BS=100), but this lineage was nested within the unresolved clade of section *Phyllotheca*. Among species in section *Heterotheca*, there was strong support for a lineage with *H. subaxillaris* (PP=0.95, BS=77). However, *H. inuloides* appeared to be polyphyletic and *H. grandiflora* is unresolved among species of *H. inuloides*. The relationships of most species within section *Phylloteca* was ambiguous. Also in this clade, there was strong support (PP=0.97, BS=55) for a lineage with *H. shevockii* (rare native of California found at lower elevations) and *H. jonesii* (native to Utah). Both of these species occur sympatrically with varieties of *H. villosa*. The relationship of section *Phyllotheca* and section *Heterotheca* was inconsistent with Semple's (1996) study of *Heterotheca.*

Subtribal Affinities of Chrysopsidinae

Outgroup taxa were selected based on analysis of nuclear data from close and distant relatives of the Chrysopsidinae. Preliminary analysis with *Pseudognaphalium* (*nr*DNA, *ETS* and *ITS* sequences from GenBank®) provided the basis for outgroup selection (Figs. 1.1-1.3). In our analysis of the outgroup taxa using data from *nr*DNA, we observed two distinct groups of Hinterhuberinae (Figs. 1.1-1.3). Species of *Hinterhubera* and *Blakiella* (both South American (SA) Hinterhuberinae) had strong support (PP=1.0). Interestingly, the lineage of North American (NA) Hinterhuberinae was more closely associated with Solidagininae than to SA Hinterhuberinae (Fig. 1.3). Among the NA Hinterhuberinae, *Ericameria parishii* was sister taxon of *E. pinifolia* and *E. cuneata* occurs at the base of this lineage (Fig. 1.3), supporting a similar observation by Roberts and Urbatsch (2003).

Ericameria served as the assigned outgroup for Bayesian analysis of combined *nr*DNA and *cp*DNA data (Fig. 1.8). Two distinct clades of Solidagininae were observed, similar to findings in Funk et al. (2009). In one lineage of subtribe Solidagininae (PP=1.0, BS=100), *Columbiadoria hallii* was the sister taxon of *Chrysothamnus*. *Bigelowia* and *Euthamia* formed the other lineage of Solidagininae (PP=1.0, BS=100). *Erigeron strigosus*, representing subtribe Conyzinae appeared to be within this lineage of Solidagininae. It appears that subtribe Solidagininae, as currently defined is polyphyletic and warrants a closer evaluation. Also, there is need for extensive sampling and investigation of the relationship of Conyzinae and Solidagininae. This study provided support for the sister relationship of Boltoniinae to Chrysopsidinae (PP=0.93, BS=86).

Morphological Character Evolution

The present study supported the hypothesis of a monophyletic Chrysopsidinae based on morphological criteria (Fig. 1.8). An evaluation of the evolutionary history of several morphological characters commonly used to delimit species and genera revealed some common patterns of inheritance, several traits displayed multiple independent origins (Fig. 1.9). Pappus traits are widely used as key characters among the Chrysopsidinae and are considered to be among the most important diagnostic features. Semple (2006), however, concedes that pappus alone is not useful in diagnosing individual genera. Two pappus traits were traced across the phylogeny (Fig. 1.9). Among the Chrysopsidinae, multiseriate pappus occurred in the lineages following divergence from *Osbertia.* Uniseriate pappus occurred as an independent event in the *Croptilon* lineage. A similar event appeared to have resulted in uniseriate pappus in *Heterotheca* section *Ammodia.* Another pappus trait ‘scaled pappus’ was also traced on the phylogeny.

Scales were first observed in the ancestral population after the divergence of the rest of the Chrysopsidinae from the *Osbertia* lineage. Within the Chrysopsidinae, this feature was lost independently in *Croptilon* and *Heterotheca* section *Ammodia*. Some species of outgroup lineage *Erigeron*, represented in this phylogeny by *E. strigosus*, also had scales. Species of *Croptilon* and *Heterotheca* section *Ammodia* share both uniseriate and bristle-like pappus traits, important identifying features for the lineages. Ribs on the cypsela, is another important diagnostic character among taxa within Chrysopsidinae. All species of Chrysopsidinae have ribs, however, some are more pronounced and colorful. Among the taxa in this study, two independent events resulted in the conspicuous ribs that are observed in *Chrysopsis* and *Noticastrum macrocephalum*. *Chrysopsis highlandsensis* appeared to have lost the pronounced ribs. Cypsela ribs, though useful for confirming identification of a few species, appeared to be of limited use in the identification of monophyletic lineages of Chrysopsidinae.

Among floral characters, solitary capitulescence evolved independently in lineages of *Noticastrum*, *Osbertia* and *Tomentaurum*. *Heterotheca brandegei* and *Ericameria suffructicosa* are also known to have solitary capitula, but they were not included in this study. Of note here was that all of these taxa of Chrysopsidinae with solitary capitula occur either in Central America or South America; these taxa do not share similar habitats and they are not reported to share similar pollination strategies. The capitula of all species of Chrysopsidinae have ray florets except for *Heterotheca oregona* (the only species in *H.* section *Ammodia*). The absence of this feature is shared with species of *Chrysothamnus* and *Bigelowia* members of the outgroup lineage. Nesom (1994) noted that subtribe Chrysopsidinae is among the few subtribes of tribe Astereae

that have “primitive yellow rays.” When tracing this character, we observed the independent loss of this character in the ingroup genus *Noticastrum*. The outgroup lineage *Erigeron* also has white ray florets.

The presence of trichomes on the phyllaries within Chrysopsidinae can be useful in delimiting many species or varieties; but rarely is this character suitable for delimiting genera. There seemed to be no discernable pattern to the evolution of glandular or stipitate-glandular trichomes. When mapped on the phylogeny independent origins of these characters were observed. With reference to leaf venation pattern, the character state of parallel venation on the leaves appeared as a derived character, observed only in the *Pityopsis* lineage. The uniqueness of *Heterotheca* section *Ammodia* was also conspicuous in this analysis of characters. Unlike the rest of *Heterotheca*, this lineage was characterized by a large number of ancestral characters. While all of these characters are used to delimit genera and species, there seems to be very little similarity in their pattern of evolution. This observation was confounded by what appears to be little evidence of a system driven by common environmental factors; species with a particular character appear to have disparate distribution. It is highly likely that microhabitat features and parallelism might be the determining forces in this system. Additionally, little is known about the pollination strategies or any other factors that may be driving the evolution of these traits. A more comprehensive study, at the tribal or subfamily level, may shed light on the evolution of traits in this group of plants.

Conclusion

The most comprehensive assessment of the status of Astereae, its constituent subtribes, its biogeography and character evolution has been informed by *nr*DNA *ITS*

data (Brouillet et al. 2009). The current study explored the utility of a combination of *nr*DNA (*ETS, ITS*) and *cp*DNA (*ycf1, psbA-trnH*) data to address these similar questions in subtribe Chrysopsidinae and found that these data were sufficient for resolving evolutionary relationships among genera of Chrysopsidinae. Additionally, this study provided evidence for new subtribal classification for *Ericameria* and the lineage composed of *Euthamia* and related genera.

Findings in this study unequivocally supported the monophyly of subtribe Chrysopsidinae. Urbatsch et al. (2003) reported a close relationship among subtribes Conyzinae and Chrysopsidinae with *nr*DNA sequence data. This study shed more light on the relationship of Chrysopsidinae to other Astereae subtribes. Lineages of *Bradburia*, *Chrysopsis*, *Croptilon*, *Heterotheca*, *Noticastrum*, *Osbertia* and *Pityopsis* were also strongly supported. While all known species of subtribe Chrysopsidinae were not included, there was sufficient evidence to support the current composition of most genera in this group *sensu* Semple. This investigation has confirmed relationships among genera but, in some cases, there was insufficient resolution to meaningfully discuss relationships among species. There was evidence for the possible radiation from South to North America among genera of Chrysopsidinae, much like the *ITS* based studies supporting the hypothesized radiation at the tribal level reported in Brouillet et al. (2009). The most likely reason for the lack of resolution among species is the relative recency of the radiation of the subtribe. A need for validation through use of additional molecular data was expressed by Brouillet et al. (2009), particularly with regard to subtribal composition and relationships. While this study added information on the Chrysopsidinae, there is still a need to evaluate other subtribes using molecular data, as a step in assessing

relationships within tribe Astereae, providing a framework for the evaluation of character evolution and the biogeography.

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Chapter 2

Techniques for improving the quality and quantity of DNA extracted from herbarium specimens

ABSTRACT

There is a need to modify DNA extraction methods to obtain amplifiable DNA from herbarium specimens that are relatively old — we explored several methods to improve both quality and quantity of DNA obtained from a range of specimens of Asteraceae. Leaf tissue was sampled from herbarium specimens of varying ages. Modifications were made to the Qiagen DNeasy® Plant Mini Kit and the CTAB Extraction protocols. Comparison of the results of both protocols for recently collected and older specimens demonstrated that better quality and greater quantity of DNA was obtained with the modified DNeasy® protocol. The modified DNeasy® protocol was more consistent in yielding amplifiable DNA from herbarium specimens older than 20 years. Modification and optimization of two currently used DNA extraction protocols were successful in yielding quality amplifiable DNA from herbarium specimens of Asteraceae, tribe Astereae, that were up to 127 years old. The concentration and quality of DNA was comparable to that obtained from specimens less than 20 years old.

INTRODUCTION

The use of natural history resources in phylogenetics, biogeography, and population biology, among other areas of biodiversity research, justifies investments in their security and accessibility by federal, state, and private funding agencies. Among the collections supported by these agencies are herbaria, repositories of millions of plant specimens, representing documented snapshots of plant diversity and distribution in

space and time. Many have called for increased use of natural history resources for research into species discovery. It is proposed that large numbers of unknown and/or undescribed species are part of these collections (Bebber et al. 2010). In addition to prospects of new species discovery, it is argued that these resources should be utilized in molecular-based studies, justifying financial and time investments in them (Drábková et al. 2002), while protecting wild populations from further degradation. However, while many institutions house valuable plant resources, seldom are researchers able to utilize older plant specimens for molecular investigation. A major obstacle is the securing of high quality, amplifiable DNA from older herbarium specimens (Telle & Thines 2008). Molecular studies, particularly among taxa of Asteraceae – the largest family of flowering plants – can benefit from the use of herbarium resources specifically in cases where taxa are rare or their distribution remote. Also, use of these resources supports current and future investments in them and contributes to the conservation and protection of wild populations. However, despite the abundance of resources in plant collections, researchers are usually restricted to using more recently collected specimens, those collected within the last 20 years. For older specimens, the limiting factor is obtaining high quality, amplifiable DNA.

The problems encountered when using older herbarium specimens for molecular studies stem primarily from specimen collection and processing practices. Post collection processing of specimens, including alcohol treatment, time between collection and drying, and method of drying can have adverse effects on the quantity and quality of DNA obtained (Staats et al. 2011). In addition, the age of the specimens (time since collection) may impede DNA retrieval due to natural DNA degradation over time (Staats

et al. 2011). This combination of natural DNA degradation and post collection processing practices that accelerate DNA degradation compounds the problem encountered when older plant specimens are used. Damaged or degraded DNA hinders PCR-based molecular studies and next-generation sequencing (Lindahl 1993). Obtaining quality DNA from old herbarium specimens usually require several modifications to commonly used protocols (Drábková et al. 2002 and citations therein). Drábková et al. (2002) recommended the use of Qiagen DNeasy® Plant Kit or the extraction methods from Doyle and Doyle (1987, 1990) as the most satisfactory extraction methods, albeit for specimens of graminoids only. The resulting extracts from these protocols must also be purified before they are used as PCR template. These extracts did not, however, consistently produce amplicons that were larger than 350 bp. Because DNA extraction requires the destructive sampling of preserved plant specimens, it is imperative that the extraction techniques employed result in high quality amplifiable DNA, reducing the frequency of sample removal from specimens, thus preserving the specimens for posterity. In addition to the challenges outlined above, products of plant secondary chemistry, concentrated by the drying of plant tissues, may have a greater impact on the quality of DNA recovered from older herbarium specimens. These secondary compounds can impede downstream use of extracted DNA as they inhibit reactions and processes in which the DNA extract is utilized.

Here we address some of the issues associated with obtaining high quality, amplifiable DNA from older herbarium specimens of Asteraceae taxa. We discuss modifications to two commonly used extraction protocols and their utility among herbarium specimens of varying ages. The modifications employed are relatively cheap

and easy to implement, without substantial increases in processing time. Specifically, we utilized strategies to increase the concentration of DNA while reducing the levels of impurities in the DNA extracted from herbarium specimens that are older than 20 years.

MATERIALS AND METHODS

Plant samples for this study were obtained from herbarium specimens. Sampled taxa included species from Asteraceae, tribe Astereae (Appendix 1). The collection year of specimens ranged from 2013 to 1887, 0–127 years old (at the time of this study). Two methods of DNA extraction were explored and modified several times to optimize for DNA quantity and quality. Samples of desiccated leaves collected close to the apex of specimens were stored in silica gel prior to DNA extraction. For both methods of extraction, 20-40 mg of leaf tissue was pulverized with a Mini-Beadbeater-8 (Bio Spec Products Inc. Bartlesville, OK).

Optimization of the hexadecyltrimethylammonium bromide (CTAB) method (modified from Doyle and Doyle 1987 & 1990) involved reduction of the volume of all reagents to facilitate the use of micro-centrifuge tubes, a useful though not novel strategy. To the pulverized tissue, 600 µL of 2X CTAB and 20mg polyvinyl polypyrolidone (PVP) were added and mixed by vortexing. The samples were incubated for 1 hr 30 min at 70°C, ensuring a more thorough lysis of cells to increase quantity of DNA obtained from the tissue. Following incubation, 600 µL chloroform:isoamyl alcohol (24:1) was added to the samples and mixed. Samples were centrifuged for 20 min at 13,000 RPM. The top aqueous phase was collected for DNA isolation while discarding pellets with cellular debris. In order to degrade RNA, 2 µL of RNaseA (New England BioLabs Inc., Ipswich,

MA) was added to samples and incubated for 30 min at 37°C. DNA from samples was precipitated with 540 µL of isopropanol (-20°C), incubated for 15 min at 25°C, then centrifuged for 4 min at 13,000 RPM. Pelleted DNA was washed with 500 µL of 75% ethanol, centrifuged for 4 min at 13,000 RPM and dried for 5 min in a Savant SpeedVac® (Thermo Fisher Scientific Inc., Maddison, WI). Dried pellets were resuspended in 80 µL 1X TE buffer (10mM Tris pH 7.4, 1mM EDTA). To this, 8 µL of 7.5M ammonium acetate and 180 µL of 100% ethanol were added and mixed. This mixture was incubated for 30 min at 25°C then centrifuged for 4 min at 13,000 RPM. Pellets were isolated and washed with 500 µL of 75% ethanol, centrifuged for 4 min at 13,000 RPM, then dried in a SpeedVac® for 5 min. The pelleted-DNA was resuspended in 100 µL 1X TE and stored at -20°C.

DNA was also extracted from pulverized leaf tissue using the Qiagen DNeasy® Plant Mini Kit and protocol. The protocol was modified and optimized for extraction of DNA from recently collected and ancient herbarium specimens. Pulverized tissue was mixed with 450 µL of Buffer AP1, 4 µL of RNaseA and mixed vigorously by vortexing. The mixture was then incubated for 60 min at 70°C to lyse the cells. After lysing, 130 µL of Buffer AP2 was added and the lysate incubated for 60 min at 4°C to precipitate detergent, proteins and polysaccharides. After this precipitation step, the DNeasy® protocol was followed as prescribed by the manufacturer until the DNA elution step. To elute the DNA 50 µL of Buffer AE was added to the spin column followed by an extended incubation time of 10 min, instead of the recommended 5 min. This step was repeated to reach final volume of 100µL.

DNA quality and concentration were evaluated using a NanoDrop Lite (NanoDrop Products, Wilmington, Delaware). We recorded A260/A280 ratios as indicative of DNA purity (1.7-1.9 indicating pure DNA) and the concentration of DNA in each sample. Extracted DNA samples were stored as stock solutions at -20°C. Subsequently, working solutions were prepared through serial dilutions for use as template in polymerase chain reactions (PCR). PCR utilized nuclear ribosomal DNA (nrDNA), spanning external transcribed spacer (ETS) and internal transcribed spacer (ITS) and chloroplast DNA, spanning psbA-trnH and ycf1 3300-4280 for amplification of DNA. In order to access the effectiveness of these extracts in amplifying DNA we used regions of variable lengths, 350-550 bp in ETS, 600-900 bp in ITS, 250-400 bp in psbA-trnH and 700-1000 bp in ycf1 3300-4280.

