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The South American radiation of *Lepechinia* (Lamiaceae): phylogenetics, divergence times and evolution of dioecy

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Lepechinia (Lamiaceae) is a diverse and widespread genus that is important in indigenous New World culture. We present a phylogenetic analysis of 72 accessions representing 31 species of *Lepechinia* using data from three plastid (*trnL-F*, *ycf1*, *ycf1-rps15* spacer) and four nuclear [internal transcribed spacer (ITS), external transcribed spacer (ETS), granule-bound starch synthase I (GBSS)I, pentatricopeptide repeat region (*PPR*)-*AT3G09060*] DNA regions. Data were analysed using parsimony, maximum likelihood and Bayesian approaches. Divergence time estimation using BEAST shows *Lepechinia* had a mid/late Miocene origin, perhaps as a response to global cooling patterns. Cladogenesis in most South American *Lepechinia* is shown to have occurred within the past 5 Myr, presumably as a response to climatic and orogenic events. Dioecy has arisen multiple times in *Lepechinia*, once in North America and at least twice in South America, and not necessarily involving gynodioecy as an intermediary step. Dioecy and gynodioecy are demonstrated to be associated with several floral characters, including flower size, number and colour. *Botanical Journal of the Linnean Society*, 2013, **171**, 171–190.

ADDITIONAL KEYWORDS: *ycf1* – gynodioecy – BEAST.

INTRODUCTION

Lepechinia Willd. is a diverse genus in the mint family (Lamiaceae), consisting of approximately 43 species that range from Northern California in the western USA to Central Argentina in South America (Fig. 1). Outside the main distribution of *Lepechinia*, disjuncts occur in the Dominican Republic (1), Hawai'i (1) and La Réunion in the Indian Ocean (1). This improbable distribution made it the first example Croizat used to illustrate a track in his theory of panbiogeography (Croizat, 1962). Since the work of Croizat, however, the occurrences in Hawai'i and La Réunion have been proposed as human introductions (Hart, 1983; Harley *et al*., 2004). In Lamiaceae, *Lepechinia* is nested in subtribe Salviinae, tribe Mentheae and subfamily Nepetoideae (Harley *et al*., 2004; Drew & Sytsma, 2011). Because of its high morphological diversity and putative ancient divergence, no synapomorphies for the genus have been found other than a potentially distinctive leaf odour. Several *Lepechinia* spp. are valued in the horticultural trade, and North and South American indigenous groups commonly use *Lepechinia* for medicinal and antiseptic purposes. The chemical compounds that give most *Lepechinia* a distinct odour may also be responsible for their medicinal and cleansing properties.

Although *Lepechinia* is not especially species-rich in South America compared with some other well-known genera (e.g. *Salvia* L., *Calceolaria* L., *Puya* Molina), it is of interest because of its distribution and diversity in the continent. *Lepechinia* occurs virtually throughout the western highlands of the Andes and into the highlands of north–central Venezuela, but is noticeably absent from the Guiana Shield. Besides the main Andean distribution of *Lepechinia* in South America, the genus also occurs in eastern Brazil and along the coast of central Chile. The distribution in Brazil is especially interesting because

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Figure 1. Distribution of *Chaunostoma, Lepechinia* and *Neoeplingia*.

it is represented by a single narrowly distributed species (*L. speciosa* (A.St.-Hil. ex Benth.) Epling) found several hundred kilometers away from the nearest other occurrence of the genus. Besides having an intriguing geographical distribution, South American *Lepechinia* spp. exhibit striking floral, morphological, breeding system and habit diversity. Floral size (2–40 mm) and leaf length (2.5–25.0 cm) can vary by an order of magnitude, and there is a wide range of floral colours, including blue, pink, purple, red and white. In association with the tremendous range of floral features in the genus, *Lepechinia* spp. are visited by a diverse group of pollinators. The South American species also exhibit rare (especially in Lamiaceae) breeding system diversity: gynodiocey, dioecy and hermaphroditism. In terms of habit, South American *Lepechinia* spp. range from perennial herbs to small trees.

