Testing the Monophyly and Placement of *Lepechinia* in the Tribe Mentheae (Lamiaceae)

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**Abstract**—*Lepechinia* (Lamiaceae subf. Nepetoideae) is a New World genus composed of about 42 species distributed primarily from Northern California to Central Argentinia. Previous morphological and molecular studies on *Lepechinia* have raised questions on the monophyly of the genus and its placement within the tribe Mentheae. In this paper the phylogenetic placement and monophyly of *Lepechinia* is examined within the context of the tribe Mentheae using cpDNA (yeast and trnL-F) and nrDNA (ITS and ETS) markers. *Melissa* is shown to be sister to *Lepechinia* in both cpDNA and nrDNA analyses, and the monotypic genera Chaunostoma and Neoeplingia are found to be embedded within *Lepechinia*. The subtribe Menthinae is shown to be paraphyletic, with several genera needing to be reassigned. In particular, Neoeplingia should be included within the subtribe Salviinae. The genera *Heterolamium* and *Melissa*, both previously unplaced with regard to subtribe, are now clearly assigned to the subtribes Nepetinae and Salviinae, respectively. The cpDNA marker *trnL-F* has great phylogenetic utility, and is shown to be 50% more informative than *trnL-F* for the taxa used in this study.

**Keywords**—Chaunostoma, cpDNA, Melissa, molecular phylogeny, Neoeplingia, yeast.

The Lamiaceae is the sixth largest family of flowering plants, containing about 236 genera and over 7,000 species divided into seven subfamilies (Harley et al. 2004). This nearly cosmopolitan family is diverse in habitat and ranging, from tropical trees and lianas to annual temperate herbs and occurring in most terrestrial habitats. Synapomorphies for the family include hypogynous flowers, a quadrangular stem, opposite leaves, and indumentum, although there are rare inconsistencies in the latter three traits (Harley et al. 2004).

With a few notable exceptions (e.g. *Clerodendrum* L. and other genera formerly placed in Verbenaceae), the Lamiaceae as a family has been well-circumscribed historically (Bentham 1876; Briquet 1895–1897; Wunderlich 1967; Wagstaff et al. 1998), but subfamilial, tribal, and generic delimitations have been less satisfactory (Harley et al. 2004). During the past fifteen years the mints have been the focus of numerous molecular phylogenetic studies (Wagstaff et al. 1995; Wagstaff and Olmstead 1997; Wagstaff et al. 1998; Prather et al. 2002; Paton et al. 2004; Trusty et al. 2004; Walker et al. 2004; Bräuchler et al. 2005; Edwards et al. 2006; Walker and Sytsma 2007; Bramley et al. 2009; Bräuchler et al. 2010; Scheen et al. 2010; Yuan et al. 2010). These efforts have spurred taxonomic revisions at several levels (Cantino and Wagstaff 1998; Harley et al. 2004; Walker et al. 2004; Bräuchler et al. 2005; Yuan et al. 2010), and have led to an unprecedented understanding of relationships within the Lamiaceae, especially in regards to subfamilial and tribal designations (Fig. 1). However, despite this recent progress the relationships between many genera remain unclear, especially within the subfamily Nepetoideae (Cantino et al. 1992; Wagstaff et al. 1995; Paton et al. 2004; Walker et al. 2004; Bräuchler et al. 2010).

The subfamily Nepetoideae consists of about 105 genera (Harley et al. 2004) and is the largest and best-supported subfamily in the Lamiaceae (Wagstaff et al. 1995; Wagstaff et al. 1998; Paton et al. 2004). Notable synapomorphies include hexacolpate pollen, presence of rosmarinic acid, an investing embryo, gynobasic style, and exalbuminous seeds (Cantino and Sanders 1986; Harley et al. 2004). Within the Nepetoideae, three tribes are currently recognized (Elsholtzieae, Mentheae, and Ocimeae) with the Mentheae the largest, containing about 65 genera (Harley et al. 2004). A number of molecular studies have been conducted within the Nepetoideae (Wagstaff et al. 1995; Prather et al. 2002; Paton et al. 2004; Trusty et al. 2004; Walker et al. 2004; Bräuchler et al. 2005; Edwards et al. 2006; Walker and Sytsma 2007; Bräuchler et al. 2010). While the Mentheae is monophyletic (Trusty et al. 2004; Walker et al. 2004; Walker and Sytsma 2007; Bräuchler et al. 2010), generic relationships within the tribe remain rather murky.

*Lepechinia* Willd. (Lamiaceae subf. Nepetoideae) is a New World genus composed of about 42 species that have a primary distribution from Northern California to Central Argentinia, with disjuncts in the Dominican Republic (1), Hawaii (1), Socorro Island (1) and Reunion Island (1). The occurrences in Hawaii and Reunion Island are probably human introductions, however (Hart 1983; Harley et al. 2004; B. Drew, unpublished data). Carl Epling (Epling 1926; Epling 1948; Epling and Mathias 1957; Epling and Jativa 1968) was the first researcher to conduct a thorough treatment of *Lepechinia*. Prior to Epling, various *Lepechinia* species had been assigned to distant genera such as *Hyiptis* Jacq., *Stachys* L., *Hornimum* L., *Dracocephalum* L., *Rosmarinus* L., *Sidertis* L., *Gardoquia* Ruiz & Pav., and *Buddleja* L. (Epling 1948). While much of Epling’s taxonomic work at the species level remains the standard, Hart (1983) made substantial revisions to South American nomenclature and to Epling’s sectional assignments. Hart (1983) performed a thorough revision of the genus including a cladistic analysis based on morphological characters. He also documented the occurrence of dioecy within some South American *Lepechinia*, a rare feature within the Lamiaceae (Hart 1983; Harley et al. 2004). Apparently most, if not all, *Lepechinia* are diploid with chromosome numbers of 2n = 32 (Harley and Heywood 1992; Hickman 1993; Harley et al. 2004). No molecular phylogenetic analysis of the genus has been attempted.