Results and Discussion

Extracts obtained using the unmodified CTAB protocol produced high concentrations of DNA, on average 234 ng/µL (mean A260/A280 ratio = 1.63). However, we were unable to obtain amplicons with these extracts when used as PCR template. The same taxa were sampled using the unmodified DNeasy® protocol, which produced on average 92.5 ng/µL (mean A260/A280 ratio = 1.24). These extracts also failed to produce amplicons for most DNA regions tested. Extracts obtained from modifications to the CTAB protocol displayed 519.8 ng/µL mean DNA concentration and mean A260/A280 ratio of 1.32. When the samples were subjected to the modified DNeasy® protocol the DNA concentration of the extracts was on average 87.9 ng/µL (mean A260/A280 ratio = 1.38). Comparisons of extracts obtained from the modified DNeasy® and CTAB protocols showed that extracts obtained from the modified

DNeasy® protocol were more consistent in amplicon production when used as PCR template. This was the case despite the tendency of the modified CTAB protocol to yield higher concentrations of DNA compared to the modified DNeasy® protocol. As a result, we continued to refine and optimize the DNeasy® protocol for extraction of DNA from older herbarium specimens, resulting in amplifiable DNA from specimens collected 127 years ago (Table 2.1).

The optimized DNeasy® protocol consistently resulted in amplifiable DNA in more cases than the CTAB protocol. In particular, regions of chloroplast DNA extracted using the modified DNeasy® protocol could be amplified from specimens that were over 100 years old. We recovered, on average, 60 ng/µL of DNA (mean A260/A280 ratio = 1.54) using the modified DNeasy® protocol (Table 2.1). The total time required for this method increased to approximately 3 hr compared to the original 1 hr. We recovered an average of 406 ng/µL of DNA (mean A260/A280 ratio = 1.4) using the modified CTAB method, and decreased time required for this protocol from ~8 hr to <4 hr (Table 2.1). The quality of DNA extracted using the modified DNeasy® protocol were significantly better than those from the modified CTAB extractions ($p=0.048$), where seven of the nine sampled taxa produced DNA of higher purity (Table 2.2).

The potential benefits of the extracting DNA from old herbarium specimens are great, as this adds to the value of millions of specimens currently housed in natural history collections. We have increased both the quantity and quality of DNA extracted from herbarium specimens of Asteraceae dating up to 127 years old, a large improvement when compared to the oldest specimen, 71 years, in Drábková et al. (2002). We increased DNA concentration using DNeasy® Plant Mini Kit by modifying lysis time for cells, the

volume of Buffer AP1 used per reaction, and increased incubation time prior to DNA elution. These modifications also resulted in increased DNA purity (Table 2.2). Although the overall time required for this protocol has increased, we believe that the yield of amplifiable DNA from relatively old herbarium specimens outweighs the increase in extraction time.

While Drábková et al. (2002) suggested their methods as useful for specimens of graminoids, we have demonstrated a wider application of these extraction methods for specimens of Asteraceae, which have vastly different secondary chemistry and anatomy. We have also reduced the amount of material needed for extractions (between 20-40 mg instead of 100-500 mg). Additionally, DNA extracts from the same specimen or related taxa, ranging from 0 to 127 years old, were used to evaluate the efficacy of the modified and optimized DNeasy® protocol. Interestingly, unlike findings in Drábková et al. (2002), our extracts resulted in successful amplification of DNA from both nuclear and chloroplast regions, ranging from 350 to 1000 bp. These modified protocols were also successful when used for recently collected specimens, increasing the range of specimens that can be harnessed for molecular studies.

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Table 1.1.

Species Name	Collector	Locality	Herbarium
Amphiachyris dracunculoides	Ballester 10	DE: Brazos Co.	DOV
Bigelowia nudata	Kral 90535	AL: Washington Co.	DOV
Bigelowia nuttallii	Demaree 46288	AL: DeKalb Co.	DOV
Boltonia asteroides	McAvoy 5306	DE: Sussex Co.	DOV
Boltonia diffusa	Bryson 18882	MS: Jones Co.	DOV
Bradburia hirtella	McCrarry 72	TX: Nacogdoches Co.	BALT
Bradburia hirtella	Fisher 5008	TX: Houston Co.	US
Bradburia hirtella	Lewis 7658	TX: Fayette Co.	US
Bradburia hirtella	Jones & Jones 455	TX: Brazos Co.	US
Bradburia pillosa	Mcgregor 15020	KS: Chautauqua Co.	US
Bradburia pillosa	Cory 56944	TX: Morris Co.	US
Bradburia pilosa	Allen 8361 Vincent 1685	LA: Union Parish	BALT
Bradburia pilosa	Thomas & Thomas	TX: Smith Co.	TENN
Bradburia pilosa	Estes 2842	TN: Giles Co.	TENN
Chrysoma pauciflosculosa	Bates 8851	GA: Wheeler Co.	LSU
Chrysopsis delaneyi	Wunderlin & DeLaney 10736	FL: Martin Co.	US
Chrysopsis delaneyi	DeLaney 4081	FL: Polk Co.	US
Chrysopsis delaneyi	Delaney 4076A	FL: Martin Co.	US
Chrysopsis floridana	Grey 94-124	FL: Hillsborough Co.	US
Chrysopsis floridana	Dress 10350	FL: Hillsborough Co.	US
Chrysopsis floridana	Wunderlin 5658	FL: Hillsborough Co.	LSU
Chrysopsis godfreyi	Uhler sn.	FL: Okaloosa Co.	US
Chrysopsis godfreyi	Harper 4228	AL: Balwin Co.	US
Chrysopsis gossypina	Pittillo 2807	GA: Burke Co.	BALT
Chrysopsis gossypina	Rothwell C-60	NC: Jones Co.	BALT
Chrysopsis gossypina	Longbottom & Williams 4667	SC: Kershaw Co.	DOV
Chrysopsis highlandsensis	DeLaney 5113	FL: Polk Co.	US
Chrysopsis highlandsensis	DeLaney 4065	FL: Polk Co.	US
Chrysopsis lanuginosa	Godfrey 83616	FL: Liberty Co.	US
Chrysopsis lanuginosa	Godfrey 61630	FL: Bay Co.	US
Chrysopsis lanuginosa	McDaniel 7092	FL: Calhoun Co.	TENN
Chrysopsis latisquamea	Godfrey et al. 53960	FL: Madison Co.	TENN
Chrysopsis latisquamea	O'Neill sn.	FL: Orlando Co.	US
Chrysopsis latisquamea	Pollard 10358	FL	US
Chrysopsis linearifolia	King & Gholson Jr. 10306	FL: Liberty Co.	US
Chrysopsis linearifolia	Godfrey 83581	FL: Wakulla Co.	US

Chrysopsis linearifolia ssp. linearifolia	Davis & Davis 15565	FL: Walton Co.	BALT
Chrysopsis mariana	Redman 1986	MD: Baltimore Co.	BALT
Chrysopsis mariana	D'Arcy 2219	FL: Alachua Co.	BALT
Chrysopsis mariana	Strong 3787	FL: Marion Co.	US
Chrysopsis mariana	Michael s.n.	NC: Durham Co.	BALT
Chrysopsis mariana	Redman 7134	MD: Baltimore Co.	BALT
Chrysopsis scabrella	DeLaney 3003	FL: Citrus Co.	US
Chrysopsis scabrella	Godfrey 83657	FL: Taylor Co.	US
Chrysopsis scabrella	Ward & Ward 1807	FL: Collier Co.	US
Chrysopsis scabrella	Kral 59244	FL: Marion Co.	TENN
Chrysopsis scabrella	Kral 59260	FL: Marion Co.	TENN
Chrysopsis subulata	Strong 2844	FL: Lake Co.	US
Chrysopsis subulata	Kral 7672	FL: Marion Co.	US
Chrysopsis subulata	Fishbein 4778	FL: Osceola Co.	LSU
Chrysopsis subulata	Kral 52717A	FL: Polk Co.	TENN
Chrysothamnus greenei	Urbatsch & Eifer 1329	CO: Mt. Rose Co.	LSU
Chrysothamnus viscidiflorus ssp. *lanceolatus*	Urbatsch 7063	WY: Lincoln Co.	LSU
Columbiadoria hallii	Urbatsch 7692	OR: Wasco Co.	LSU
Conyza canadensis	Costa & Roberts 2013-002	MD: Baltimore Co.	BALT
Conyza canadensis	Ahles 81272	MA: Hampshire Co.	BALT
Conyza canadensis	Pancer-Kotejowa sn.	Poland	BALT
Conyza canadensis	Henrickson 5296	CA: Orange Co.	BALT
Conyza crispa	Pereira 58	Portugal	BALT
Conyza ramosissima	Evers 106608	IL: Whiteside Co.	BALT
Croptilon divaricatum	Allen 8357 Vincent 1682	LA: Union Parish	BALT
Croptilon divaricatum	Herndon 2971	FL: Leon Co.	LSU
Croptilon divaricatum	Chapman sn.	FL	DOV
Croptilon hookerianum	Higgins 6259	TX: Cottle Co.	BALT
Croptilon hookerianum	Raven & Gregory 19449	TX: Torrant Co.	US
Croptilon hookerianum	Horr 3531	KS: Meade Co.	US
Croptilon hookerianum	Carleton 292	KS: Reno Co.	DOV
Croptilon rigidifolium	Palmer 475	TX: Aransas Co.	US
Croptilon rigidifolium	Lynch & Kutac 7847	TX: Lee Co.	LSU
Croptilon rigidifolium	Lievens 82TX66	TX: Alascosa Co.	LSU
Croptilon rigidifolium	O'neill 30	TX: Grimes Co.	LSU
E. cuneata ssp. *spathalata*	Urbatsch 7092	CA: Riverside Co.	LSU
Ericameria brachylepis	Urbatsch 7090	CA: San Diego Co.	LSU
Ericameria parishii	Urbatsch 7082	CA: San Diego Co.	LSU
Ericameria pinifolia	Urbatsch 7084	CA: San Diego Co.	LSU
Erigeron philadelphicus	Redman 6098	MD: Harford Co.	BALT
Erigeron pulchellus	Redman 11053	MD: Baltimore Co.	BALT

Erigeron pulchellus	Michael 89	NC: Orange Co.	BALT
Erigeron strigosus	DeWitt 2010-24	MD: Harford Co.	BALT
Erigeron strigosus	Ahles 85417	MA: Hampshire Co.	BALT
Erigeron untermannii	Atwood & Furniss 29666	UT: Duchesne Co.	BALT
Euthamia caroliniana	McAvoy 4586	MD: Wicomico Co.	DOV
Euthamia graminifolia	Naczi 8917	DE: Kent Co.	DOV
Euthamia sp.	Raven & Gregory 19298	TX: Garza Co.	US
Heterotheca brandegei	Thorne et al. 60870	Mexico: Baja California	TEX/LL
Heterotheca brandegei	Moran & Thorne 14257	Mexico	TEX/LL
Heterotheca camporum var. *camporum*	Semple et al. 9902	MO: Wayne Co.	BALT
Heterotheca camporum var. *glandulissimum*	Semple et al. 10178	AL: Colbert Co.	BALT
Heterotheca canescens	Semple & Love 236	OK: King Fisher Co.	BALT
Heterotheca canescens	Nighswonger & Wilson 700	OK: Woods Co.	BALT
Heterotheca canescens	Semple & Shea 1538	TX: Brown Co.	BALT
Heterotheca fulcrata var. *fulcrata*	Simple 2707	CO: Jefferson Co.	BALT
Heterotheca fulcrata var. *fulcrata*	Semple & Heard 8149	NM: Otero Co.	BALT
Heterotheca grandiflora	Henrickson 4977	CA: Orange Co.	BALT
Heterotheca grandiflora	Semple & Semple 10481	CA: Kern Co.	BALT
Heterotheca grandiflora	Semple & Semple 5621	CA: Los Angeles Co.	BALT
Heterotheca grandiflora	Adee sn.	HI: Pohakaloa Burn	US
Heterotheca gypsophila	Hinton et al. 22864	Mexico: Galeana	TEX/LL
Heterotheca gypsophila	Hinteon et al. 25511	Mexico: Galeana	TEX/LL
Heterotheca inuloides	Duke & Jansen 399	Mexico	LSU
Heterotheca inuloides var. *inuloides*	Osorio 162	Mexico: Oaxaca	TEX/LL
Heterotheca inuloides var. *inuloides*	King 3609	Mexico	US
Heterotheca inuloides var. *rosei*	Cronquist & Fay 10815	Mexico	US
Heterotheca inuloides var. *rosei*	McVaugh 13078	Mexico	US
Heterotheca inuloides var. *viridis*	Holmes 10335	Mexico: Jalisco	TEX/LL
Heterotheca inuloides var. *viridis*	Martin 543	Mexico	US
Heterotheca inuloides var. *viridis*	King 2897	Mexico	US
Heterotheca inuloides var. *viridis*	Breedlove 12203	Mexico	US
Heterotheca jonesii	Semple & Chimielewski	UT: Garfield Co.	BALT

	8898		
Heterotheca leptoglossa	McVaugh 16688	Mexico	US
Heterotheca marginata	Semple 10385	AR: Maricopa Co.	BALT
Heterotheca marginata	Semple & Semple 10499	AZ: Maricopa Co.	BALT
Heterotheca mexicana	Lane & Longstreth 2720	Mexico: Durango	TEX/LL
Heterotheca mexicana	Sundberg & Lavin 2897	Mexico: Durango	TEX/LL
Heterotheca mucronata	Gomez & Garcia 262	Mexico: Coahuila	TEX/LL
Heterotheca mucronata var. *mucronata*	Hinton et al. 24484	Mexico: Galeana	TEX/LL
Heterotheca oregona var. *compacta*	Semple & Semple 5691	CA: Humboldt Co.	BALT
Heterotheca oregona var. *compacta*	Semple & Semple 5693	CA: Trinity Co.	BALT
Heterotheca oregona var. *oregona*	Semple & Heard 8526	CA: Del Norte Co.	BALT
Heterotheca oregona var. *oregona*	Semple & Heard 8566	CA: Napa Co.	BALT
Heterotheca oregona var. *rudis*	Semple & Heard 8472	CA: Humboldt Co.	BALT
Heterotheca oregona var. *rudis*	Semple & Heard 8567	CA: Napa Co.	BALT
Heterotheca oregona var. *scaberrima*	Semple & Chimielewski 8942	CA: San Benito Co.	BALT
Heterotheca oregona var. *scaberrima*	Semple & Heard 8588	CA: Santa Clara Co.	BALT
Heterotheca oregona var. *scaberrima*	Semple & Heard 8600	CA: Santa Clara Co.	BALT
Heterotheca pumila	Semple & Zhang 10453	CO: Pitkin Co.	BALT
Heterotheca pumila	Semple & Heard 8362	CO: Eagle Co.	BALT
Heterotheca rutteri	Semple 10386	AR: Santa Cruz Co.	BALT
Heterotheca rutteri	Semple & Chimielewski 9030	AZ: Santa Cruz Co.	BALT
Heterotheca sessiliflora ssp. *echioides*	Semple & Heard 8603	CA: Monterey Co.	BALT
Heterotheca sessiliflora var. *bolanderioides*	Semple et al. 9339	CA: Conta Costa Co.	BALT
Heterotheca shevockii	Semple et al. 9361	CA: Kern Co.	BALT
Heterotheca shevockii	Semple & Chimielewski 8954	CA: Kern Co.	BALT
Heterotheca stenophylla	Higgins 5675	TX: Randall Co.	BALT
Heterotheca stenophylla var. *angustifolia*	Semple & Brammall 2693	SD: Mellette Co.	BALT
Heterotheca subaxillaris	Baltzell 3303	FL: Marion Co.	BALT
Heterotheca subaxillaris	Longbottom 6604	MD: Wicomico Co.	DOV
Heterotheca subaxillaris ssp. *latifolia*	Semple et al. 9498	NJ: Salem Co.	BALT

Heterotheca subaxillaris ssp. *latifolia*	Higgins 6050	TX: Knox Co.	BALT
Heterotheca subaxillaris ssp. *subaxillaris*	Semple, Shea & Wunderlin 1667	FL: Pinellas Co.	BALT
Heterotheca subaxillaris ssp. *subaxillaris*	Semple &Suripto 10561	FL: Okaloosa Co.	BALT
Heterotheca villosa var. *foliosa*	Pinkava et al. 5838	AZ: Coconico Co.	BALT
Heterotheca villosa var. *minor*	Semple 4256	Canada: Saskatchewan	BALT
Heterotheca villosa var. *pedunculata*	Semple et al. 9373	NM: McKinley Co.	BALT
Heterotheca villosa var. pedunculata	Semple 10508	NM: Dona Ana Co.	BALT
Heterotheca viscida	Bennett 8306	NM: Santa Fe Co.	US
Heterotheca viscida	Hinckley 2085	TX: Presidio Co.	US
Heterotheca viscida	Worthington 19594	NM: Luna Co.	TEX/LL
Heterotheca viscida	Carr & Karges 19141	TX: Jeff Davis Co.	TEX/LL
Heterotheca zionensis	Semple & Chimielewski 8884	UT: Utah Co.	BALT
Heterotheca zionensis	Semple & Semple 10494	AZ: Gila Co.	BALT
Noticastrum acuminatum	Zardini & Villate 46343	Paraguay	US
Noticastrum acuminatum	Zardini 1329	Uruguay	US
Noticastrum acuminatum	Burkart & Gamerro 21974	Argentina	US
Noticastrum acuminatum	Quintana et al. 146	Paraguay	MO
Noticastrum acuminatum	Zuloaga 5316	Argentina	MO
Noticastrum acuminatum	Tressens et al. 6592	Argentina	TEX/LL
Noticastrum adcendens	Claude-Joseph 1592	Chile	US
Noticastrum adcendens	Claude-Joseph 574	Chile	US
Noticastrum album	Eyerdam 10709	Chile	US
Noticastrum album	Philippi sn.	Chile	US
Noticastrum argenteum	Venturi 6219	Argentina	US
Noticastrum argenteum	Venturi 9428	Argentina	US
Noticastrum argentinense	Pedersen 9053	Argentina	US
Noticastrum argentinense	Pedersen 8160	Argentina	US
Noticastrum calvatum	Scur 479	Brazil	US
Noticastrum calvatum	Butzke & Nodari 11483	Brazil	US
Noticastrum calvatum	Hatschbach & Cordeiro	Brazil	US