Of the 43 *Lepechinia* spp., *c*. 30 occur in South America. Most species of *Lepechinia* in South America are found at high elevations, from 1500 to 4000 m, but within this altitudinal range the genus occupies a fairly broad range of habitats. The majority of South American *Lepechinia* spp. occur in relatively dry open habitats of the Andean highlands, but some species (e.g. *L. bullata* (Kunth) Epling, *L. vesiculosa* (Benth.) Epling) are found in relatively moist sites at the edges of forests and some are found in paramo (*L. rufocampii* Epling & Mathias) or puna (*L. meyenii* (Walp.) Epling) environments. When present, *Lepechinia* is often abundant along roads and in disturbed fields and secondary vegetation. Many South American *Lepechinia* spp., especially from section *Parviflorae* Epling (Table 1), occur in open areas near the upper limits of cloud forests or in sub-paramo shrubland. The two Chilean *Lepechinia* spp. are unusual in that they occur in mattoral vegetation from 500 m in elevation down to almost sea level, a similar pattern to that seen in *Lepechinia* spp. found in the California Floristic Province. At least 13 of the 30 South American *Lepechinia* spp. belong to section *Parviflorae* as described by Hart (1983; but see Wood, 1988). Section *Parviflorae* is morphologically distinct from the rest of the genus because of the following combination of characters: a robust shrubby habit; the presence of large panicled inflorescences of densely clustered small white flowers; and a gynodioecious or dioecious breeding system. The presence of dioecy is particularly notable because, although it is rare in flowering plants in general (~6% of species, ~7% of genera; Renner & Ricklefs, 1995), it is especially rare in Lamiaceae (~2.5% of genera; Harley *et al*., 2004).

Epling (Epling, 1926, 1937, 1948; Epling & Mathias, 1957; Epling & Jativa, 1968) conducted the first thorough treatment of *Lepechinia*. Prior to Epling, various *Lepechinia* spp. had been assigned to distant genera now placed in other subtribes, tribes or even families (Epling, 1948). Briquet (1895–1897), and initially Epling (1926) treated *Lepechinia* as two distinct genera: *Lepechinia* and *Sphacele* Benth. Epling (1937, 1948) later combined the two genera, with the comment that the only consistent alternative to recognizing one genus would be to recognize eight. As part of his treatment of Lamiaceae for the *Flora of Peru*, Macbride (1960) reverted to using *Sphacele*, and not *Lepechinia*, for most of his descriptions involving *Lepechinia*. Strangely, Macbride did not include *Lepechinia marica* Epling & Mathias or *L. scobina* Epling as part of his resurrected *Sphacele*, but instead maintained them as *Lepechinia*. This is especially curious in the case of *L. scobina* because it is quite similar morphologically to other species in Peru (e.g. *L. radula* (Benth.) Epling and *L. mollis* Epling) that Macbride included in *Sphacele*. Likewise, Epling considered *L. marica* to be part of section *Campanulatae* Epling (Epling, 1948; Epling & Mathias, 1957), which also contains *L. chamaedryoides* (Balb.) Epling, a species also known as *Sphacele chamaedryoides* (Balb.) Briq. The reason Macbride did not include the two aforementioned species in *Sphacele* is unclear. Macbride's treatment has led to confusion in some South American (and to a lesser extent elsewhere) herbaria, some of which still include many of their *Lepechinia* accessions in a folder labelled *Sphacele*. To

Table 1. Species and distribution of *Lepechinia* section *Parviflorae* as defined by Hart (1983). Taxon in brackets was described by Wood (1988). An asterisk indicates taxa that were included in this study

Species	Distribution	Species	Distribution
**Lepechinia betonicifolia*	Colombia, Ecuador	**Lepechinia mutica*	Ecuador
**Lepechinia bullata*	Colombia, Ecuador, Venezuela	**Lepechinia paniculata*	Ecuador
Lepechinia conferta	Colombia	**Lepechinia radula*	Ecuador, Peru
**Lepechinia dioica*	Ecuador	**Lepechinia scobina*	Peru
**Lepechinia graveolens*	Argentina, Bolivia	**Lepechinia vesiculosa*	Colombia
**Lepechinia heteromorpha*	Argentina, Ecuador, Peru	[*Lepechinia vulcanicola*]	Colombia, Ecuador
**Lepechinia mollis*	Peru		

further complicate matters, publications (generally focusing on chemical compounds) involving the two Chilean *Lepechinia* spp., *L. salviae* (Lindl.) Epling and *L. chamaedryoides*, routinely use *Sphacele*.