Historically, the placement of *Lepechinia* within the Nepetoideae has been uncertain, at times even being placed in a tribe of its own (Epling 1948; Wunderlich 1967). The most recent treatment of the family places *Lepechinia* in the tribe Mentheae, subtribe Salviinae (Harley et al. 2004). Recent molecular studies (Wagstaff et al. 1995; Walker and Sytsma 2007) suggest the closest relatives to *Lepechinia* within the Mentheae are the Eurasian genus *Melissa* L. and the large “Salvia” clade (over 1000 species), which contains three lineages of *Salvia* L. and the small genera *Dorystachys* Boiss. & Heldr., *Meriandra* Benth., *Perovskia* Kar., *Rosmarinus*, and...
Zhumeria Rech. f. & Wendelbo (Walker et al. 2004; Walker and Sytsma 2007). However, these studies used only three to four species of Lepechinia, and did not always support monophyly of the genus, nor consistent relationships to other genera. Importantly, Chaunostoma Donn. Sm., a monotypic genus historically considered closely allied with Lepechinia, has never been included in any molecular phylogenetic analysis.

Despite the work of Epling, Hart, and subsequent molecular analyses, many questions remain with Lepechinia. Where does Lepechinia fit within the tribe Mentheae? Is Lepechinia monophyletic? How is Chaunostoma related to Lepechinia? Where did Lepechinia likely originate? We address these questions using a robust molecular phylogenetic framework based on cpDNA (ycf1, the ycf1-rps15 spacer, and trnL-F) and nrDNA comprising both ITS and ETS (external transcribed spacer). The lack of resolution in previous phylogenies is due in part to a reliance on a few molecular markers (e.g. only trnL-F and ITS) that appear insufficient to resolve relationships within the tribe Mentheae with convincing support. We also demonstrate the phylogenetic utility of the large chloroplast gene,ycf1 - a gene that has not been used previously in eudicot phylogenies (other than as part of whole plastome phylogenies).

Materials and Methods

Sampling and Outgroups—The phylogenetic analyses involved two separate but nested taxon sampling strategies (Appendix 1). The larger and taxonomically broader cpDNA phylogenetic framework contained 74 total taxa with 65 from the tribe Mentheae, including good generic coverage across the three subtribes of subf. Nepetoideae (Mentheinae, Nepetinae, Salviinae). Sampling within the subf. Nepetoideae outside the Mentheae included the tribes Elsholtzieae (two) and Ocimeae (five); Lamium L. (subf. Lamioideae) and Caryopteris Bunge. (subf. Aguioideae) served as outgroups (monophyletic). Selection of outgroups was based on Wagstaff et al. (1995) and our unpublished data. The smaller, more taxonomically focused nrDNA analysis of the subtribe Salviinae included 31 taxa. All but one (Salvia sclarea L.) of these 31 formed a subset of the larger cpDNA sampling. Of these taxa, 29 were from the subtribe Salviinae, with Hor nimium and Hedeoma Pers. (subtribe Mentheae) serving as outgroups (monophyletic). A representative from each of the eight sections of Lepechinia as outlined by Epling (Epling 1948; Epling and Mathias 1957) was included in both the cpDNA and nrDNA analyses. Due to some preliminary and unexpected results, two allopatric accessions of Lepechinia mexicana (S. Schauer) Epling were included in both data sets. Additionally, 13 out of the 14 subclades of “Salvia” as defined by the staminal lever mechanism (Walker and Sytsma 2007) were sampled for the cpDNA data set, and all 14 Salvia clades were sampled for the nrDNA data set. Lophanthus Adans. (14%), Meriandra (19%), and heterolanium C. Y. Wu (75%) were missing various amounts of cpDNA data due to DNA extraction from degraded herbarium specimens.

DNA Extraction, Amplification, and Sequencing—DNA was extracted from silica-dried plant material and herbarium specimens using the DNeasy™ plant mini kit (Qiagen, Valencia, California) according to manufacturer’s specifications. The one modification to the protocol involved heating the extracts at 65°C for 30 min (instead of 10) to break down secondary compounds that might interfere with subsequent amplifications. The PCR procedures were similar to those described in Sytsma et al. (2002), although some DNA samples (from subtribe Menthinae) were diluted in water 5 x prior to sequencing. The PCR products, obtained with TaKaRa Ex Taq (Otsu, Shiga, Japan), were diluted 30 x in water prior to cycle sequencing and subsequently cleaned using Agencourt magnetic beads (Agencourt, Beverly, Massachusetts). Cycle sequencing reactions used the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California). Samples were electrophoresed on an Applied Biosystems 3730xl automated DNA sequencing instrument, using 50 cm capillary arrays and POP-7 polymer. Data were analyzed using PE-Biosystems version 3.7 of Sequencing Analysis at the University Wisconsin-Madison Biotechnology Center.