	52819		
Noticastrum calvatum	Ribas & Silva 74	Brazil	MO
Noticastrum decumbens	Hatschbach & Ribas 61343	Brazil	US
Noticastrum decumbens	Hatschback et al. 78266	Brazil	US
Noticastrum diffusum	Abbiatti 4313	Argentina	US
Noticastrum diffusum	Osten 6630	Uruguay	US
Noticastrum diffusum	Zardini 1330	Argentina	MO
Noticastrum diffusum	Zardini 1094	Argentina	TEX/LL
Noticastrum diffusum	Irving & Irving U-36	Uruguay	TEX/LL
Noticastrum gnaphalioides	Ribas et al. 5098	Brazil	US
Noticastrum gnaphalioides	Dusen 9812	Brazil	US
Noticastrum gnaphalioides	Zardini & Gamarra 55551	Paraguay	MO
Noticastrum hatschbachii	Hatschbach & Zardini 41024	Brazil	US
Noticastrum hatschbachii	Hatschbach 44907	Brazil	MO
Noticastrum jujuyense	Nee 46650	Bolivia	US
Noticastrum jujuyense	Nee 46650	Bolivia	TEX/LL
Noticastrum macrocephalum	Pedersen 2654	Argentina	US
Noticastrum macrocephalum	Zardini & Villate 46225	Paraguay	US
Noticastrum macrocephalum	Schinini et al. 29157	Argentina	MO
Noticastrum macrocephalum	Morrone et al. 1069	Argentina	MO
Noticastrum macrocephalum	Krapovickas et al. 18302	Argentina	TEX/LL
Noticastrum macrocephalum	Neffa et al. 208	Argentina	TEX/LL
Noticastrum macrocephalum (image)	Egea et al. 287	Paraguay	MO
Noticastrum macrocephalum (image)	Zardini & Villate 46225	Paraguay	MO
Noticastrum malmei	Reitz & Klein 642	Brazil	US
Noticastrum malmei	Smith & Klein 11106	Brazil	US
Noticastrum marginatum	King & Guevara 5664	Colombia	US
Noticastrum marginatum	MacDougal & Roldan 3587	Colombia	US
Noticastrum marginatum	Norrbom et al. 11-PE-17	Peru	US

Noticastrum marginatum	Olsen & Escobar 576	Colombia	TEX/LL
Noticastrum marginatum	Bacon & Bohnstedt 1524	Argentina	TEX/LL
Noticastrum marginatum(image)	Serrano et al. 4815	Bolivia	MO
Noticastrum marginatum(image)	Villalobos et al. 1206	Bolivia	MO
Noticastrum montevidense	Pedersen 3822	Argentina	US
Noticastrum psammophilum	Dusen 8421	Brazil	US
Noticastrum sericeum	Conrad 2347	Argentina	US
Noticastrum sericeum	Gunckel 15564	Chile	US
Noticastrum sericeum	Villamil 5782	Argentina	MO
Noticastrum(image)	Peña-Chocarro et al. 1786	Paraguay	MO
Osbertia bartlettii	Nesom et al. 6296	Mexico: San Carlos	TEX/LL
Osbertia bartlettii	Patterson 6436	Mexico: Nuevo Leon	TEX/LL
Osbertia bartlettii	Meyer & Rogers 2827	Mexico	US
Osbertia chihuahuana	Garcia & Acevedo 349	Mexico	TEX/LL
Osbertia stolonifera	Wells & Nesom 412	Mexico: Nuevo Leon	TEX/LL
Osbertia stolonifera	Estrada & Kasey 1153	Mexico: Oaxaca	TEX/LL
Osbertia stolonifera	Spooner & Triplehorn 2111	Mexico	US
Osbertia stolonifera	Lyonnet 2599	Mexico	US
Pityopsis aspera	Unk. 10371	MS: Pearl River Co.	BALT
Pityopsis aspera	Uttal 8372	VA: Henry Co.	BALT
Pityopsis aspera	Rugel 484	FL	DOV
Pityopsis aspera var. *adelolepis*	Bowers & Bowers 45554	NC: More Co.	TENN
Pityopsis aspera var. *adelolepis*	Diamond 12180	AL: Conecuh Co.	TENN
Pityopsis falcata	Reese et al.	MA: Truro Co.	BALT
Pityopsis falcata	Naczi 10067	NJ: Burlington Co.	DOV
Pityopsis falcata	Murray sn.	NY: Suffolk Co.	DOV
Pityopsis falcata	Morton s.n.	NJ: Ocean Co.	TENN
Pityopsis falcata	Bowers & Bowers 72-210	NJ: Atlantic Co.	TENN
Pityopsis flexuosa	Godfrey 70072	FL: Wakulla Co.	BALT
Pityopsis flexuosa	Godfrey 83983	FL: Leon Co.	US
Pityopsis flexuosa	Godfrey 84404	FL: Leon Co.	US
Pityopsis flexuosa	Bowers & Bowers 70-484	FL: Leon Co.	TENN
Pityopsis flexuosa	Bowers & Bowers 45593	FL: Leon Co.	TENN
Pityopsis graminifolia	Windler 4155	MD: Worcester Co.	BALT
Pityopsis graminifolia	Martin 140	LA: Jackson Pa.	BALT

Pityopsis graminifolia	Kral 93330	MS: Pearl River Co.	DOV
Pityopsis graminifolia	Phillips sn.	NC: Dare Co.	DOV
Pityopsis graminifolia	Costa & Roberts 2013-001	VA: Kent Co.	BALT
Pityopsis graminifolia var. *graminifolia*	Spongberg et al. 17175	SC: Charleston Co.	TENN
Pityopsis oligantha	Kelloff et al. 1462	FL: Liberty Co.	US
Pityopsis oligantha	Correll & Correll 51609	FL: Liberty Co.	US
Pityopsis oligantha	Bowers & wofford 72-120	FL: Liberty Co.	TENN
Pityopsis oligantha	Godfrey 84808	FL: Liberty Co.	TENN
Pityopsis oligantha	Chapman sn.	FL	DOV
Pityopsis pinifolia	Gibson 208	NC: Harnett	BALT
Pityopsis pinifolia	Sorrie 11709	NC: Lee Co.	US
Pityopsis pinifolia	Dress 10170	NC: Wayne Co.	DOV
Pityopsis ruthii	Wofford et al. 79-289	TN: Polk Co.	TENN
Pityopsis ruthii	Wofford et al. 79-288	TN: Polk Co.	TENN
Pityopsis ruthii	Ruth 1501	TN: Hiawassee Valley	US
Pityopsis ruthii	Ruth 622	TN: Hiawassee Valley	US
Pityopsis ruthii	Schilling (Aug. 2013)	TN: Polk Co.	TENN
Solidago sempervirens	Moldenke 31249	NJ: Ocean Co.	LSU
Tomentaurum niveum	Mexia 2598	Mexico	US
Tomentaurum niveum	Pringle sn.	Mexico	US
Tomentaurum niveum	Van Dervender et al. 87-165	Mexico	TEX/LL

Table 1.2

Primer Designation	Primer Sequence 5′-3′	Author(s), year of publication
*nr*DNA *ETS*		
18S-2L	TGA CTA CTG GCA GGA TCA ACC AG	Linder et al., 2000
Ast-1	CGT AAA GGT GCA TGA GTG GTG T	Markos & Baldwin, 2001
18S-ETS	ACT TAC ACA TGC ATG GCT TAA TCT	Baldwin & Markos, 1998
Ast-8	TTC TCT TCG TAT CGT GCG GT	Markos & Baldwin, 2001
*nr*DNA *ITS*		
ITS-1	TCC GTA GGT GAA CCT GCG G	White et al. 1990
ITS-4	TCC TCC GCT TAT TGA TAT GC	White et al. 1990
ITS-20	TCG CGT TGA CTA CGT CCC TGC C	Urbatsch et al. 2000
*cp*DNA *ycf1 3300-4280*		
ycf1 3300F	GCT TTT GAT AAT CTT AGA AAT AGT AAG	Neubig et al., unpublished
ycf1 4280R	GCT TGG RAT AAA CCA AGG TTT CTC	Neubig et al., unpublished
ycf1 TU F	GAA ATW GTA AGC GAA AGT CAC ATA	Costa & Roberts, (this publication)
*cp*DNA *psbA-trnH*		
psbA F	GTT ATG CAT GAA CGT AAT GCT C	Shaw et al., 2005
trnH R	CGC GCA TGG TGG ATT CAC AAT CC	Shaw et al., 2005
psbATU-FDeg	GTT ATG CAN NNA CGT AAT GCT C	Costa & Roberts, (this publication)2014
psbATU-F	ACG TAA TGC TCA CAA TTT	Costa & Roberts, (this publication)

Table 1.3

Gene - Model of Nucleotide Evolution	Description
Individual Dataset	
ETS - TPM3uf+I+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT;AG=CT
ITS - SYM+I+G	Base frequencies – equal Substitution rates – AC; AG; AT; CG; CT; GT
psbA-trnH - HKY+G	Base frequencies – unequal Substitution rates – AC=AT=CG=GT; AG=CT
ycf1 - TIM3+I+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT; AG; CT
Nuclear Dataset	
ETS - TVM+G	Base frequencies – unequal Substitution rates – AC; AT; CG; GT; AG=CT
ITS - SYM+I+G	Base frequencies – equal Substitution rates – AC; AG; AT; CG; CT; GT
Chloroplast Dataset	
psbA-trnH - TPM3uf+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT;AG=CT
ycf1 - TPM3uf+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT;AG=CT
Combined Dataset (*ETS*, ycf1 3300F-4280R, psbA-trnH)	
ETS - TPM3uf+I+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT;AG=CT
ycf1 - TPM3uf+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT;AG=CT
psbA-trnH - HKY+I	Base frequencies – unequal Substitution rates – AC=AT=CG=GT; AG=CT
Combined Dataset with all genes	
ETS - TPM3uf+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT;AG=CT
ITS - SYM+I+G	Base frequencies – equal

	Substitution rates – AC; AG; AT; CG; CT; GT
psbA-trnH - HKY+G	Base frequencies – unequal Substitution rates – AC=AT=CG=GT; AG=CT
ycf1 - TPM3uf+G	Base frequencies – unequal Substitution rates – AC=CG; AT=GT;AG=CT

Table 1.4

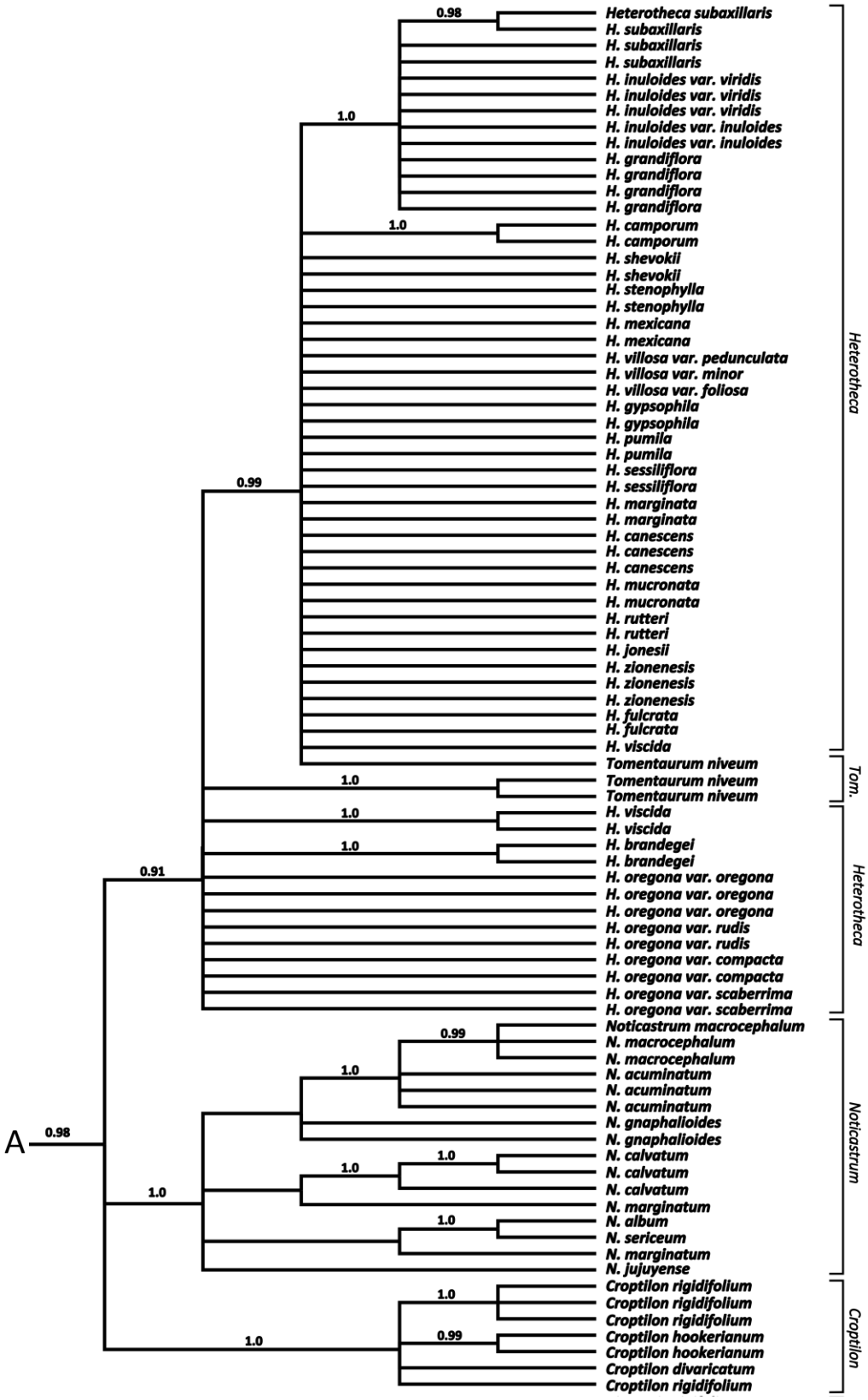
Character	Traits
Pappus	Uniseriate, Multiseriate
Pappus scales	Absent, Present
Cypsela rib	Thin, Pronounced
Capitulescence	Solitary, Non-solitary
Ray florets	Absent, Present
Ray floret color	Yellow, White
Leaf vein	Reticulate, Pseudo-parallel
Trichomes on phyllaries	Eglandular, Glandular, Stipitate-glandular

Table 2.1

Taxon Name	Year collected	A260/A280	[DNA] (ng/μL)
Chrysopsis mariana	1967	1.65	71.1
Chrysopsis mariana	2008	1.65	79.6
Pityopsis ruthii	1900	1.40	44.8
Pityopsis ruthii	2013	1.46	55.0
Heterotheca viscida	1963	1.48	37.9
Heterotheca viscida	2000	1.44	16.4
Noticastrum marginatum	1977	1.51	9.0
Noticastrum marginatum	2011	1.25	94.6