Using a variety of morphological characters in a cladistic analysis as a phylogenetic framework, Hart undertook a monographic revision of the genus as part of his PhD thesis (Hart, 1983). He made thorough collections of most of the South American species and discovered that seven species are dioecious and heteromorphic with respect to corolla and calyx features. Hart also thoroughly documented the occurrence of gynodioecy in the genus. Based on his cladistic analysis, Hart (1985a) hypothesized that dioecy arose in *Lepechinia* multiple times. As part of his work, Hart (1983) divided *Lepechinia* into two sections, *Parviflorae* and *Lepechinia*, the latter of which he considered non-monophyletic. After the work of Hart, Wood (1988) described three new species from Colombia (all gynodioecious) and provided detailed habitat and range information for all *Lepechinia* spp. growing in Colombia. Recently, Henrickson, Fishbein & Van Devender (2011) described a new species of *Lepechinia* from northern Mexico (*L. yecorana* Henrickson, Fishbein & Van Devender) and documented the occurrence of dioecy in that species and in *L. mexicana* (S.Schauer) Epling. This is an exciting and unexpected discovery because of the wide geographical separation of these two species from the other dioecious *Lepechinia* spp. and the fact that a close relationship between *L. mexicana* and *Lepechinia* section *Parviflorae* has never been postulated. No formal analysis of breeding system evolution in a molecular phylogenetic context has been carried out in *Lepechinia*, and there has been no assessment of correlative evolution with other traits often proposed to be ecologically linked with dioecy (Bawa, 1980; Givnish, 1980; Fox, 1985; Renner & Ricklefs, 1995; Sakai *et al*., 1995; Weiblen, Oyama & Donoghue, 2000; Vamosi, Otto & Barrett, 2003; Vamosi & Vamosi, 2004; Vary *et al*., 2011; but see also Soza & Olmstead, 2010). The occurrence of both gynodioecy and dioecy in *Lepechinia* also provides an opportunity to examine whether the former is a precursor of the latter, as has sometimes been proposed (Bawa, 1980; Hart, 1985a; Ainsworth, Parker & Buchanan-Wollaston, 1998; Weller & Sakai, 1999; Barrett, 2002), or whether other models of the origin of dioecy are supported (see Soza & Olmstead, 2010 for recent review).

Here, we present a phylogenetic, evolutionary and biogeographic perspective on the origin and diversification of *Lepechinia* in South America using a time-calibrated phylogenetic tree as a framework. Recently, we definitively placed *Lepechinia* in subtribe Salviinae of tribe Mentheae (based upon limited species sampling in the genus) and documented the relationship of the monotypic genera *Neoeplingia* Ramamoorthy, Hiriart & Medrano and *Chaunostoma* Donn. Sm. to some members of *Lepechinia* (Drew & Sytsma, 2011). We present here a more comprehensive phylogenetic analysis across *Lepechinia* and related genera based on three plastid (*trnL-F* spacer and intron, *ycf1*, *ycf1-rps15* spacer), two nuclear ribosomal (nr: ITS and ETS – internal transcribed and external transcribed spacers, respectively) and two low-copy nuclear gene regions [granule-bound starch synthase I (GBSSI or *waxy*) and a pentatricopeptide repeat region, *PPR-AT3G09060*]. Key biogeographic and evolutionary questions in *Lepechinia* examined here include: (1) What are the relationships among the South American *Lepechinia* spp.? (2) When did *Lepechinia* radiate in South America? (3) Are Hart's (1983, 1985a) hypotheses on the origin of dioecy and gynodioecy in *Lepechinia* supported? (4) Does the newly discovered occurrence of dioecy in species from northern Mexico represent an independent origin of dioecy in the genus? (5) Does breeding system (hermaphroditism, gynodioecy or dioecy) evolve in *Lepechinia* in a correlated fashion with other traits (e.g. floral colour, size and number)?

MATERIAL AND METHODS

Sampling and outgroups

A total of 72 accessions were included in this study (Appendix 1). The larger and more taxonomically broad plastid DNA phylogenetic framework contained all 72 accessions and included 31 *Lepechinia* spp. Of the remaining 41 samples, 11 species were from subtribe Salviinae, 21 species were from subtribes Lycopinae, Menthinae Nepetinae and Prunellinae (subtribes *sensu* Drew & Sytsma, 2012) and seven species were from subfamily Nepetoideae tribes Ocimeae and Elsholtzieae. *Lamium* L. (subfamily Lamiodeae) and *Caryopteris* Bunge (subfamily Ajugoideae) were used as a monophyletic outgroup. The smaller, more taxonomically focused nuclear DNA analysis of subtribe Salviinae included 44 taxa. These 44 accessions formed a subset of the larger plastid DNA sampling. Of these taxa, 42 were from subtribe Salviinae, with *Agastache pallida* (Lindl.) Cory and *Hedeoma piperitum* Benth. (subtribes Nepetinae and Menthinae, respectively) serving as outgroups (monophyletic). At least one representative from each of the eight sections of *Lepechinia* as outlined by Epling (Epling, 1948; Epling & Mathias, 1957) was included in both the plastid DNA and nuclear DNA analyses.