Ycf1 is a variable coding region of unknown function (Drescher et al. 2000; Kleine et al. 2009) situated near the border of the inverted repeat (IR) and the small single copy region (SSC) of the chloroplast genome (Fig. 2). It is roughly 5,600 base pairs in length, and differs widely in variability depending on how much resides within the inverted repeat. Ycf1 has been shown to be rapidly evolving in orchids (Neubig et al. 2008), even eclipsing the rapidly evolving matK. Because the first ~1,000 base pairs of ycf1 lie within the inverted repeat in the mint family (see Fig. 2; B. Drew, unpubl. data) and are relatively uninformative (Perry and Wolfe 2002), only about 100 bp of this region was amplified. The remaining ~4,600 nucleotides of ycf1 and 500 nucleotides of the ycf1-rps15 spacer were amplified and sequenced primarily by using a series of 14 overlapping primers (Table 1; Fig. 2). The chloroplast region trnL-F was amplified primarily by using the ‘C’ and ‘F’ primers, but the internal ‘D’ and ‘E’ primers were necessary to amplify and sequence some herbarium specimens.
ITS was amplified using the primers Lue1 (Baldwin 1992) and ITS4 (White et al. 1990) for most taxa. The internal primers ITS2 and ITS3 (White et al. 1990) were used to amplify material from herbarium specimens. Combinations of these primers were used for sequencing. ETS was first amplified using 18S-IGS (Baldwin and Markos 1998) and ETS-B (Beardsley and Olmstead 2002), but a Nepetoideae specific primer (ETS-bdfl–GTGAGTGGGTGTTGCGGTYGT) was designed and used for the majority of PCR reactions. The primers 18S-E (Baldwin and Markos 1998) and ETS-bdfl (initially ETS-B) were used for sequencing.

**Phylogenetic Analyses**—Sequences of ycf1, the ycf1-rps15 spacer, trnL-F, ITS and ETS were manually edited in Sequencher 4.7 (Gene Codes, Ann Arbor, Michigan) and the resulting sequences were manually aligned in Se-Al v2.0a7b (Rambaut 2003) and/or MacClade 4.08 (Maddison and Maddison 2005). Maximum parsimony (MP) was performed in PAUP* 4.0b10 (Swofford 2002) by sampling 1,000 random addition replicates with TBR branch swapping and Multrees On. Bootstrap (Felsenstein 1985) values were obtained by performing 1,000 heuristic searches using all characters, with 10 TBR branch swapping replicates per bootstrap, and saving no more than 5,000 trees per replicate. In the cpDNA dataset nucleotide positions coded for multiple states were treated as uncertainties, but for the nrDNA dataset they were treated as polymorphisms. Maximum likelihood (ML) analysis was conducted in GARLI v1.0 (Zwickl 2006) using default parameters and a model of evolution (TVM + G for chloroplast; GTR + G + I for nuclear) inferred from Modeltest v.3.7 (Posada and Crandall 1998). One hundred bootstrap repetitions were conducted using the same ML settings as the initial search. Bayesian analysis was performed in MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001) and implemented on the Cyberinfrastructure for Phylogenetic Research (CIPRES) cluster (http://www.phylo.org/). For both the nuclear and chloroplast data sets analyses were run for two million generations using the default settings. The first 25% of trees were discarded as burnin. Gaps were treated as missing data in all analyses.
Separate analyses were conducted with single and combined datasets. For the broader Mentheae-wide cpDNA study, the three regions ycf1, ycf1-rps15, and trnL-F were concatenated into a single data matrix, although ycf1 was evaluated separately to compare its phylogenetic signal to the combined set. For the narrower Salviinae-wide study, the three datasets ITS, ETS, and cpDNA were examined separately. In addition, nrDNA (ITS and ETS) combined and total evidence (nrDNA and cpDNA combined) data sets were analyzed. Alignments are available at TreeBASE (study number SI0999). Congruence between the ITS and ETS datasets and between the cpDNA and nrDNA datasets (with only taxa in common) was assessed using the ILD test (Farris et al. 1995) as implemented in PAUP. Shortcomings of the ILD test are known, especially a rejection of the null hypothesis of combinability when in fact the combined data outperforms the individual data sets (e.g. Yoder et al. 2001; Barker and Lutzeni 2002). However, further analyses indicate that the ILD is useful as a first examination of congruence (Hipp et al. 2004). Incongruent data sets, as suggested by the ILD test, were further explored in two ways. First, nodes in disagreement between data sets were examined for support values [MP and ML bootstrap, and Bayesian posterior probabilities (PP)] to find strong discordance, if any. Second, one or more taxa were removed in an iterative process prior to phylogenetic analysis and the ILD test was implemented to find taxa, if any, contributing to the discordance.

### Results

**Phylogenetic Analyses within Tribe Mentheae**—The variability of the three cpDNA regions sampled is summarized in Table 2. The combined cpDNA data matrix was 6,949 bp when aligned. The majority of the data set came from ycf1 with an aligned length of 5,014 bp, of which 65 bp were excluded due to ambiguity. The portion of the sequence that straddled the inverted repeat and the SSC region was particularly recalcitrant (Logacheva et al. 2009) in terms of alignment. The ycf1-rps15 spacer region had a length of 869 aligned nucleotides, of which 159 characters were excluded. The trnL-F data set was 1,066 bp, with 106 bp excluded. Most of the excluded characters from the ycf1-rps15 spacer and the trnL-F data matrices were due to long uninformative or ambiguous insertions. Parsimony analysis of the concatenated set of cpDNA regions found four MP trees of length 7,048 (CI = 0.608, RI = 0.759, RC = 0.461). The ML tree is shown in Fig. 3a to illustrate relative branch lengths, and the strict consensus of the four MP trees is shown in Fig. 3b. The ML and Bayesian trees were topologically similar to the parsimony tree, differing only in the placement of Elsholtzia Willd. + Callistonia C. L. clade (sister to the rest of subfamily in the MP trees) and in the placement of the two clades Lycopus L. and Horminum + Cleonia L. + Prunella L. (order switched). Most of the branches have high support values (MP and ML bootstrap, PP; Fig. 3). Two of the subtribes within Mentheae are strongly monophyletic (Salviinae and Nepetinae). The third, Mentheinae, is paraphyletic relative to Nepetinae and Salviinae due to the placement of Hyssopus L., Lycopus, Horminum, Cleonia, Prunella, and Neoplingia Ramamoorthy, Hiriart & Medrano, (Fig. 3), genera that were included in the subtribe Mentheinae by Harley et al. (2004). The subtribes Nepetinae and Mentheinae (together with the first five aforementioned genera) form a strongly monophyletic group that is sister to Salviinae.