Table 2.2

Taxon Name	Year collected	CTAB A260/A280	QIAGEN A260/A280	CTAB [DNA] (ng/μL)	QIAGEN [DNA] (ng/μL)
Chrysopsis mariana[f]	1967	1.50	1.65	417.0	71.1
Chrysopsis gossypina	1974	1.27	1.71	188.5	70.1
Heterotheca oregona	1986	1.43	1.48	466.7	41.3
Croptilon divaricatum	1987	1.42	1.43	133.0	25.3
Tomentaurum niveum	1929	1.57	1.65	702.3	59.0
Tomentaurum niveum[f]	1887	1.30	1.14	414.7	14.8
Heterotheca inuloides[f]	1970	1.35	1.53	470.9	66.8
Biglowia nudata	2000	1.19	1.56	161.1	47.5
Euthamia graminifolia	2001	1.58	1.73	705.1	150.1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1.0
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H. inuloides var. viridis
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H. inuloides var. inuloides
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H. grandiflora
H. grandiflora
H. grandiflora
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H. camporum
H. shevokii
H. shevokii
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H. stenophylla
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H. mexicana
H. villosa var. pedunculata
H. villosa var. minor
H. villosa var. foliosa
H. gypsophila
H. gypsophila
H. pumila
H. pumila
H. sessiliflora
H. sessiliflora
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H. marginata
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H. canescens
H. canescens
H. mucronata
H. mucronata
H. rutteri
H. rutteri
H. jonesii
H. zionensis
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H. fulcrata
H. viscida
Tomentaurum niveum
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Tomentaurum niveum
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H. viscida
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H. brandegei
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Noticastrum macrocephalum
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N. macrocephalum
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N. acuminatum
N. acuminatum
N. gnaphalioides
N. gnaphalioides
N. calvatum
N. calvatum
N. calvatum
N. marginatum
N. album
N. sericeum
N. marginatum
N. jujuyense
Croptilon rigidifolium
Croptilon rigidifolium
Croptilon rigidifolium
Croptilon hookerianum
Croptilon hookerianum
Croptilon divaricatum
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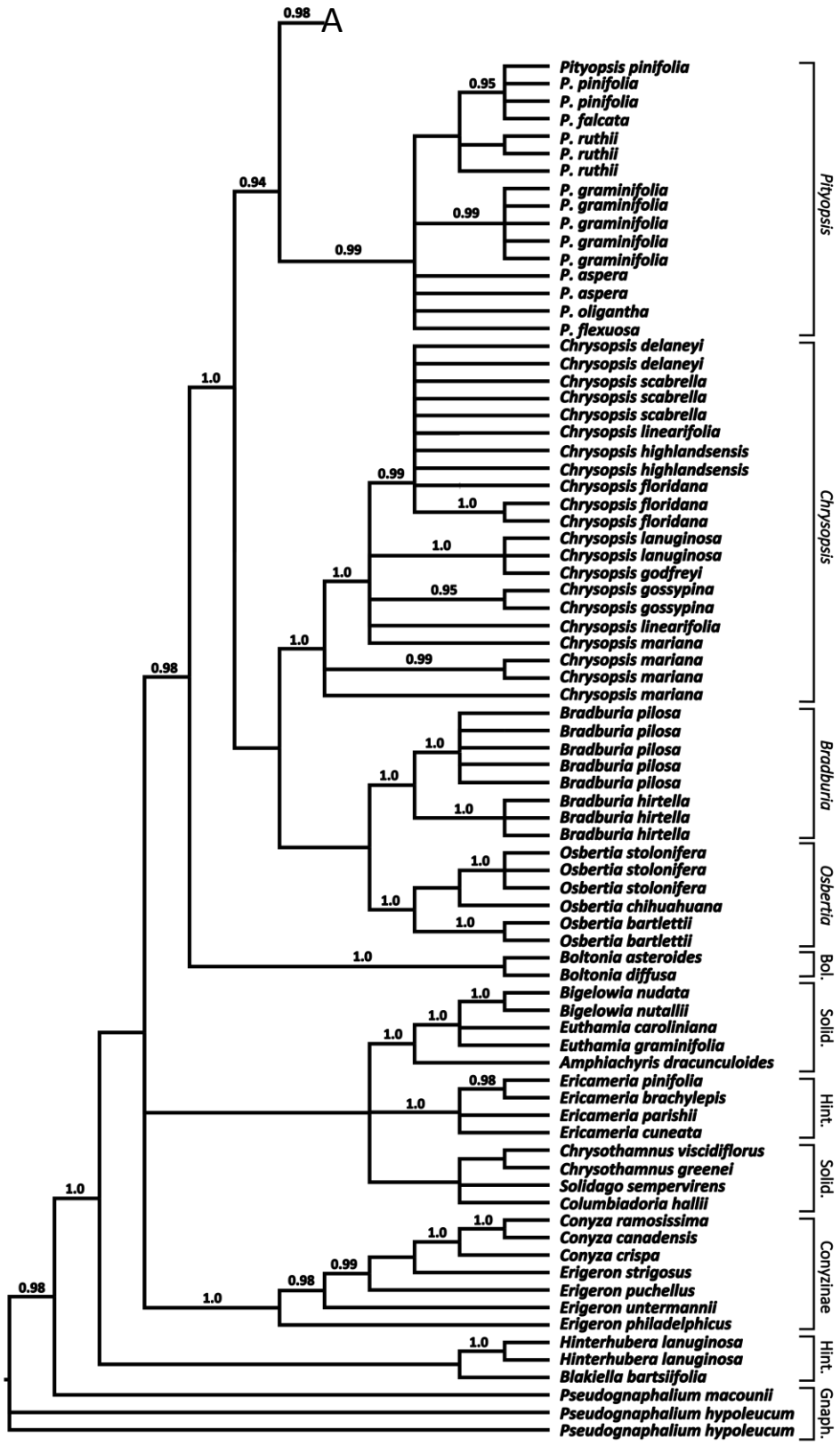
Heterotheca
Tom.
Heterotheca
Noticastrum
Croptilon

Figure 1.1.1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P. graminifolia
P. aspera
P. aspera
P. oligantha
P. flexuosa
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Chrysopsis delaneyi
Chrysopsis scabrella
Chrysopsis scabrella
Chrysopsis scabrella
Chrysopsis linearifolia
Chrysopsis highlandsensis
Chrysopsis highlandsensis
Chrysopsis floridana
Chrysopsis floridana
Chrysopsis floridana
Chrysopsis lanuginosa
Chrysopsis lanuginosa
Chrysopsis godfreyi
Chrysopsis gossypina
Chrysopsis gossypina
Chrysopsis linearifolia
Chrysopsis mariana
Chrysopsis mariana
Chrysopsis mariana
Chrysopsis mariana
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Bradburia pilosa
Bradburia pilosa
Bradburia pilosa
Bradburia pilosa
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Bradburia hirtella
Bradburia hirtella
Osbertia stolonifera
Osbertia stolonifera
Osbertia stolonifera
Osbertia chihuahuana
Osbertia bartlettii
Osbertia bartlettii
Boltonia asteroides
Boltonia diffusa
Bigelowia nudata
Bigelowia nutallii
Euthamia caroliniana
Euthamia graminifolia
Amphiachyris dracunculoides
Ericameria pinifolia
Ericameria brachylepis
Ericameria parishii
Ericameria cuneata
Chrysothamnus viscidiflorus
Chrysothamnus greenei
Solidago sempervirens
Columbiadoria hallii
Conyza ramosissima
Conyza canadensis
Conyza crispa
Erigeron strigosus
Erigeron puchellus
Erigeron untermannii
Erigeron philadelphicus
Hinterhubera lanuginosa
Hinterhubera lanuginosa
Blakiella bartsiifolia
Pseudognaphalium macounii
Pseudognaphalium hypoleucum
Pseudognaphalium hypoleucum
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Osbertia
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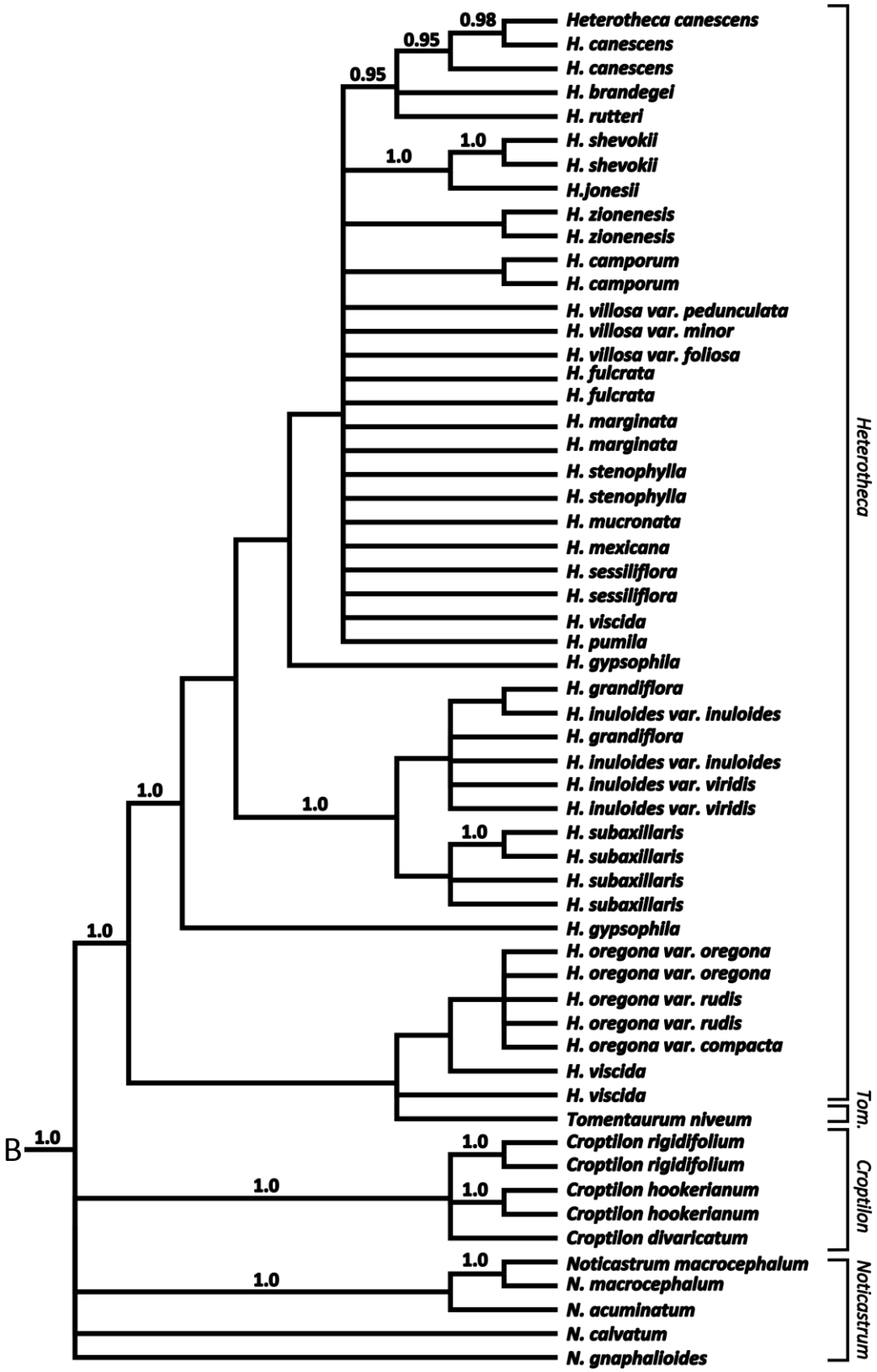
Figure 1.1.2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H. oregona var. compacta
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H. viscida
Tomentaurum niveum
Croptilon rigidifolium
Croptilon rigidifolium
Croptilon hookerianum
Croptilon hookerianum
Croptilon divaricatum
Noticastrum macrocephalum
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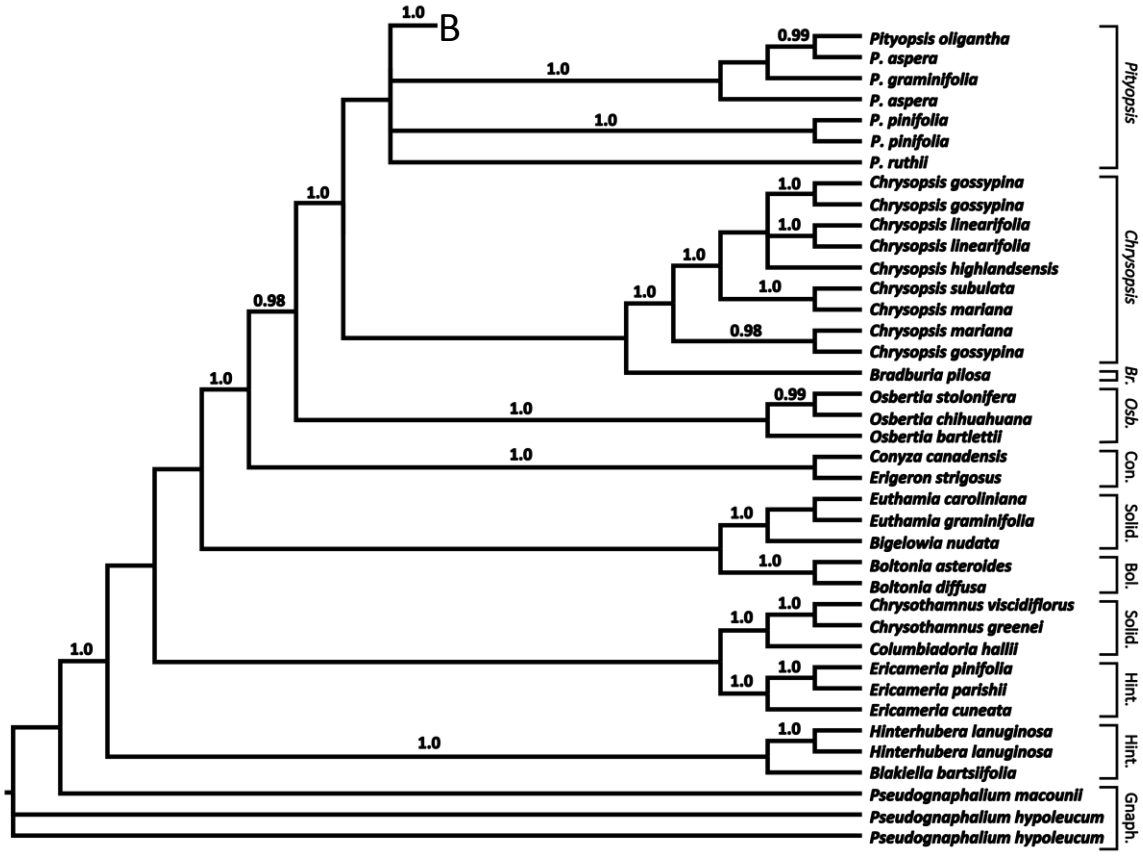
N. calvatum
N. gnaphalioides
Heterotheca
Tom.
Croptilon
Noticastrum

Figure 1.2.1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Chrysopsis gossypina
Chrysopsis linearifolia
Chrysopsis linearifolia
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Chrysopsis subulata
Chrysopsis mariana
Chrysopsis mariana
Chrysopsis gossypina
Bradburia pilosa
Osbertia stolonifera
Osbertia chihuahuana
Osbertia bartlettii
Conyza canadensis
Erigeron strigosus
Euthamia caroliniana
Euthamia graminifolia
Bigelowia nudata
Boltonia asteroides
Boltonia diffusa
Chrysothamnus viscidiflorus
Chrysothamnus greenei
Columbiadoria hallii
Ericameria pinifolia
Ericameria parishii
Ericameria cuneata
Hinterhubera lanuginosa
Hinterhubera lanuginosa
Blakiella bartsiifolia
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Pseudognaphalium hypoleucum
Pseudognaphalium hypoleucum
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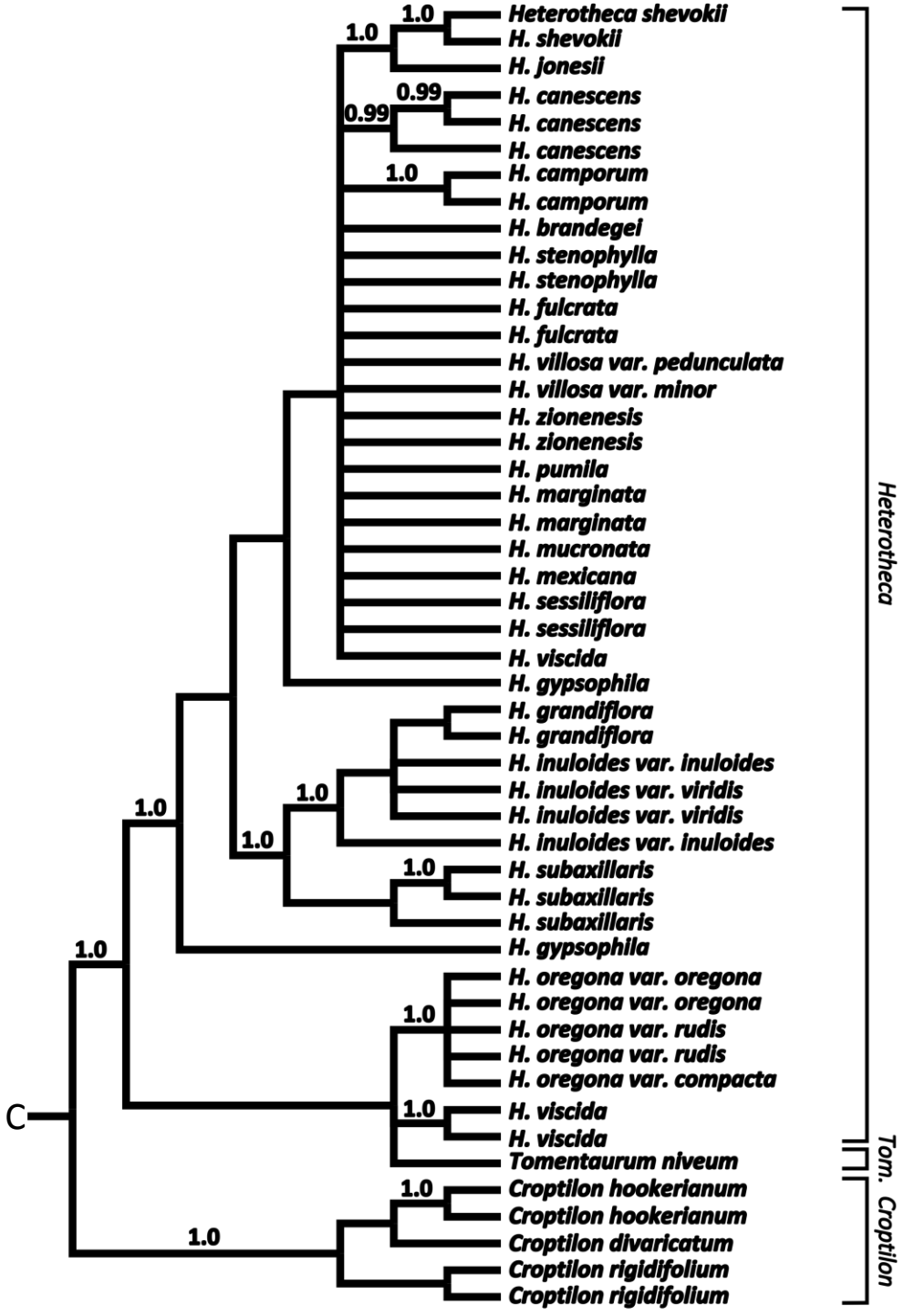
Bol.
Solid.
Hint.
Hint.
Gnaph.