DNA extraction, amplification and sequencing

DNA was extracted from silica-dried plant material and herbarium specimens using the DNeasy Plant

Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's specifications. PCR thermal cycler settings for the plastid and nrDNA regions were similar to those described in Sytsma *et al*. (2002). PCR thermal cycling conditions for the nuclear gene regions were as used in Yuan *et al*. (2010) for *PPR-AT3G09060*. PCR products, obtained with TaKaRa Ex Taq (Otsu, Shiga, Japan), were diluted 30 × in water prior to cycle sequencing and subsequently cleaned using Agencourt magnetic beads (Agencourt, Beverly, MA, USA). Cycle sequencing reactions used the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Samples were electrophoresed on an Applied Biosystems 3730xl automated DNA sequencing instrument, using 50-cm capillary arrays and POP-7 polymer. Data were analysed using PE-Biosystems version 3.7 of Sequencing Analysis at the University Wisconsin–Madison Biotechnology Center.

Approximately 4600 nucleotides of *ycf1* and 500 nucleotides of the *ycf1-rps15* spacer were amplified and sequenced, primarily by using a series of 14 overlapping primers (Drew & Sytsma, 2011). The plastid 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 (Taberlet *et al*., 1991). ITS was amplified using the primers Leu1 (Andreasen, Baldwin & Bremer, 1999) 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 older herbarium specimens. Combinations of these primers were used for sequencing. The nrDNA ETS region was amplified and sequenced as described in Drew & Sytsma (2011). For the GBSSI gene, we used a nested PCR approach to amplify the region between (and including parts of) exons 7–11. For the initial PCR reaction we used the primers bd7f and bd12r (Table 2). The PCR product from the above amplification was then used (after 20 × dilution) as a template for two additional PCR reactions, one using primers bd7f and bd10r, the other using primers bd9f and bd11r. Products of these two amplifications were then sequenced with the same primers used in the nested PCR. The pentatricopeptide repeat (*PPR-AT3G09060*) gene was amplified using a similar nested approach. For *PPR-AT3G09060* the 930f and 2080r primers from Yuan *et al*. (2010) were used for the initial amplification. The PCR product was then re-amplified using the *Lepechinia* specific primer pair (bd1000f and bd2040r). The resulting PCR product was sequenced using the two aforementioned specific primers in combination with bd1244f and bd1760r.

For all nuclear regions except ETS, a subset of taxa was evaluated for copy number and allelic variation either by cloning or single-strand conformational polymorphism (SSCP) techniques (Sunnucks *et al*., 2000). For cloning, the initial PCR product was obtained as described above. The PCR product was then gel purified with QIAquick Gel Extraction Kit (Qiagen), ligated into a pGEM T-Vector (Promega, Madison, WI, USA), cloned in *Escherichia coli* DHB-5α competent cells (Invitrogen, Carlsbad, CA, USA), re-amplified and sequenced. SSCP protocols followed Rodriguez *et al*. (2011). Taxa were cloned if they displayed high numbers (greater than approximately 5/1000 nucleotides) of polymorphisms (but otherwise had clear single bands) after direct sequencing. The vast majority of sequences had clear single bands and few if any double peaks. From six to ten clones were amplified from the following regions and taxa: ITS – *Lepechinia betonicifolia* (Lam.) Epling, *L. chamaedryoides* and *L. dioica* Hart; *PPR-AT3G09060-Hedeoma piperitum*, *Dorystaechas hastata* Boiss. & Heldr. ex Benth., *Lepechinia bella* Epling, *L. calycina* (Benth.) Epling ex Munz, *Salvia greatae* Brandegee, *Salvia patens* Cav. and *Zhumeria majdae* Rech.f. & Wendelbo. For GBSSI, *Lepechinia calycina*, *L. hastata* (A.Gray) Epling, *Lepechinia caulescens* (Ortega) Epling and *L. mexicana* were sequenced using bands derived from SSCPs. We were unable to amplify *Agastache pallida* for the GBSSI region, but we had full taxon/character sampling for all other nuclear DNA and plastid DNA regions.