**Phylogenetic Analyses within Subtribe Salviinae**—The more taxonomically focused analyses of subtribe Salviinae included ITS and ETS data sets examined separately and combined, and finally these nrDNA data sets were combined with cpDNA. The ITS region had an aligned length of 706 bp (after the exclusion of six characters from the ITS2 region), while ETS was 450 bp. For the combined dataset of 1,156 bp, 561 (ITS-274; ETS-287) characters were parsimony-informative. The MP analysis of ITS alone gave four trees of length 813 (CI = 0.517, RI = 0.530, RC = 0.274). The MP analysis of ETS alone generated 28 trees (length = 749 CI = 0.538, RI = 0.541, RC = 0.291). The ILD test indicated that there was significant discordance between the ITS and ETS datasets (p < 0.001). Iterative removal of taxa prior to the ILD test provided no evidence for “rogue” taxa contributing to the discordance. The removal of Chaunostoma and Salvia patens Cav. from both ITS and ETS data sets allowed passing (although barely) of the ILD test. An examination of the topologies of the datasets revealed most differences occurred within the Salvia clade, but some minor differences also occurred within Lepechinia. However, all discordances in topology involved weak branch support (BS < 60) and thus no hard incongruencies existed between the datasets. Based on these results, the ITS and ETS data sets were considered non-discordant and subsequently combined.

The ML tree of the subtribe Salviinae with nodal support (MP and ML bootstrap, and Bayesian PP) is shown in Fig. 4. Parsimony analysis of the combined nrDNA found two MP trees of length 1,739 (CI = 0.561, RI = 0.512, RC = 0.287). The ML and Bayesian topologies were similar to the consensus parsimony tree, with differences only weakly supported in all three analyses. Both the backbone of the subtribe Salviinae and early branching events in Lepechinia are weakly supported at a number of nodes with the nrDNA data set, giving rise to most of the differences in topology relative to cpDNA (i.e. that seen in Fig. 3 when taxa not sampled in the Salviinae study are removed). When the cpDNA data set

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Chloroplast phylogeny of subfamily Nepetoideae, with Lamium and Caryopteris as outgroups. A. ML phylogram. B. ML cladogram with support values indicated (MP and ML bootstrap values above branches, Bayesian PP below). Asterisks indicate full support in all three analyses, dashes indicate <50% support, branches with an X collapse in the parsimony strict consensus tree. The M to the right of some taxa signifies that these genera were placed in the Menthinae by Harley et al. (2004). The ? indicates genera whose subtribal placement is uncertain.
was tested against the combined nrDNA, the ILD test showed significant discordance \((p < 0.001)\). The cpDNA and nrDNA data sets passed the ILD test \((p = 0.115)\) with the exclusion of Rosmarinus and Lepechinia chamaedryoides (Balb.) Epling. This suggests that the two data sets are not fundamentally incongruent. When cpDNA and nrDNA data sets were combined, the resulting trees under MP, ML, and Bayesian returned the topology based on cpDNA (see Fig. 3).

**Placement and Relationships of Lepechinia**—All cpDNA and nrDNA support the placement of Lepechinia within the subtribe Salviinae of the Mentheae. The Salviinae in turn is sister to the rest of the tribe Mentheae (subtribes Nepetinae and Menthinae). This position of Salviinae is well supported in all cpDNA analyses (Fig. 3). Within the Salviinae, Melissa is well supported as sister to a clade containing Lepechinia, Neoeplingia, and Chaunostoma in both cpDNA (Fig. 3) and nrDNA (Fig. 4) analyses. The cpDNA evidence is strong for a sister relationship of Melissa, Lepechinia, Neoeplingia, and Chaunostoma to the “Salvia” clade (Fig. 3). However, nrDNA neither supports nor discounts this relationship due to the lack of resolution in the backbone of the Salviinae (Fig. 4).

All analyses indicate that Lepechinia is not monophyletic as presently circumscribed. With both the cpDNA and nrDNA data sets the monotypic genera Neoeplingia and Chaunostoma are embedded within Lepechinia. In the cpDNA analysis, Neoeplingia emerges as sister to two populations of the Mexican Lepechinia mexicana, and these two species form a well-supported clade with Chaunostoma (Fig. 5). Together, these three taxa are resolved as sister to the rest of Lepechinia with full support in MP, ML, and Bayesian trees (Fig. 3). In the nrDNA analysis, Neoeplingia, Chaunostoma, and Lepechinia mexicana also form a clade embedded within Lepechinia, but this clade is not strongly supported (<50% and 75% bootstrap for MP and ML, respectively, 1.00 PP; see Fig. 4). Due to lack of resolution at
the base of Lepechimia in the nrDNA tree (Fig. 4), the nrDNA data neither support nor discount the cpDNA finding that Neoeplingia, Chaunostoma, and Lepechimia mexicana are sister to the remainder of Lepechimia. The cpDNA and total evidence trees for the subtribe Salviinae do support a biogeographical scenario for Lepechimia diversifying in Mesoamerica. The central Mexican clade of Neoeplingia, Chaunostoma, and Lepechimia mexicana is sister to a clade with two subclades, one Mexican (with two later dispersal events to Mediterranean California and northern South America) and the other South American.