Figure 1.2.2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H. oregona var. rudis
H. oregona var. rudis
H. oregona var. compacta
H. viscida
H. viscida
Tomentaurum niveum
Croptilon hookerianum
Croptilon hookerianum
Croptilon divaricatum
Croptilon rigidifolium
Croptilon rigidifolium
1.0
0.99
C
Heterotheca
Tom.
Croptilon

Figure 1.3.1

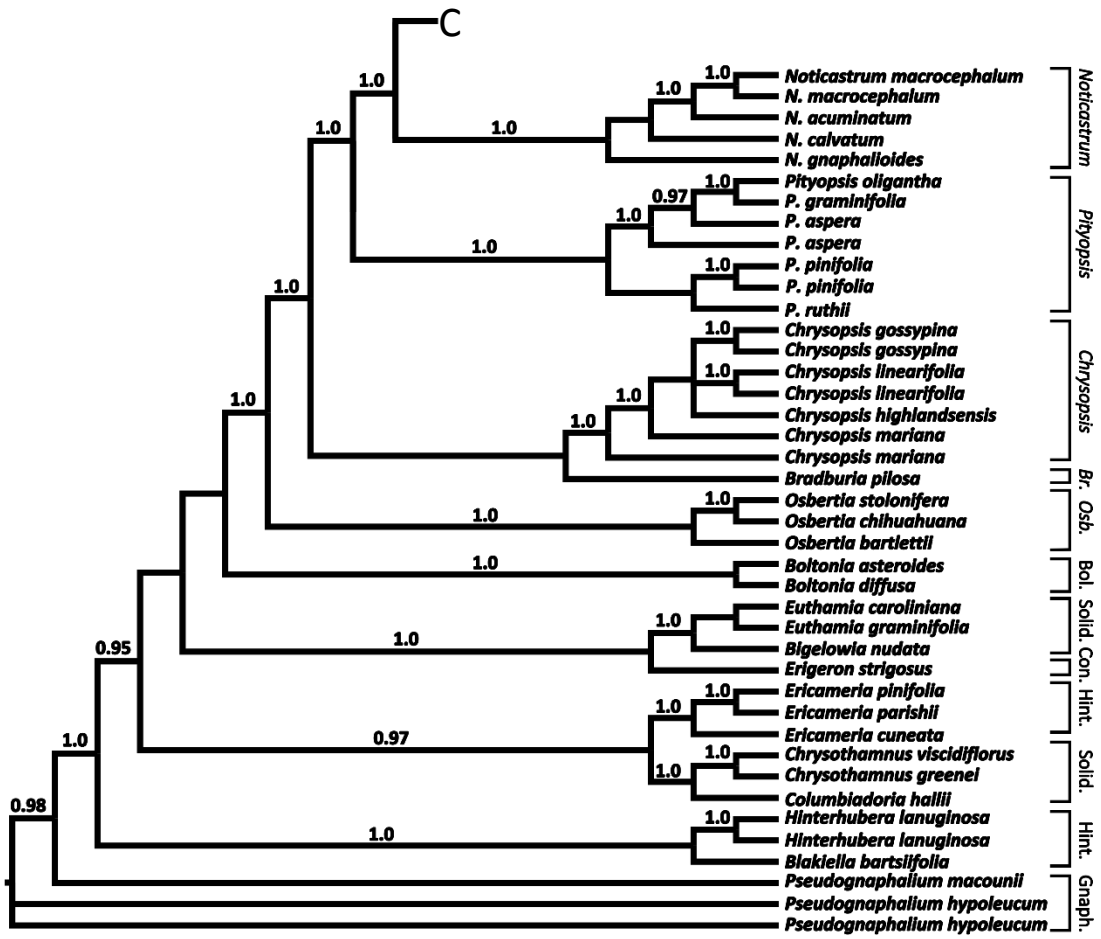
C
Noticastrum macrocephalum
N. macrocephalum
N. acuminatum
N. calvatum
N. gnaphalioides
Pityopsis oligantha
P. graminifolia
P. aspera
P. aspera
P. pinifolia
P. pinifolia
P. ruthii
Chrysopsis gossypina
Chrysopsis gossypina
Chrysopsis linearifolia
Chrysopsis linearifolia
Chrysopsis highlandsensis
Chrysopsis mariana
Chrysopsis mariana
Bradburia pilosa
Osbertia stolonifera
Osbertia chihuahuana
Osbertia bartlettii
Boltonia asteroides
Boltonia diffusa
Euthamia caroliniana
Euthamia graminifolia
Bigelowia nudata
Erigeron strigosus
Ericameria pinifolia
Ericameria parishii
Ericameria cuneata
Chrysothamnus viscidiflorus
Chrysothamnus greenei
Columbiadoria hallii
Hinterhubera lanuginosa
Hinterhubera lanuginosa
Blakiella bartsiifolia
Pseudognaphalium macounii
Pseudognaphalium hypoleucum
Pseudognaphalium hypoleucum
Noticastrum
Pityopsis
Chrysopsis
Br. Osb.
Bol.
Solid. Con.
Hint.
Solid.
Hint.
Gnaph.

Figure 1.3.2

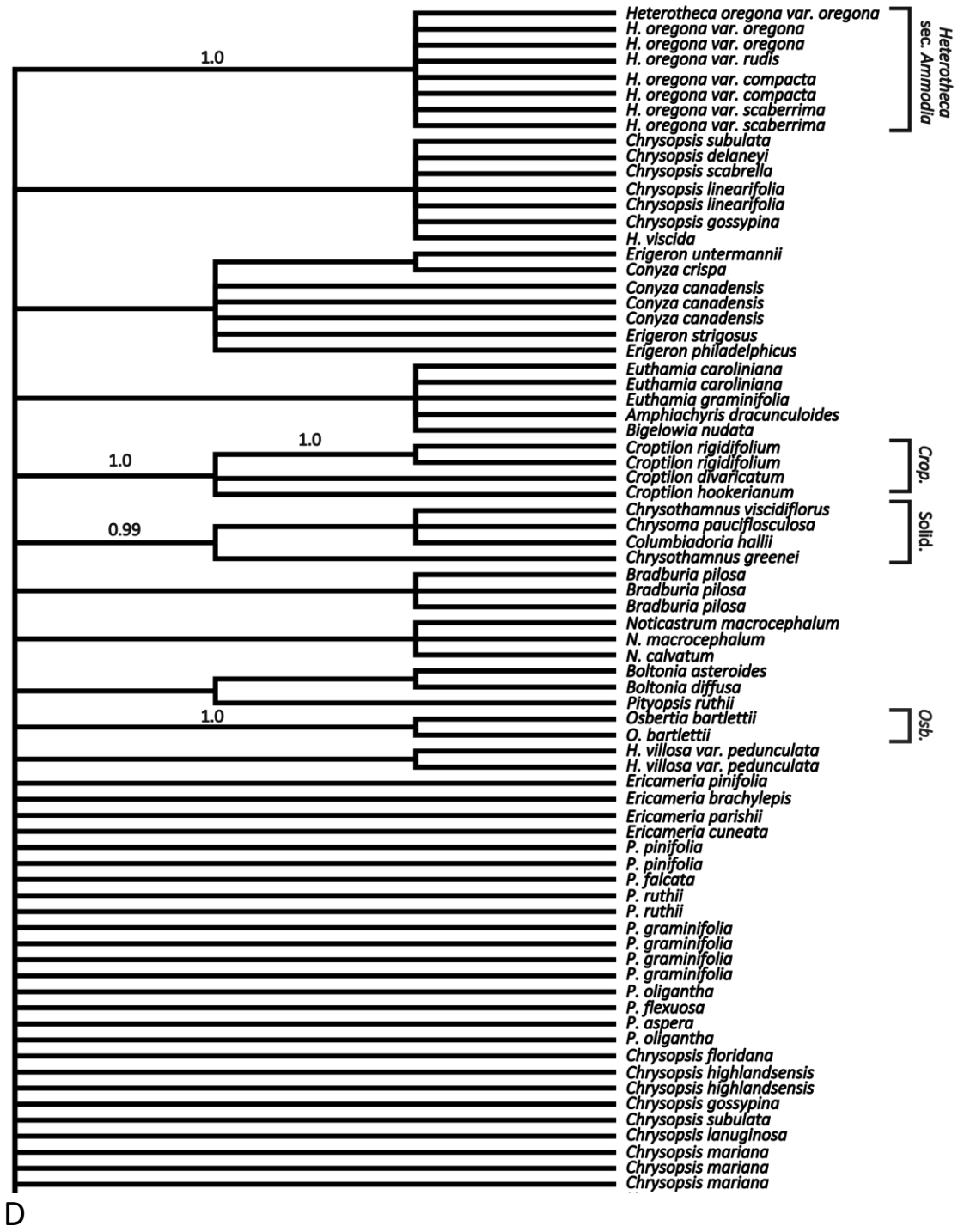
Heterotheca oregona var. oregona
H. oregona var. oregona
H. oregona var. oregona
H. oregona var. rudis
H. oregona var. compacta
H. oregona var. compacta
H. oregona var. scaberrima
H. oregona var. scaberrima
Heterotheca sec. Ammodia
Chrysopsis subulata
Chrysopsis delaneyi
Chrysopsis scabrella
Chrysopsis linearifolia
Chrysopsis linearifolia
Chrysopsis gossypina
H. viscida
Erigeron untermannii
Conyza crispa
Conyza canadensis
Conyza canadensis
Conyza canadensis
Erigeron strigosus
Erigeron philadelphicus
Euthamia caroliniana
Euthamia caroliniana
Euthamia graminifolia
Amphiachyris dracunculoides
Bigelowia nudata
Croptilon rigidifolium
Croptilon rigidifolium
Croptilon divaricatum
Croptilon hookerianum
Crop.
Chrysothamnus viscidiflorus
Chrysoma pauciflosculosa
Columbiadoria hallii
Chrysothamnus greenei
Solid.
Bradburia pilosa
Bradburia pilosa
Bradburia pilosa
Noticastrum macrocephalum
N. macrocephalum
N. calvatum
Boltonia asteroides
Boltonia diffusa
Pityopsis ruthii
Osbertia bartlettii
O. bartlettii
Osb.
H. villosa var. pedunculata
H. villosa var. pedunculata
Ericameria pinifolia
Ericameria brachylepis
Ericameria parishii
Ericameria cuneata
P. pinifolia
P. pinifolia
P. falcata
P. ruthii
P. ruthii
P. graminifolia
P. graminifolia
P. graminifolia
P. graminifolia
P. oligantha
P. flexuosa
P. aspera
P. oligantha
Chrysopsis floridana
Chrysopsis highlandsensis
Chrysopsis highlandsensis
Chrysopsis gossypina
Chrysopsis subulata
Chrysopsis lanuginosa
Chrysopsis mariana
Chrysopsis mariana
Chrysopsis mariana
1.0
1.0
1.0
0.99
1.0
D

Figure 1.4.1

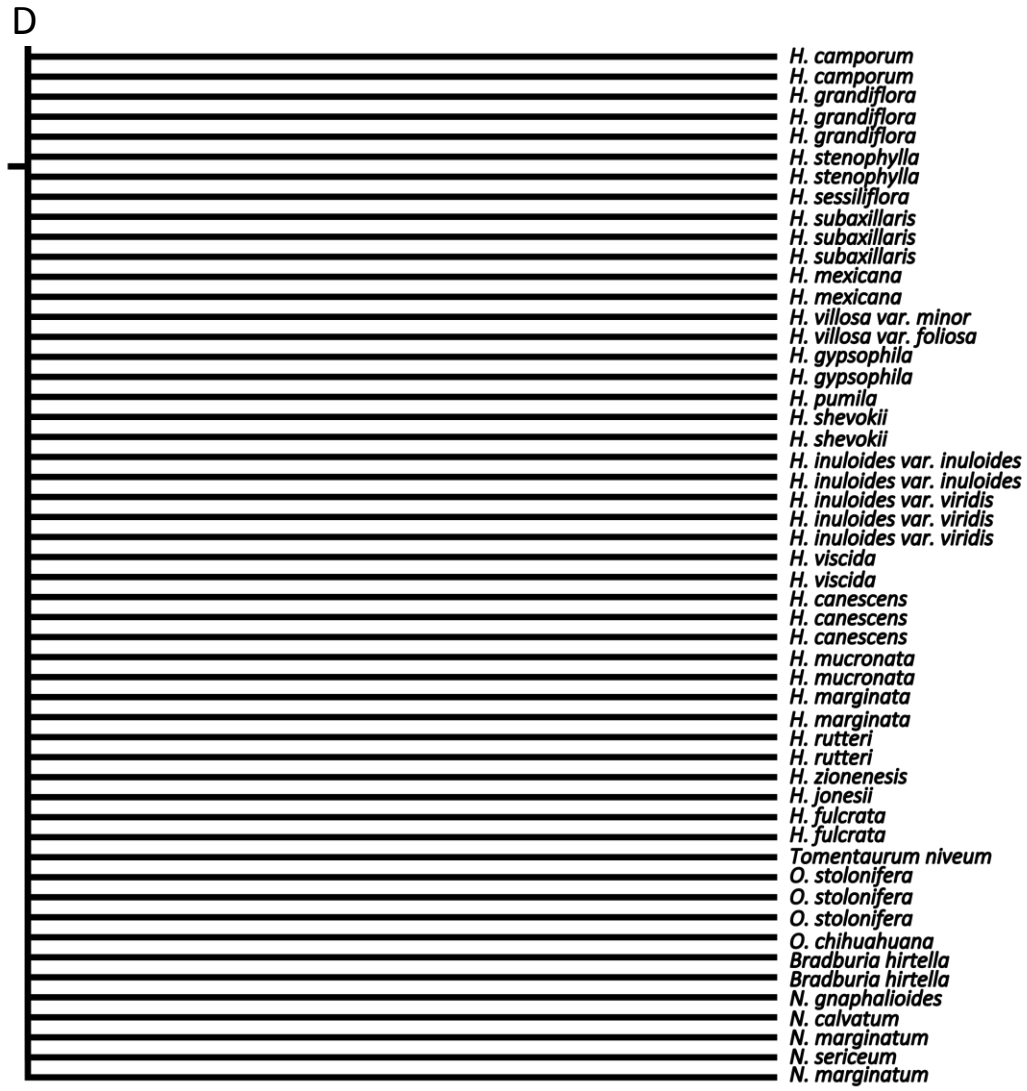
D
H. camporum
H. camporum
H. grandiflora
H. grandiflora
H. grandiflora
H. stenophylla
H. stenophylla
H. sessiliflora
H. subaxillaris
H. subaxillaris
H. subaxillaris
H. mexicana
H. mexicana
H. villosa var. minor
H. villosa var. foliosa
H. gypsophila
H. gypsophila
H. pumila
H. shevokii
H. shevokii
H. inuloides var. inuloides
H. inuloides var. inuloides
H. inuloides var. viridis
H. inuloides var. viridis
H. inuloides var. viridis
H. viscida
H. viscida
H. canescens
H. canescens
H. canescens
H. mucronata
H. mucronata
H. marginata
H. marginata
H. rutteri
H. rutteri
H. zionenesis
H. jonesii
H. fulcrata
H. fulcrata
Tomentaurum niveum
O. stolonifera
O. stolonifera
O. stolonifera
O. chihuahuana
Bradburia hirtella
Bradburia hirtella
N. gnaphalioides
N. calvatum
N. marginatum
N. sericeum
N. marginatum

Figure 1.4.2

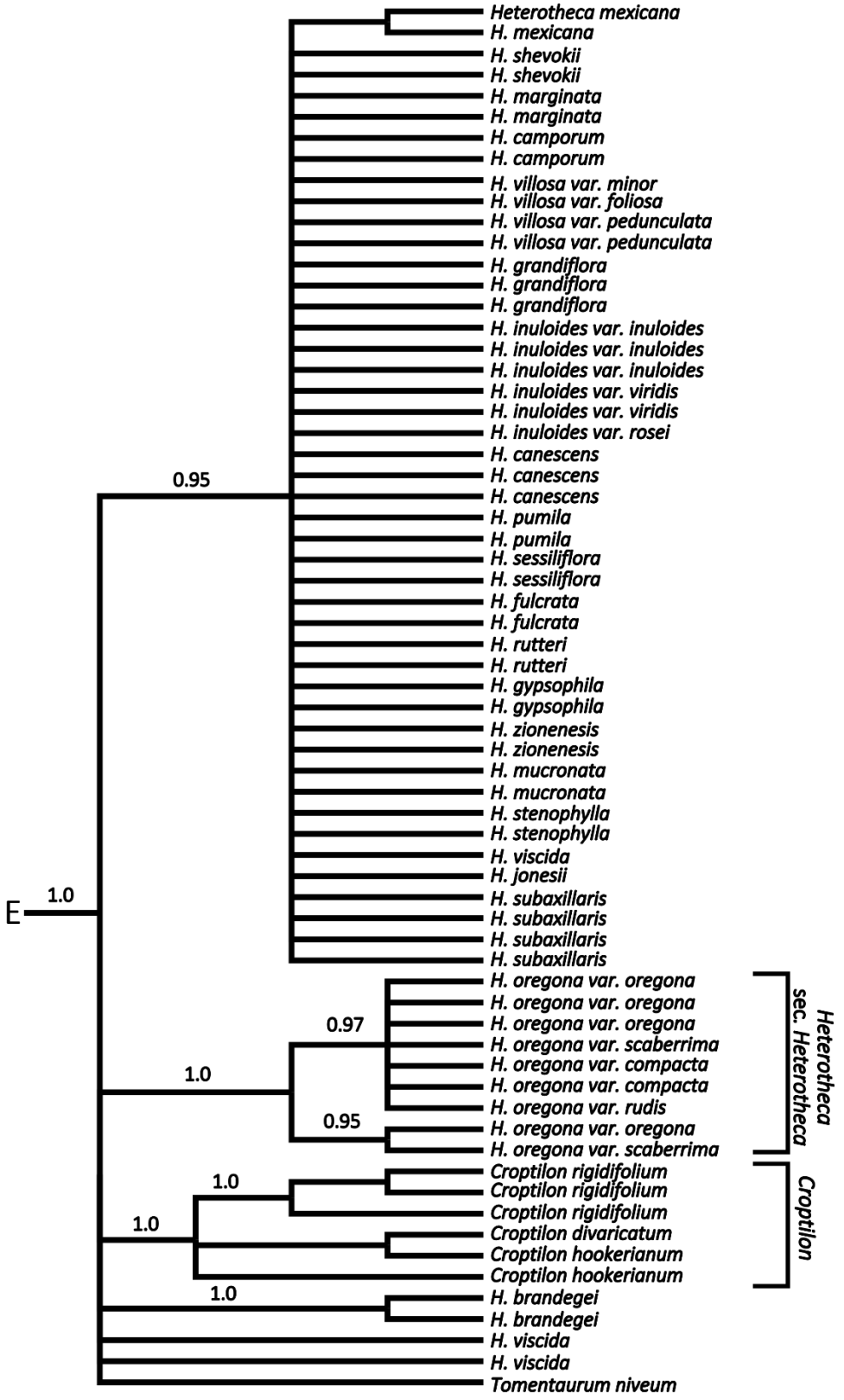
Heterotheca mexicana
H. mexicana
H. shevokii
H. shevokii
H. marginata
H. marginata
H. camporum
H. camporum
H. villosa var. minor
H. villosa var. foliosa
H. villosa var. pedunculata
H. villosa var. pedunculata
H. grandiflora
H. grandiflora
H. grandiflora
H. inuloides var. inuloides
H. inuloides var. inuloides
H. inuloides var. inuloides
H. inuloides var. viridis
H. inuloides var. viridis
H. inuloides var. rosei
H. canescens
H. canescens
H. canescens
H. pumila
H. pumila
H. sessiliflora
H. sessiliflora
H. fulcrata
H. fulcrata
H. rutteri
H. rutteri
H. gypsophila
H. gypsophila
H. zionenesis
H. zionenesis
H. mucronata
H. mucronata
H. stenophylla
H. stenophylla
H. viscida
H. jonesii
H. subaxillaris
H. subaxillaris
H. subaxillaris
H. subaxillaris
H. oregona var. oregona
H. oregona var. oregona
H. oregona var. oregona
H. oregona var. scaberrima
H. oregona var. compacta
H. oregona var. compacta
H. oregona var. rudis
H. oregona var. oregona
H. oregona var. scaberrima
Croptilon rigidifolium
Croptilon rigidifolium
Croptilon rigidifolium
Croptilon divaricatum
Croptilon hookerianum
Croptilon hookerianum
H. brandegei
H. brandegei
H. viscida
H. viscida
Tomentaurum niveum
E
1.0
0.95
0.97
1.0
0.95
1.0
1.0
1.0
Heterotheca sec. Heterotheca
Croptilon

Figure 1.5.1

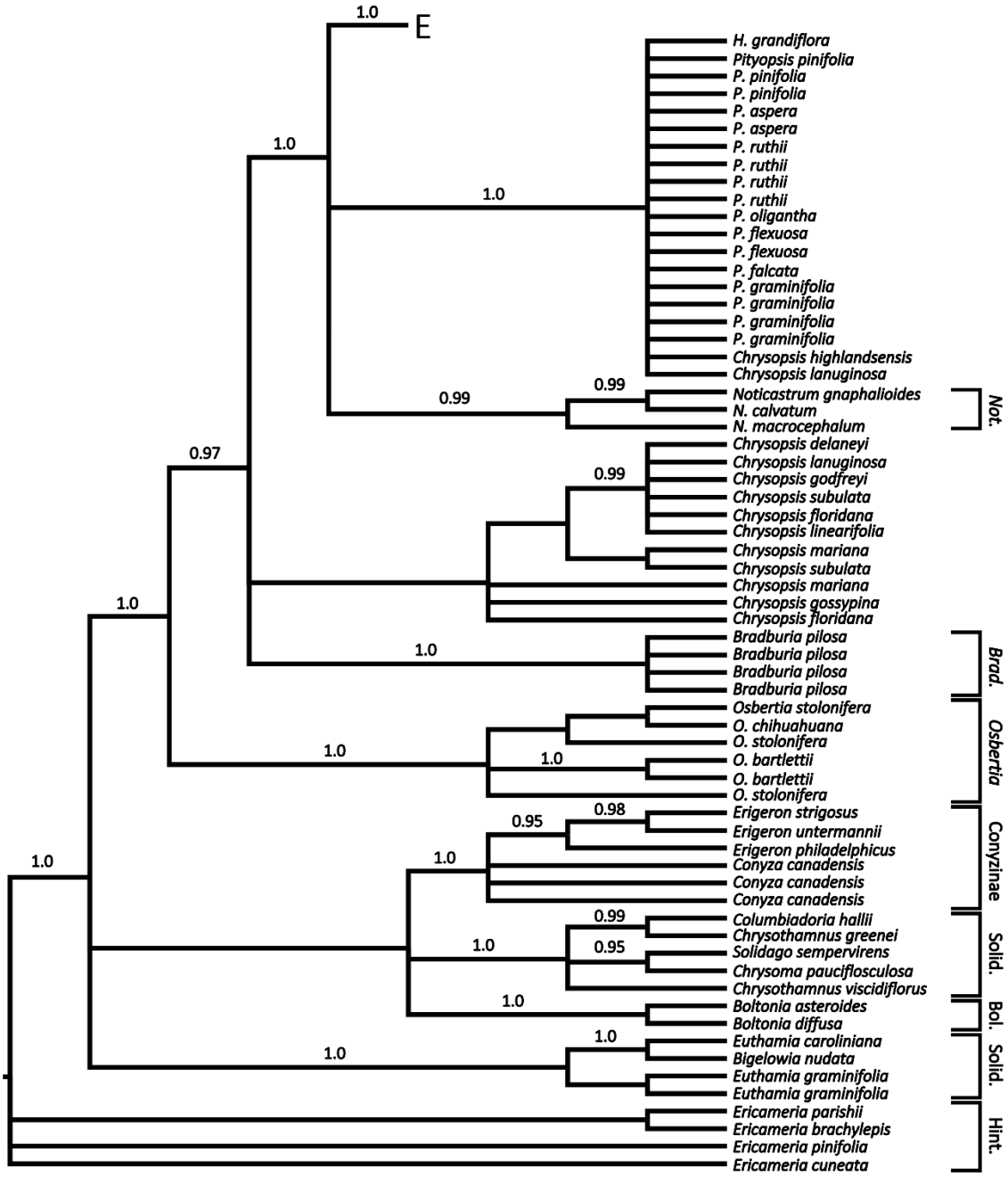
E
H. grandiflora
Pityopsis pinifolia
P. pinifolia
P. pinifolia
P. aspera
P. aspera
P. ruthii
P. ruthii
P. ruthii
P. ruthii
P. oligantha
P. flexuosa
P. flexuosa
P. falcata
P. graminifolia
P. graminifolia
P. graminifolia
P. graminifolia
Chrysopsis highlandsensis
Chrysopsis lanuginosa
Noticastrum gnaphalioides
N. calvatum
N. macrocephalum
Not.
Chrysopsis delaneyi
Chrysopsis lanuginosa
Chrysopsis godfreyi
Chrysopsis subulata
Chrysopsis floridana
Chrysopsis linearifolia
Chrysopsis mariana
Chrysopsis subulata
Chrysopsis mariana
Chrysopsis gossypina
Chrysopsis floridana
Bradburia pilosa
Bradburia pilosa
Bradburia pilosa
Bradburia pilosa
Brad.
Osbertia stolonifera
O. chihuahuana
O. stolonifera
O. bartlettii
O. bartlettii
O. stolonifera
Osbertia
Erigeron strigosus
Erigeron untermannii
Erigeron philadelphicus
Conyza canadensis
Conyza canadensis
Conyza canadensis
Conyzinae
Columbiadoria hallii
Chrysothamnus greenei
Solidago sempervirens
Chrysoma pauciflosculosa
Chrysothamnus viscidiflorus
Solid.
Boltonia asteroides
Boltonia diffusa
Bol.
Euthamia caroliniana
Bigelowia nudata
Euthamia graminifolia
Euthamia graminifolia
Solid.
Ericameria parishii
Ericameria brachylepis
Ericameria pinifolia
Ericameria cuneata
Hint.
1.0
1.0
1.0
0.99
0.99
0.97
0.99
1.0
1.0
1.0
1.0
0.98
0.95
1.0
0.99
1.0
0.95
1.0
1.0
1.0

Figure 1.5.2

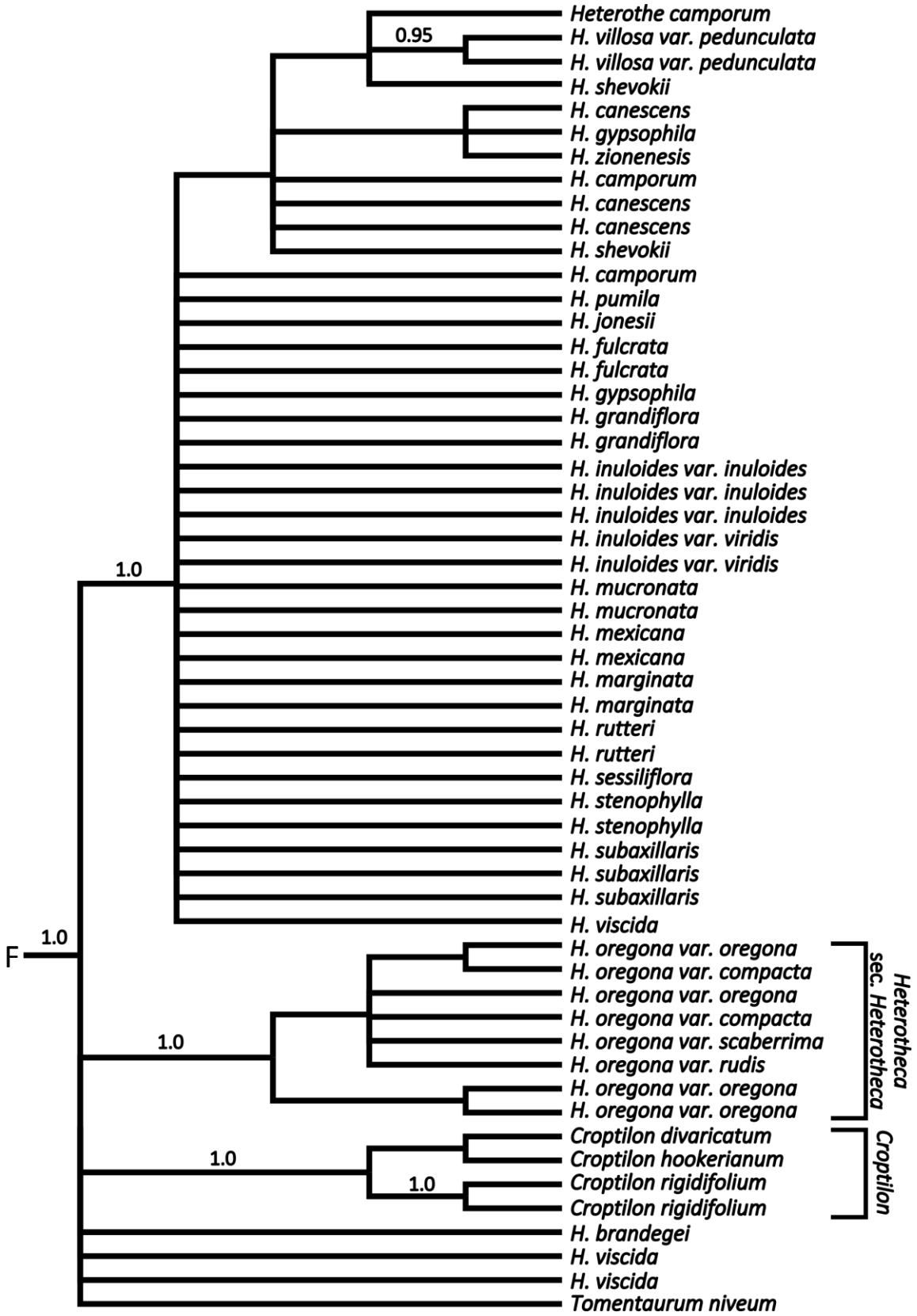
Heterothe camporum
0.95
H. villosa var. pedunculata
H. villosa var. pedunculata
H. shevokii
H. canescens
H. gypsophila
H. zionenesis
H. camporum
H. canescens
H. canescens
H. shevokii
H. camporum
H. pumila
H. jonesii
H. fulcrata
H. fulcrata
H. gypsophila
H. grandiflora
H. grandiflora
H. inuloides var. inuloides
H. inuloides var. inuloides
H. inuloides var. inuloides
H. inuloides var. viridis
H. inuloides var. viridis
1.0
H. mucronata
H. mucronata
H. mexicana
H. mexicana
H. marginata
H. marginata
H. rutteri
H. rutteri
H. sessiliflora
H. stenophylla
H. stenophylla
H. subaxillaris
H. subaxillaris
H. subaxillaris
H. viscida
1.0
F
H. oregona var. oregona
H. oregona var. compacta
H. oregona var. oregona
H. oregona var. compacta
1.0
H. oregona var. scaberrima
H. oregona var. rudis
H. oregona var. oregona
H. oregona var. oregona
Heterotheca sec. Heterotheca
Croptilon divaricatum
1.0
Croptilon hookerianum
1.0
Croptilon rigidifolium
Croptilon rigidifolium
Croptilon
H. brandegei
H. viscida
H. viscida
Tomentaurum niveum