**Discussion**

The Phylogenetic Utility of ycf1—The largest open reading frames in land plant chloroplast genomes are ycf1 and ycf2 with putative protein products of around 1,901 and 2,280 amino acids, respectively (Drescher et al. 2000; De Las Rivas et al. 2002; Morris and Duvall 2010). Although the function of ycf1 is uncertain and debated (De Las Rivas et al. 2002), the ycf1 product was found to be essential for cell survival in Nicotiana L. (Drescher et al. 2000) and the gene to be under selective pressure in Pinus L. (Parks et al. 2009). This study demonstrates the utility of the cpDNA gene ycf1 in phylogenetic studies at different taxonomic levels in Lamiaceae from closely related species to between subfamilies. This is the first explicit use of ycf1 in phylogenetic studies of eudicots as previous phylogenetic studies have been restricted to Orchidaceae (Neubig et al. 2008; Chase et al. 2009) and Pinaceae (Parks et al. 2009). In the tribe Mentheae, ycf1 was considerably more variable and informative than trnL:F (Table 2). The ycf1 data matrix yielded 4,949 aligned characters, of which almost 29% were parsimony-informative. The trnL:F alignment had 960 characters, of which only 17% were parsimony-informative. The ycf1 gene was also much easier to align than either trnL:F or the ycf1-rps15 spacer region. Likewise, Neubig et al. (2008) demonstrated the ease of aligning ycf1 across the family Orchidaceae and that ycf1 was more variable than matK both in total number of parsimony-informative characters and in percent variability. In addition, nearly 100 insertion/deletion events ranging from three to 12 bp (in multiples of 3s) were evident in our Mentheae-wide data set. The MP phylogenetic analyses of these scored indels as an appended set of binary characters to the cpDNA data set (following the method of Baum et al. 1994) indicated little homoplasy in these indels and increased branch support for many nodes (trees not shown). Though these indels are easily treated in parsimony, there is presently no widely accepted model to evaluate this information in a ML or Bayesian framework (but see Bräuchler et al. 2010), so these characters were left out of the final analyses.

Several aspects of ycf1 structure, placement, and evolution, however, should be viewed with caution. First, its placement at the intersection of the IR and SSC regions (Fig. 2) can affect its structure. In most plastid genomes of land plants examined to date, ycf1 spans the junction of the IR and one end of the SSC (as shown in Fig. 2). Thus, a small portion of the 5′ end is duplicated on both ends of the IR, a region that has a slower rate of molecular evolution relative to the SSC region (Wolfe et al. 1987). However, the well-known expansion or contraction of the IR (Palmer 1991; Goulding et al. 1996) can cause ycf1 to become imbedded within the IR (e.g. Jasminum L. in Oleaceae, Lee et al. 2007). Second, ycf1 is known to be independently lost in some land plant plastid genomes (Roper et al. 2007; Cai et al. 2008; Wu et al. 2009; Gao et al. 2010; Morris and...
Duvall 2010). The Poales exhibit an interesting loss of ycf1. It is found intact in Typhaceae (Gusinger et al. 2010), nearly intact but as a pseudogene in an early diverging grass (Anomochloa Bronn., Morris and Duvall 2010), and essentially lost, except for a small remnant in the IR, of all other grasses examined (Gusinger et al. 2010; Morris and Duvall 2010).

When ycf1 is present, its large size, ease of alignment at least up to the family level in both monocots and eudicots, and relatively high numbers of phylogenetically informative characters (and indels) should make ycf1 an ideal new cpDNA gene region for phylogenetic studies. With low-copy nuclear regions becoming more readily available and entire plastome sequencing becoming more common (De Las Rivas et al. 2002; Jansen et al. 2007; Moore et al. 2007; Gao et al. 2010), one might question the continued utility of relying on multiple, but individual small chloroplast genes or spacers. We argue that phylogenetic inferences will continue to rely on using relatively small parts of the chloroplast genome because this approach (1) will remain cheaper than whole plastome sequencing for the foreseeable future, (2) is much less labor and time-intensive for phylogenetic analyses with many taxa, (3) is more feasible with herbarium and/or less-than pristine plant material, (4) can achieve resolution comparable to entire plastome gene datasets in some studies, for example using ycf1 at almost 6,000 base pairs (Parks et al. 2009), and (5) can readily provide (in conjunction with nuclear DNA data) valuable maternal genome information when hybridization/introggression is likely or suspected in the histories of species (e.g. Jabaily and Sytsma 2010).

Systematic Implications for the Tribe Mentheae—Generic sampling within the Mentheae, with 65 recognized genera, was not comprehensive for the cpDNA analysis, but did span all major groupings proposed within the three subtribes (Harley et al. 2004). Although it was not the intent of this study to address systematic issues within the tribe Mentheae, five significant issues are resolved. First, the subtribes Salviniae (including Melissa and Neoeplingia) and Nepetinae (including Hyssopus) as sampled are monophyletic, whereas subtribe Mentheae is paraphyletic on account of the placement of Neoeplingia, Hyssopus, Horminum, Cleonia, Prunella, and Lycopus (Fig. 3). The Salviniae are strongly supported as sister to the remainder of the tribe. Second, the genera Horminum, Cleonia, Prunella, and Lycopus, are problematic in regards to subtribe delimitation. The cpDNA tree (Fig. 3) strongly unites the first three genera as a clade. Together with Lycopus, they form a weakly supported grade leading to a strongly supported clade comprising both the subtribe Nepetinae and the remainder of subtribe Mentheae. These four genera were placed in the Mentheae by Harley et al. (2004), but phylogenetic (Wagstaff et al. 1995; Trusty et al. 2004; Walker and Sytsma 2007) and morphological (Moon et al. 2009; Ryding 2010) studies have consistently suggested they should be placed elsewhere. We agree with the explicit recommendations of Ryding (2010) that the monophyletic clade of Horminum, Cleonia, and Prunella should be segregated into a distinct subtribe, and the subtribal assignment of the enigmatic genus Lycopus should be treated as incertae sedis until greater support for its placement is found.

Third, the Chinese monotypic genus Heterolamium was previously unplaced within the subfamily Nepetoideae (Harley et al. 2004), but is nested well within subtribe Nepetinae based on these cpDNA analyses (Fig. 3). A placement of Heterolamium within Nepetinae was also suggested by Moon et al. (2008) based on nutlet morphology, but this is the first molecular phylogenetic study to clarify its position. Heterolamium is strongly placed within a clade of the circumboreal Mechnia and Eurasian Glechoma. Preliminary analysis of this group with ITS and ITS data in the context of a larger Nepetoideae study also supports this finding (B. Drew, unpub. data). Fourth, the small genus Hyssopus should be placed within the subtribe Nepetinae, not within the Mentheae as suggested by Harley et al. (2004). The placement of Hyssopus within the Nepetinae was first shown by Trusty et al. (2004). And fifth, the widely distributed North (nine species) and South American (ten species) Cunila D. Royen ex L. is not monophyletic according to cpDNA data presented here with two species sampled (Fig. 3). The two species are each sister to Glechon marfolia Benth. or Rhabdocaulon strictus (Benth.) Epling, respectively, but with all four taxa forming a strongly supported clade. The non-monophyly of Cunila mirrors the findings of Walker and Sytsma (2007). However, as only two South American accessions of Cunila were included in this study, it is premature at this time to suggest taxonomic changes.

Lepechinia Is Placed Within Subtribe Salviniae—Both cpDNA (Fig. 3) and nrDNA (Fig. 4) data strongly confirm previous findings (Harley et al. 2004; Walker and Sytsma 2007) that Lepechinia falls within the subtribe Salviniae. Additionally, these molecular results support Gentry and Vasquez’s (1993) suggestion that (the Andean upland) Lepechinia is “essentially a small-flowered 4-stamened version of Salvia, but neither calyx nor corolla very bilabiate.” Within the subtribe Salviniae, six genera (Salvia, Meriandra, Dorystaechas, Zhumeria, Rosmarinus and Perovskia) possess only two fertile stamens and are strongly monophyletic based on cpDNA (Fig. 3). Salvia, as demonstrated earlier (Walker et al. 2004; Walker and Sytsma 2007), is polyphyletic (Fig. 3) with at least three independent origins within subtribe Salviniae of the unusual “Salvia” stamen morphology (Walker and Sytsma 2007), presumably a “key innovation” permitting pronounced species diversifications in each of the three relative to their sister taxa. The clade comprising Lepechinia, Chaunostoma, Neoeplingia and Melissa, all possessing four rather than two fertile stamens, is strongly monophyletic and sister to the larger “Salvia” clade (Fig. 3). Although stamen number appears to be fairly homoplasious within the tribe Mentheae (Harley et al. 2004), it does appear that the shift to two stamens is a synapomorphic character for the “Salvia” clade within subtribe Salviniae.

The sister relationship of Melissa to Lepechinia (including Chaunostoma and Neoeplingia, see below) is consistent with previous preliminary molecular phylogenetic findings (Walker and Sytsma 2007), but is by no means universally accepted. Melissa is a genus of four species distributed across the Eurasian subcontinent, Northern Africa, and Macronesia. Harley et al. (2004) placed Melissa within the tribe Mentheae, but did not assign it to a subtribe. This treatment was influenced by results of an earlier and preliminary cpDNA restriction site analysis (Wagstaff et al. 1995) that placed Melissa outside the tribe Mentheae and sister to a clade consisting of the tribes Elsholtzieae and Mentheae. Ryding (2010) advocated placing Melissa outside of Salviniae based on pericarp structure and the results of Wagstaff et al. (1995). Indeed, Melissa does not appear to share any obvious morphological synapomorphy with members of the subtribe Salviniae. The genus is somewhat similar to some species of Salvia in calyx appearance and the presence of mucilaginous fruits, but clearly different in other ways (e.g. stamen number, corolla...
architecture, pericarp structure). However, chromosome number may be a synapomorphy uniting *Melissa* with *Lepechinia*. *Melissa* is known to possess 16, 17, or 32 pairs of chromosomes (Harley et al. 2004), while *Lepechinia* has been reported to have 16, 17, or 33 pairs (Epling 1948; Beaman et al. 1962; Moscone 1986; Harley and Heywood 1992; Hickman 1993; Harley et al. 2004). Unfortunately, chromosomal information is not available for *Neoeplingia* or *Chaunostoma*.

**Phylogenetic and Biogeographical Relationships Within Lepechinia—**Our sampling of *Lepechinia*, with representatives selected from each of Epling’s (Epling 1948; Epling and Mathias 1957) sections, is broad enough to provide preliminary phylogenetic and biogeographical findings. These findings, importantly, are so far consistent with ongoing analyses focusing on phylogenetic and biogeographic issues across *Lepechinia* using other gene regions (Drew et al. 2010). The most interesting and important result is that two rare, Meso-American monotypic genera, *Neoeplingia* and *Chaunostoma*, are embedded in *Lepechinia* as currently circumscribed (Figs. 3–5). In the cpDNA trees (Fig. 3), these two genera, along with *Lepechinia mexicana*, form a well-supported clade that is sister to the rest of *Lepechinia*. Of these two monotypic genera, *Neoeplingia* is sister to *Lepechinia mexicana*, with *Chaunostoma* then sister to these two. Likewise, all analyses involving nrDNA yield a clade of *Chaunostoma*, *Neoeplingia*, and *Lepechinia mexicana*, but support values for the clade and for its position within *Lepechinia* are weak (Fig. 4).