Figure 1.6.1

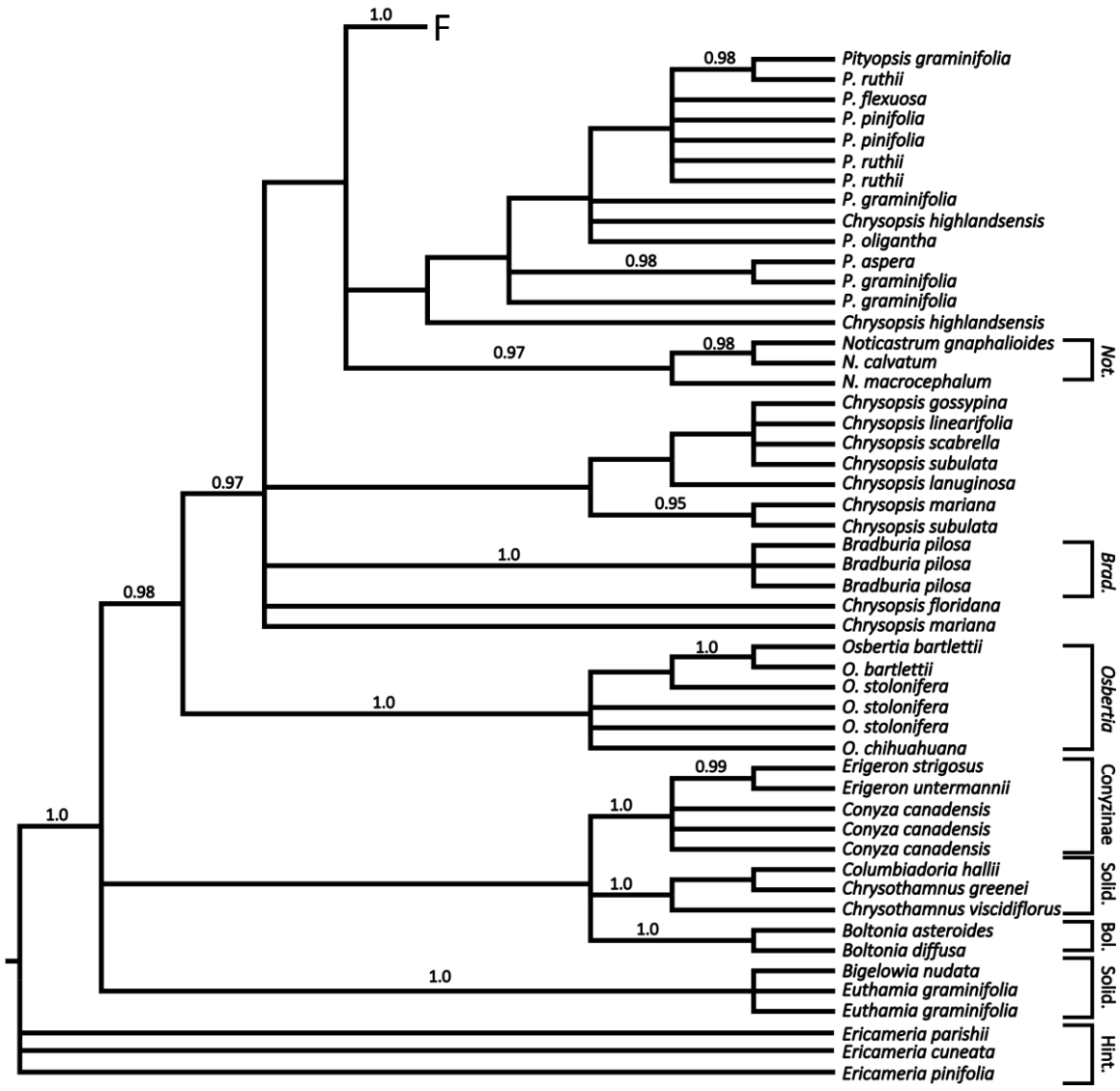
1.0
F
0.98
Pityopsis graminifolia
P. ruthii
P. flexuosa
P. pinifolia
P. pinifolia
P. ruthii
P. ruthii
P. graminifolia
Chrysopsis highlandsensis
P. oligantha
0.98
P. aspera
P. graminifolia
P. graminifolia
Chrysopsis highlandsensis
0.97
0.98
Noticastrum gnaphalioides
N. calvatum
N. macrocephalum
Not.
Chrysopsis gossypina
Chrysopsis linearifolia
Chrysopsis scabrella
Chrysopsis subulata
Chrysopsis lanuginosa
0.97
0.95
Chrysopsis mariana
Chrysopsis subulata
1.0
Bradburia pilosa
Bradburia pilosa
Bradburia pilosa
Brad.
0.98
Chrysopsis floridana
Chrysopsis mariana
1.0
Osbertia bartlettii
O. bartlettii
O. stolonifera
1.0
O. stolonifera
O. stolonifera
O. chihuahuana
Osbertia
0.99
Erigeron strigosus
Erigeron untermannii
1.0
1.0
Conyza canadensis
Conyza canadensis
Conyza canadensis
Conyzinae
Columbiadoria hallii
1.0
Chrysothamnus greenei
Chrysothamnus viscidiflorus
Solid.
1.0
Boltonia asteroides
Boltonia diffusa
Bol.
1.0
Bigelowia nudata
Euthamia graminifolia
Euthamia graminifolia
Solid.
Ericameria parishii
Ericameria cuneata
Ericameria pinifolia
Hint.

Figure 1.6.2

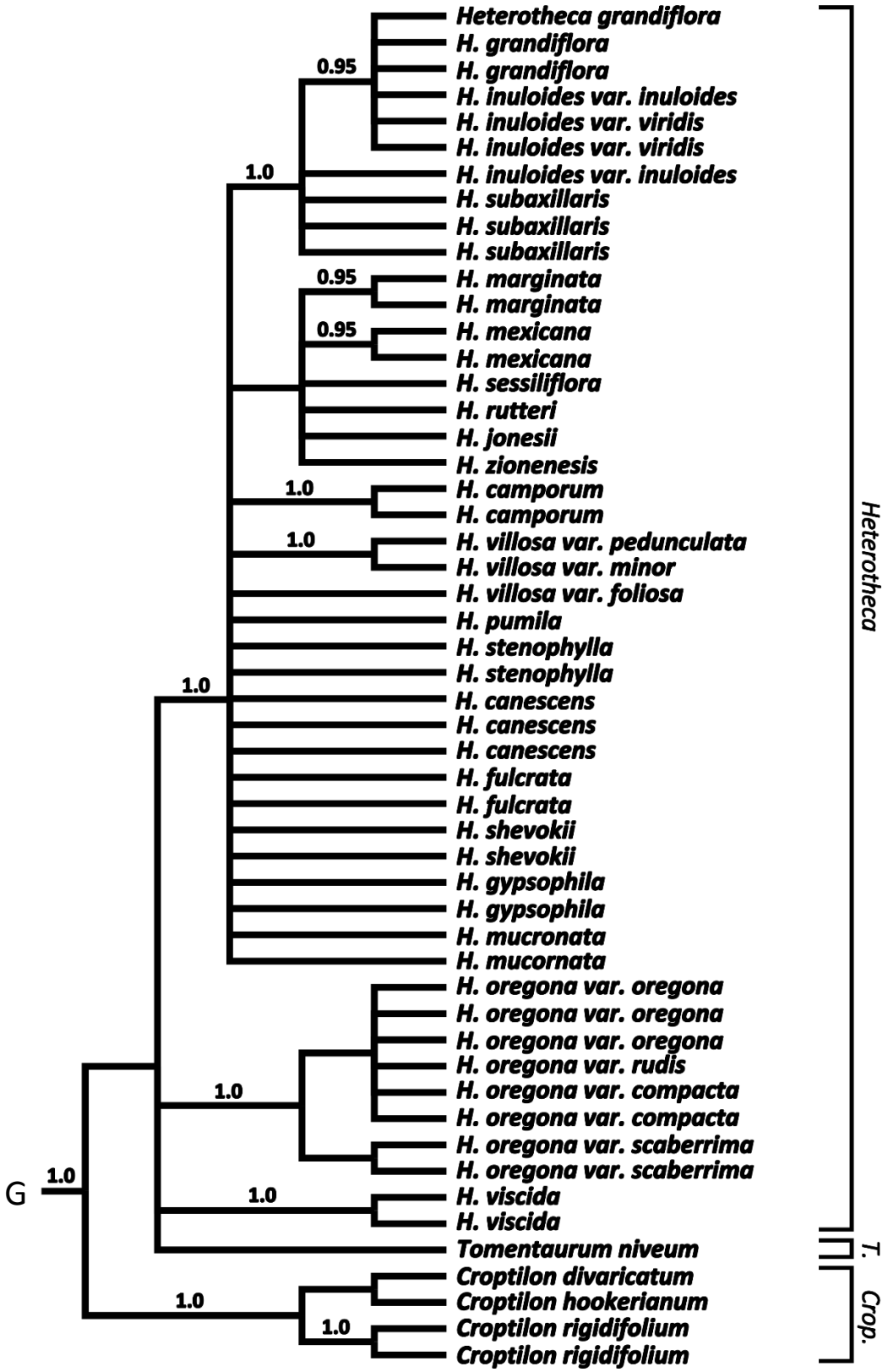
Heterotheca grandiflora
H. grandiflora
H. grandiflora
H. inuloides var. inuloides
H. inuloides var. viridis
H. inuloides var. viridis
H. inuloides var. inuloides
H. subaxillaris
H. subaxillaris
H. subaxillaris
H. marginata
H. marginata
H. mexicana
H. mexicana
H. sessiliflora
H. rutteri
H. jonesii
H. zionenesis
H. camporum
H. camporum
H. villosa var. pedunculata
H. villosa var. minor
H. villosa var. foliosa
H. pumila
H. stenophylla
H. stenophylla
H. canescens
H. canescens
H. canescens
H. fulcrata
H. fulcrata
H. shevokii
H. shevokii
H. gypsophila
H. gypsophila
H. mucronata
H. mucornata
H. oregona var. oregona
H. oregona var. oregona
H. oregona var. oregona
H. oregona var. rudis
H. oregona var. compacta
H. oregona var. compacta
H. oregona var. scaberrima
H. oregona var. scaberrima
H. viscida
H. viscida
Tomentaurum niveum
Croptilon divaricatum
Croptilon hookerianum
Croptilon rigidifolium
Croptilon rigidifolium
0.95
0.95
0.95
1.0
1.0
1.0
1.0
1.0
1.0
1.0
1.0
G
Heterotheca
T.
Crop.

Figure 1.7.1

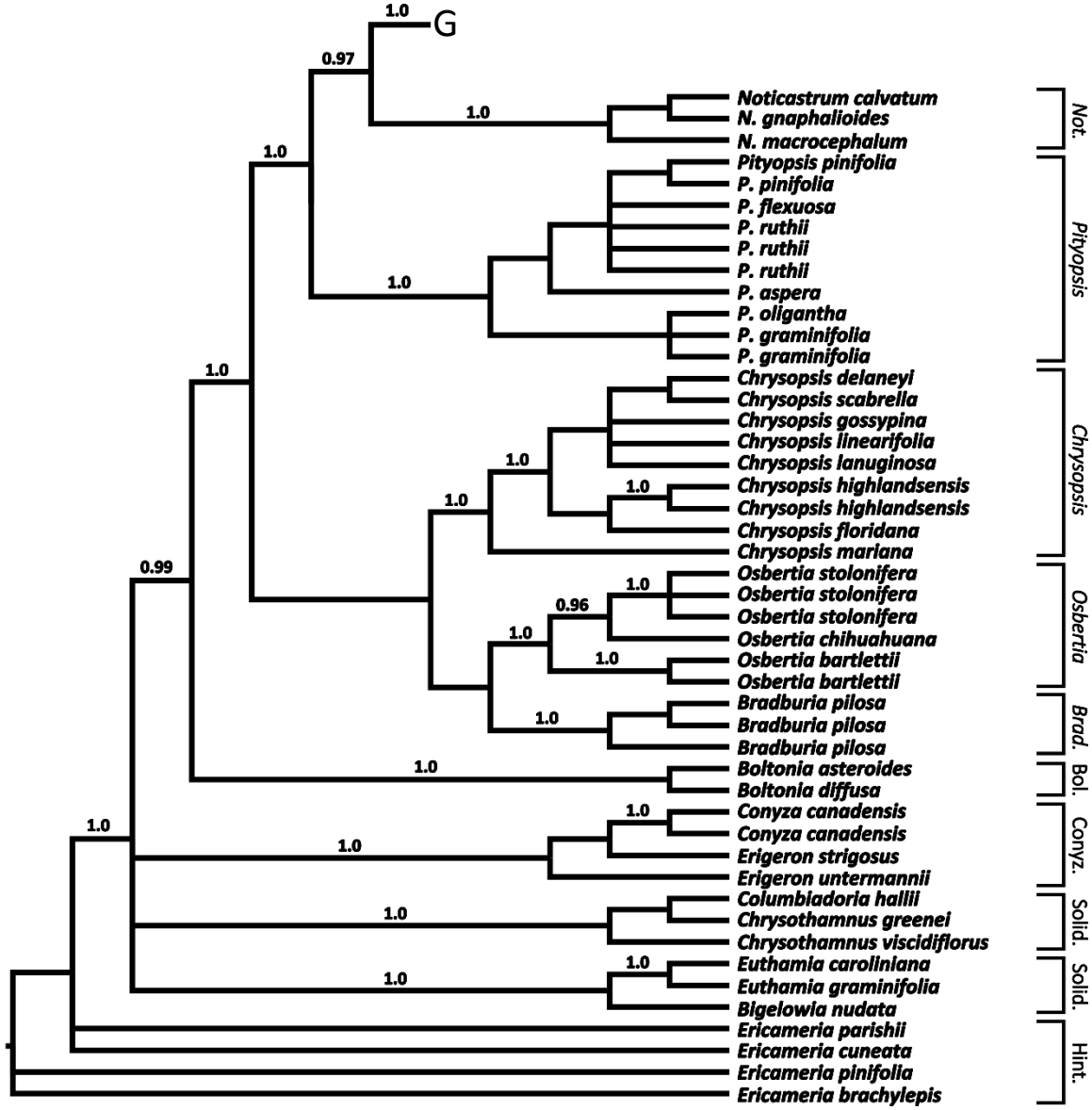
G
Noticastrum calvatum
N. gnaphalioides
N. macrocephalum
Pityopsis pinifolia
P. pinifolia
P. flexuosa
P. ruthii
P. ruthii
P. ruthii
P. aspera
P. oligantha
P. graminifolia
P. graminifolia
Chrysopsis delaneyi
Chrysopsis scabrella
Chrysopsis gossypina
Chrysopsis linearifolia
Chrysopsis lanuginosa
Chrysopsis highlandsensis
Chrysopsis highlandsensis
Chrysopsis floridana
Chrysopsis mariana
Osbertia stolonifera
Osbertia stolonifera
Osbertia stolonifera
Osbertia chihuahuana
Osbertia bartlettii
Osbertia bartlettii
Bradburia pilosa
Bradburia pilosa
Bradburia pilosa
Boltonia asteroides
Boltonia diffusa
Conyza canadensis
Conyza canadensis
Erigeron strigosus
Erigeron untermannii
Columbiadoria hallii
Chrysothamnus greenei
Chrysothamnus viscidiflorus
Euthamia caroliniana
Euthamia graminifolia
Bigelowia nudata
Ericameria parishii
Ericameria cuneata
Ericameria pinifolia
Ericameria brachylepis
Not.
Pityopsis
Chrysopsis
Osbertia
Brad.
Bol.
Conyz.
Solid.
Solid.
Hint.

Figure 1.7.2

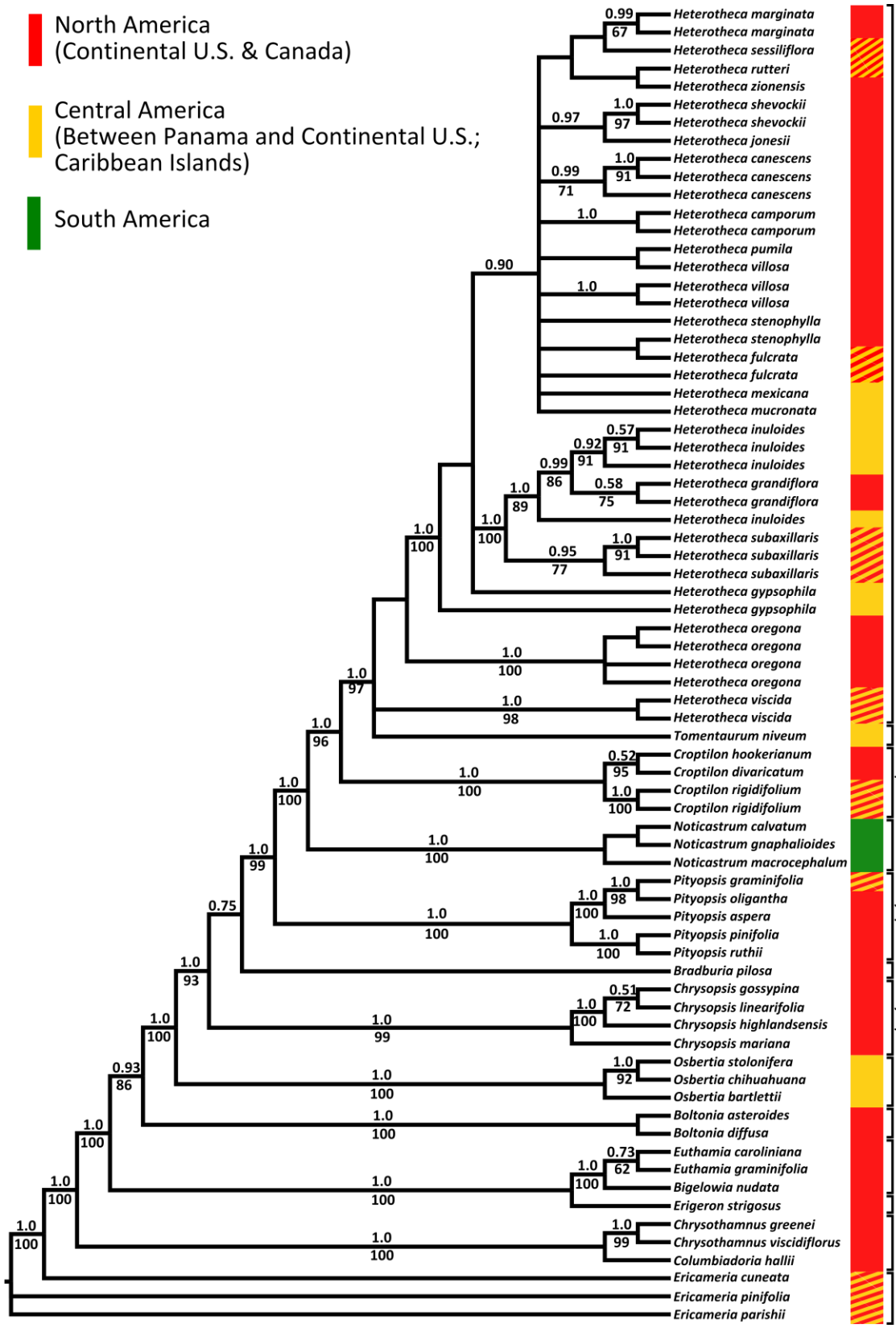
North America
(Continental U.S. & Canada)
Central America
(Between Panama and Continental U.S.;
Caribbean Islands)
South America
Heterotheca marginata
Heterotheca marginata
Heterotheca sessiliflora
Heterotheca rutteri
Heterotheca zionensis
Heterotheca shevockii
Heterotheca shevockii
Heterotheca jonesii
Heterotheca canescens
Heterotheca canescens
Heterotheca canescens
Heterotheca camporum
Heterotheca camporum
Heterotheca pumila
Heterotheca villosa
Heterotheca villosa
Heterotheca villosa
Heterotheca stenophylla
Heterotheca stenophylla
Heterotheca fulcrata
Heterotheca fulcrata
Heterotheca mexicana
Heterotheca mucronata
Heterotheca inuloides
Heterotheca inuloides
Heterotheca inuloides
Heterotheca grandiflora
Heterotheca grandiflora
Heterotheca inuloides
Heterotheca subaxillaris
Heterotheca subaxillaris
Heterotheca subaxillaris
Heterotheca gypsophila
Heterotheca gypsophila
Heterotheca oregona
Heterotheca oregona
Heterotheca oregona
Heterotheca oregona
Heterotheca viscida
Heterotheca viscida
Tomentaurum niveum
Croptilon hookerianum
Croptilon divaricatum
Croptilon rigidifolium
Croptilon rigidifolium
Noticastrum calvatum
Noticastrum gnaphalioides
Noticastrum macrocephalum
Pityopsis graminifolia
Pityopsis oligantha
Pityopsis aspera
Pityopsis pinifolia
Pityopsis ruthii
Bradburia pilosa
Chrysopsis gossypina
Chrysopsis linearifolia
Chrysopsis highlandsensis
Chrysopsis mariana
Osbertia stolonifera
Osbertia chihuahuana
Osbertia bartlettii
Boltonia asteroides
Boltonia diffusa
Euthamia caroliniana
Euthamia graminifolia
Bigelowia nudata
Erigeron strigosus
Chrysothamnus greenei
Chrysothamnus viscidiflorus
Columbiadoria hallii
Ericameria cuneata
Ericameria pinifolia
Ericameria parishii
Heterotheca
T.
Croptilon
Not.
Pityopsis
Br.
Chrysopsis
Osb.
Bol.
Solid. Con.
Solid.
Hint.