*Neoeplingia leucophylloides* is known only from the type locality in Hidalgo, Mexico (Fig. 5). *Neoeplingia* is uncommon in this rugged area with sparse vegetation, and was only observed growing in open areas on calcareous soil. The type locality for *Neoeplingia* is part of a broad but fragmented xeric floristic assemblage, with the fragments having high rates of endemism due to their geographic isolation from similar xeric habitats (Parga-Mateos et al. 1996). The species was first collected, described, and placed in a new genus in 1982 (Ramamoorthy et al. 1982). As part of the species description, the authors compared *Neoeplingia* to *Hedeoma*, *Hesperozygis* Epling and *Polomintha* A. Gray (subtribe Menthinae) for reasons that are somewhat unclear. The characters presented in their published table would indicate more similarity to *Lepechinia* as a whole than to the former three. The views of Ramamoorthy et al. (1982) led Harley et al. (2004) to place *Neoeplingia* in subtribe Menthinae (and not *Salviinae*). In overall appearance, habit, and habitat, *Lepechinia mexicana* and *Neoeplingia leucophylloides* Ramamoorthy, Hiriart & Medrano are similar in many respects (Table 3; Fig. 5): (1) flowers born in axillary cymes, (2) flowers small with blue corollas, (3) calyces nearly actinomorphic and which do not inflate much in fruit (especially when compared to other *Lepechinia* species), (4) leaves xeromorphic (small and thick), and (5) occurrences in a habitat much more xeric than the rest of *Lepechinia*.

As *Lepechinia mexicana* was found growing near individuals of *Neoeplingia leucophylloides*, a possible scenario for hybridization and/or chloroplast capture existed. Several lines of evidence indicate no history of hybridization between these two taxa. First, nrDNA and cpDNA analyses gave exactly the same relationships for the two taxa with no mosaic signal between plastid and nuclear trees (Figs. 3–4). Second, both ITS and ETS were congruent and showed no evidence of analogous copies from two parental species. Third, we included two accessions of *Lepechinia mexicana*, one sympatric with *Neoeplingia* and the other allopatric from over three hundred kilometers away (Fig. 5). The two accessions of *Lepechinia mexicana* were monophyletic in both cpDNA and nrDNA analyses (Figs. 3–5). Lastly, two widely spaced individuals of *Neoeplingia* were sampled and found to be identical with respect to nrDNA and cpDNA sequences (data not shown). We can thus strongly discount hybridization and/or chloroplast capture as explanations for the surprising close relationship of *Neoeplingia leucophylloides* to *Lepechinia mexicana*. As a result of our findings, *Neoeplingia* should clearly be treated as a member of the subtribe *Salviinae*, not the Menthinae.

In contrast to *Neoeplingia*, *Chaunostoma meciandrum* Donn, Sm. has been considered closely related to *Lepechinia* (Epling 1948; Hart 1983; Walker and Sytsma 2007), but was generally believed to be sister to *Lepechinia* as opposed to embedded within it as shown in this study (Figs. 3–5). *Chaunostoma* has been maintained as a distinct genus mainly due to its cauliflorous inflorescence type and arched exserted stamens (Epling 1948). It also differs from most *Lepechinia* by its occurrence in mesic, cloud forest habitats. *Chaunostoma meciandrum* is known from only four collection localities at similar elevations in southern Mexico (Chiapas, Guatemala, and El Salvador. *Chaunostoma* is so poorly known and collected that floral color is listed as red in the type description (Smith 1895) and in the most recent description (Harley et al. 2004). The actual floral color is light blue based on the El Salvadoran population sampled here (Fig. 5). Interestingly, *Chaunostoma meciandrum* is not morphologically similar to its sister Mexican taxa, *Lepechinia mexicana* and *Neoeplingia leucophylloides*. Rather, *Chaunostoma* is more similar to the *Lepechinia* species of California or to the Mexican *L. hastata* (A. Gray) Epling in terms of leaf appearance, leaf odor, corolla size, and habit.

The placement of this Mexican clade (*Chaunostoma meciandrum, Neoeplingia leucophylloides, and Lepechinia mexicana*) as sister to a clade of all other *Lepechinia* (strongly supported with cpDNA but unresolved with nrDNA) has interesting biogeographic implications that warrant further study. Two subclades are strongly supported in the remainder of *Lepechinia* (Fig. 3). One (*L. calycina*/*L. hastata* clade) is primarily Mexican (with two subsequent dispersal events inferred to Mediterranean California and northern South America) and the other (*L. lamifolia*/*L. chamaedryoides* clade) is strictly South American. The sister clade to *Lepechinia* is the Eurasian *Melissa*, and these two in turn are sister to the “Salvia” clade that has a clear Eurasian origin, although with subsequent dispersal(s) to western North America, Central America, and South America (Walker and Sytsma 2007). This preliminary sampling of *Lepechinia* (and other *Salviinae*) thus suggests the hypotheses that (1) the subtribe *Salviinae* originated in Eurasia, (2) *Lepechinia* s. l. first diversified in Mexico (or more broadly in Central America), (3) at least two movements out of Mexico and subsequent radiations in South America occurred, and (4) at least one radiation from Mexico to Mediterranean California occurred.