Figure 1.8

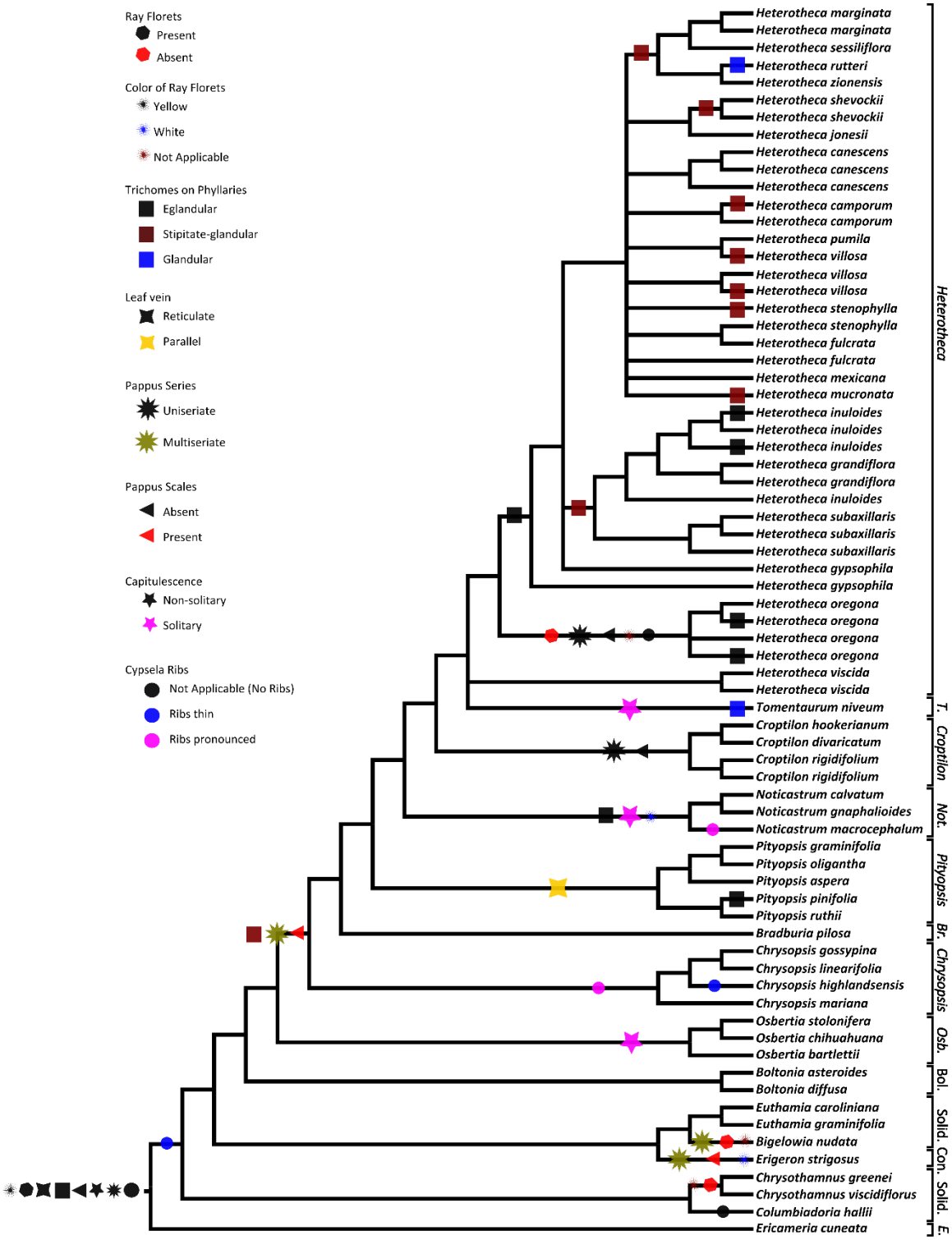
Ray Florets
Present
Absent
Color of Ray Florets
Yellow
White
Not Applicable
Trichomes on Phyllaries
Eglandular
Stipitate-glandular
Glandular
Leaf vein
Reticulate
Parallel
Pappus Series
Uniseriate
Multiseriate
Pappus Scales
Absent
Present
Capitulescence
Non-solitary
Solitary
Cypsela Ribs
Not Applicable (No Ribs)
Ribs thin
Ribs pronounced
Heterotheca marginata
Heterotheca marginata
Heterotheca sessiliflora
Heterotheca rutteri
Heterotheca zionensis
Heterotheca shevockii
Heterotheca shevockii
Heterotheca jonesii
Heterotheca canescens
Heterotheca canescens
Heterotheca canescens
Heterotheca camporum
Heterotheca camporum
Heterotheca pumila
Heterotheca villosa
Heterotheca villosa
Heterotheca villosa
Heterotheca stenophylla
Heterotheca stenophylla
Heterotheca fulcrata
Heterotheca fulcrata
Heterotheca mexicana
Heterotheca mucronata
Heterotheca inuloides
Heterotheca inuloides
Heterotheca inuloides
Heterotheca grandiflora
Heterotheca grandiflora
Heterotheca inuloides
Heterotheca subaxillaris
Heterotheca subaxillaris
Heterotheca subaxillaris
Heterotheca gypsophila
Heterotheca gypsophila
Heterotheca oregona
Heterotheca oregona
Heterotheca oregona
Heterotheca oregona
Heterotheca viscida
Heterotheca viscida
Tomentaurum niveum
Croptilon hookerianum
Croptilon divaricatum
Croptilon rigidifolium
Croptilon rigidifolium
Noticastrum calvatum
Noticastrum gnaphalioides
Noticastrum macrocephalum
Pityopsis graminifolia
Pityopsis oligantha
Pityopsis aspera
Pityopsis pinifolia
Pityopsis ruthii
Bradburia pilosa
Chrysopsis gossypina
Chrysopsis linearifolia
Chrysopsis highlandsensis
Chrysopsis mariana
Osbertia stolonifera
Osbertia chihuahuana
Osbertia bartlettii
Boltonia asteroides
Boltonia diffusa
Euthamia caroliniana
Euthamia graminifolia
Bigelowia nudata
Erigeron strigosus
Chrysothamnus greenei
Chrysothamnus viscidiflorus
Columbiadoria hallii
Ericameria cuneata
Heterotheca
T.
Croptilon
Not.
Pityopsis
Br.
Chrysopsis
Osb.
Bol.
Solid. Con.
Solid.
E.

Figure 1.9

Appendix 1: Permission for inclusion of published work from *Phytoneuron* in chapter 2 of thesis.

Roland,
Yes, email it. Make sure to check out the formatting instructions etc.

If some kind of formal permission from Phytoneuron is necessary, you have it from me (owner, editor, bottlewasher).

Guy

On Saturday, May 3, 2014 6:26 AM, "Roberts, Roland" <rroberts@towson.edu> wrote:
Hi Guy,
The manuscript is ready. Do we email it to you? Also, if this work will be a section of a MS thesis, how do we go about getting permission to include it in the thesis following publication in Phytoneuron?

Roland
****
Roland P. Roberts, Ph.D.
Associate Professor of Biology
Director TU Herbarium (BALT)
Dept. of Biological Sciences
Towson University, 8000 York Road
Towson, MD 21252-0001
email: rroberts@towson.edu
Phone: 410-704-3034 Fax: 410-704-2405

Appendix 2. CTAB extraction protocol

Sample Preparation

- Prepare 2ml tubes for tissue homogenizer with labels and white zirconia/silica beads
- Warm 2X CTAB to 60°C in water bath
- Homogenize samples in Mini Bead Beater 8 for 2min
- Spin for 1min at 13,000 RPM
- Under the hood
    - Add 600µl 2X CTAB to homogenized sample
    - Add small volume of PVP with sterilized spatula
- Vortex samples; invert and vortex again

Extraction and Isolation of DNA

- Incubate for 1hr 30min at 70°C.
    - Mix manually (DO NOT VORTEX) every 30min
- Add 600µl 24:1 chloroform: isoamyl alcohol to sample
    - Manually mix samples
- Spin for 20min at 13,000 RPM
    - Check for clear liquid and spin again if necessary
- Under the hood
    - Transfer top aqueous phase only to a new 1.5ml microfuge tube

DNA Precipitation

- Add 2µL of RNaseA and incubate 30min at 37°C
- Add 540µl cold isopropanol (-20°C)
    - Mix manually
- Incubate for 15min at 25°C
- Spin samples for 4min at 13,000 RPM

    - Decant supernatant (DNA is present in the pellet)
- Wash pellet with 500µl 75% EtOH and invert tubes or flick w/ finger several times
- Spin samples for 4min at 13,000 RPM
    - Decant supernatant and dry excess liquid by blotting with Kimwipe
- Dry with SpeedVac for 5min

Final Cleaning and Re-Suspension of DNA

- Resuspend pellet in 80µl 1X TE; break up pellet completely
- Add 8µl of 7.5M ammonium acetate, and 180µl of 100% EtOH
- Mix manually and incubate for 30min at 25°C
- Spin for 4min at 13,000 RPM, decant supernatant
- Add final wash of 500µl 75% EtOH, gently invert tubes
- Spin 4min at 13,000 RPM
    - Decant supernatant and dry excess liquid by blotting with Kimwipe
- Dry with SpeedVac for 5min
- Resuspend pelleted DNA with 100µL 1X TE and store at -20°C

Appendix 3. QIAGEN DNeasy® Plant Kit Protocol

- Disrupt samples (20-40mg desiccated plant tissue) using Mini Beadbeater-8
    - Centrifuge samples @ 14,000 RPM for 1 min
- Add 450µL Buffer AP1 and 4µL RNase A
    - Vortex samples to thoroughly mix
    - Incubate at 70°C for 60 min
    - Transfer to NEW 2mL microfuge tube
- Add 130µL Buffer P3
    - Mix by inversion
    - Incubate in ice for 60 min
- Centrifuge the lysate @ 14,000 RPM for 5 min
- Pipet clear lysate into a QIAshredder spin column (lilac) placed in a 2mL collection tube
    - Centrifuge @ 14,000 RPM for 2 min
    - Transfer the flow-through fraction (measure volume) into NEW 1.5mL microfuge tube without disturbing the cell-debris pellet
- Add 1.5 volumes of Buffer AW1. MIX IMMEDIATELY
- Pipet 650µL of the mixture into DNeasy® Mini spin column (white) placed in a 2mL collection tube
    - Centrifuge @ 8,000 RPM for 1 min
    - Discard flow-through. Blot-dry the collection tube and reuse
- Repeat step 7 with remaining sample
    - Centrifuge @ 8,000 RPM for 1 min
    - Discard flow-through and collection tube
    - Place DNeasy® Mini spin column into NEW 2mL collection tube
- Add 500µL Buffer AW2
    - Centrifuge @ 8,000 RPM for 1 min

    - Discard flow-through. Blot-dry the collection tube and reuse
- Add 500µL Buffer AW2
    - Centrifuge @ 14,000 RPM for 2 min
- Remove the spin column from collection tube carefully so that the column does not come into contact with the flow-through
    - Discard flow-through and collection tube
- Transfer the spin column to a new 1.5mL microfuge tube
- Add 50µL Buffer AE for elution
    - Incubate at room temperature for 10 min
    - Centrifuge @ 8,000 RPM for 1 min
- Repeat previous step.
- Store in -20°C

CURRICULUM VITA

NAME: CLAYTON M. COSTA

[redacted]

PROGRAM OF STUDY: BIOLOGY

DEGREE AND DATE TO BE CONFERRED: MASTER OF SCIENCE, 2014

Secondary education: Eleanor Roosevelt High, Graduated 2004

Collegiate institutions attended	Dates	Degree	Date of Degree
The Pennsylvania State University	2004-2008	Bachelor of Science	2008
Major: Science			
Towson University	2011-2014	Master of Science	2014
Program of Study: Biology			

Professional publications and presentations:

Costa, C.M. and R.P. Roberts. 2014. Techniques for improving the quality and quantity of DNA extracted from herbarium specimens. Phytoneuron 2014-48: 1–9.

Costa, C.M and Roberts, R.P. 2014. All roads lead to *Heterotheca*! Molecular phylogeny of subtribe Chrysopsidinae (Asteraceae, Astereae), based on nuclear ribosomal and chloroplast sequence data. The Association of Southeastern Biologists Meeting. Spartanburg, SC. April 2014.

Costa, C.M. and Roberts, R.P. 2013. Molecular phylogeny of the Goldenasters, subtribe Chrysopsidinae (Asteraceae, Astereae), based on nuclear ribosomal and chloroplast sequence data. Botany 2013. New Orleans, LA. July 2013.

Costa, C.M and Roberts, R.P. 2013. Molecular phylogeny of the Goldenasters, subtribe Chrysopsidinae (Asteraceae, Astereae), based on nuclear ribosomal and chloroplast sequence data. The Association of Southeastern Biologists Meeting. Charleston, WV. April 2013.

Costa, C.M. and Yang, S. 2009. Counting pollen grains using readily available, free image processing and analysis software. Annals of Botany 104 (5): 1005-1010.

Research Experience

Towson University Department of Biological Sciences

Master's Thesis Project – TU Plant Systematics Lab	Aug 2012 – 2014
Non-Thesis Research	Aug 2011 – Jul 2012

The Pennsylvania State University Department of Biology

Independent Study	Jul 2008 – Nov 2008
Internship - Laboratory of Dr. Katriona Shea	Jun 2008 – Aug 2008

Teaching Experience

Towson University – Biology I: Cellular Biology and Genetics (BIOL 201)

- Teaching Assistant – Spring 2014
- Teaching Assistant – Fall 2013
- Teaching Assistant – Spring 2013

Towson University – Biodiversity (BIOL 208)

- Guest lecture on Protostome diversity – Spring 2013
- Guest lecture on Fungal diversity – Spring 2012
- Guest lecture on Echinoderm diversity – Fall 2011

Awards and Honors

Earl Core Student Award 2013
Southern Maryland Resource Conservation and Development Scholarship 2013
Towson University Graduate Student Association Research Grant 2013
Towson University Travel Grant – Spring 2014, Summer 2013, Spring 2013

Professional Organization Memberships

Association of Southeastern Biologists (ASB)
Botanical Society of America (BSA)
The Smithsonian Institution – National Museum of Natural History (SI-NMNH)
Southern Appalachian Botanical Society (SABS)