**Taxonomic Considerations Within Lepechinia—**Since it is clear that *Neoeplingia* and *Chaunostoma* are embedded within *Lepechinia*, some taxonomic revision within the genus is needed. Considering that *Lepechinia caulescens* (Ortega) Epling is the type for the genus and given the presented molecular phylogenetic results, especially that of cpDNA and total evidence, three possible scenarios exist: (1) *Neoeplingia* and *Chaunostoma* retain their generic status and *Lepechinia mexicana* be transferred to *Neoeplingia*; (2) *Neoeplingia* and *Chaunostoma*...
retain their generic status and *Lepechinia mexicana* be elevated to its own genus; or (3) both monotypic genera *Neoeplingia* and *Chaunostoma* be subsumed within *Lepechinia*. We feel it is prudent to be cautious at this time for several reasons. First, a more thorough sampling of *Lepechinia*, now underway, is necessary before taxonomic changes are made. Second, the support values for the clade comprising *Chaunostoma meci-standrum, Neoeplingia leucophylloides*, and *Lepechinia mexicana*, and for its position relative to other *Lepechinia* were not strong in the nrDNA analyses. Third, additional single or low-copy nuclear genes will need to be sampled and their phylogenetic results compared to those of nrDNA (and cpDNA) to be certain that taxonomic changes are consistent with both genomes. Preliminary phylogenetic analyses in Mentheae using low copy nuclear markers, e.g. the nuclear pentatricopeptide repeat (PPR) gene family (Yuan et al. 2009, 2010), suggest that they may be critical in assessing relationships among species of *Lepechinia, Chaunostoma*, and *Neoeplingia* (Drew et al. 2010).

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**Literature Cited**


Appendix I. Voucher information and GenBank accession numbers for taxa used in this study. Information is as follows: taxon name and authority, collecting locality, collector(s) name and collection number (herbarium), Genbank numbers for the four loci: ycf1 and rpl15 spacer region, trnL-F, ITS, ETS, respectively. Abbreviations: RBG-Edinburgh = Royal Botanic Garden-Edinburgh, RSAGB = Rancho Santa Ana Botanical Garden, UCBG = UC-Berkeley Botanical Garden. DBG = Denver Botanical Garden.

Acanthomintha lanceolata Curran, U. S. A., Crosby & Morin 14383 (MO); JF289000, DQ667522; Agastache pallida (Lindl.) Cory, Mexico, B. Drew 118 (WIS); JF289001, JF301357; Bledilla hirsuta (Pursh) Benth., U. S. A., cultivated-UW-Madison, B. Drew 70 (WIS); JF289024, JF301360, JF301353, JF301325; Mentha arvensis L., U. S. A., B. Drew 82 (WIS); JF289023, JF301347; M. arvensis L., U. S. A., cultivated-UW-Madison, B. Drew 127 (WIS); JF289036, JF301349, JF301341; Monarda citriodora C. B. Clarke, Mexico, B. Drew 114 (WIS); JF289048, JF301329, B. Drew 13609 (WIS); JF289043, JF301334; Monarda villosa Benth., U. S. A., B. Drew 66 (WIS); JF289046, JF301389; Neoeplingia leucochrysolophoideae Ramamoorthy, Hirtiat & Medrano, Mexico, B. Drew 129 (WIS); JF289047, JF301340, JF301354, JF301327; Nepeta cataria L., U. S. A., B. Drew 72 (WIS); JF289048, JF301391; Ocimum basilicum L., cultivated-UW-Madison, B. Drew 125 (WIS); JF289051, AY570464; Origanum vulgare L., U. S. A., cultivated-UW-Madison Greenhouse, J. Walker 2557 (WIS); JF289049, AY570436; Poliomintha incana Benth., cultivated-UW-Madison Botanical Garden, J. Walker 2524 (WIS); JF289051, AY570464, DQ667223, JF301332; Plectranthus crenatus B. & J. Conn, U. S. A., cultivated-UW-CBCG, 3: 408–424.

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Verbena pyrenaicum (Hemsl.) C. Y. Wu, China, Lallemantia canescens (Ortega) Epling, Mexico, B. Drew 106 (WIS); JF289030, JF301376, JF301345, JF301316; Lepechinia chaumaeidroides (Balb.) Epling, Chile, cultivat-RSAGB, J. Walker 2537 (WIS); JF289031, AY570499, DQ667231, JF301317; Lepechinia glomerata Epling, Mexico, B. Drew 155 (WIS); JF289032, JF301377, JF301346, JF301318; Lepechinia hustata (A. Gray) Epling, Mexico, B. Drew 44 (WIS); JF289033, JF301378, JF301347, JF301319; Lepechinia lanfilifolia (Benth.) Epling, Peru, B. Drew 178 (WIS); JF289034, JF301379, JF301348, JF301320; Lepechinia mexicana (S. Schauer) Epling, Mexico, B. Drew 164 (WIS); JF289035, JF301380, JF301349, JF301321; Lepechinia mexi- cana (S. Schauer) Epling, Mexico, B. Drew 127 (WIS); JF289036, JF301381, JF301330, JF301322; Lepechinia radula (Benth.) Epling, Peru, B. Drew 185 (WIS); JF289037, JF301382, JF301331, JF301323; Lepechinia salviifolia (Kunth) Epling, Colombia, R. Jubally s. n. (WIS); JF289038, JF301383, JF301352, JF301324; Lophanthus lipoquiansis Ik.-Gal. & Nevskii, Uzbekistan, Vassiljeva (WIS); JF289039, JF301384; Lycopus uniflorus Michx., U. S. A., J. Walker 2586 (WIS); JF289040, DQ667488; Melastoma arboreum (L.) Makino, China, Lai Shushen & Shau Honrong s. n. (MO); JF289041, JF301385; Melissa officinalis L., cultivated-UW-Madison, B. Drew 70 (WIS); JF289042, JF301386, JF301335, JF301326; Mentha arvensis L., U. S. A., B. Drew 82 (WIS); JF289043, JF301347; Mentha arvensis L., U. S. A., cultivated-UW-CBCG, 3: 408–424.

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