Phylogenetic analyses and divergence time estimation

All sequences were manually edited in Sequencher 4.7 (Gene Codes, Ann Arbor, MI, USA) and the resulting

Table 2. Primers designed for this study

Forward primer	Reverse primer
GBSSI bd7f – ATGTTGTMTTYGTYGCYAATGACTG	GBSSI bd10r – ACATAAAATCAGCACCAGCAG
GBSSI bd9f – TTATCGGYAGACTTGAAGAACA	GBSSI bd11r – TCMACRAGACCACCTGTTG
PPR bd1000f – TCACCTGATTCTGTTGTRTRTAATGC	GBSSI bd12r – TTCCAGGAGAGATCGWGTGMC
PPR bd1244f – ATGGRCTTTGTGAGAAYGG	PPR bd2040r – TTRTTAGCCAASGCATTGTG
	PPR bd1760r – CCATGAATCAAAATATTRTGCA

sequences were manually aligned in MacClade 4.08 (Maddison & Maddison, 2005). The plastid DNA and nuclear DNA data sets were analysed separately as described below. Phylogenetic and divergence time analyses for each data set were performed using Garli 2.0 (Zwickl, 2006) and Bayesian Inference (BI) using BEAST v1.6.1 (Drummond & Rambaut, 2007), a program that estimates phylogenies and divergence times simultaneously. In Garli we did not partition our data sets and we ran our analyses using the GTR + G + I model. In BEAST, the plastid DNA and nuclear DNA data sets were each partitioned to accommodate sequence rate heterogeneity. The plastid DNA data set had three partitions: (1) the first and second codon positions of *ycf1*; (2) the third codon position of *ycf1*; and (3) the non-coding spacer regions. The nuclear DNA data set also had three partitions: (1) the nrDNA (ITS and ETS); (2) the GBSSI gene region; and (3) the *PPR-ATG1G09060* (hereafter PPR) gene region. For each of our data partitions, we used a model of evolution as determined by the Akaike information criterion (AIC) in ModelTest v3.7 (Posada & Crandall, 1998). For the plastid DNA partitions ModelTest suggested the GTR + G + I (*ycf1*) and TVM + G (*ycf1-rps15* spacer and *trnL-F*) models. For the nuclear partitions, ModelTest suggested the GTR + G + I model for the nrDNA and the GTR + G model for the nuclear genes. These models were then used as molecular evolution models for each respective partition in BEAST after choosing the 'unlink substitution model' option and adjusting the appropriate prior and operator settings (for the TVM model). The partitions were subsequently analysed for rate constancy among lineages using the likelihood ratio test (Felsenstein, 1988) as implemented in PAUP* (Swofford, 2002). Rate constancy was rejected for all partitions, so we used a relaxed clock model as implemented in BEAST. In order to optimize efficiency in BEAST, several trial runs of 15 million generations were undertaken and the results were analysed using Tracer v1.5 (Rambaut & Drummond, 2007). For all runs we estimated rate change using the uncorrelated log-normal model. Our trial runs were used to determine the number of generations necessary to achieve an effective sample size (ESS) of at least 200 and to optimize the operator settings for our final analyses.

For the plastid DNA analysis we constrained two nodes of Nepetoideae with log-normal priors, and constrained the root of the tree with an exponential prior distribution (see below). For the plastid DNA BEAST analysis we ran 15 million generations on three separate computers, each starting with a randomly generated tree. Samples were taken every 1000 generations and the first 1.5 million generations of each run were discarded as burn-in. The resulting 13 500 trees from each run were combined with LogCombiner v1.6.1. The trees were then interpreted by TreeAnnotator v1.6.1 prior to visualization in FigTree v1.3.1.

For the nuclear DNA data set we constrained the crown of the tree using an age range based on the 95% confidence intervals of the crown group of Mentheae from our plastid analysis, and the most recent common ancestor (MRCA) of *Lepechinia* and *Melissa* L. was constrained with a log-normal prior. For the nuclear DNA analyses we conducted three runs of 15 million generations, each starting with a randomly generated tree. Samples were taken every 1000 generations, and the first 1.5 million generations of each run were discarded as burn-in. The resulting 13 500 trees from each run were combined with LogCombiner v1.6.1. The trees were then interpreted by TreeAnnotator v1.6.1 prior to visualization in FigTree v1.3.1.

Calibration points for plastid DNA

The root of the plastid DNA tree was constrained with an exponential distribution having an offset of 49 Mya and a mean of 14. This calibration point is based on the findings of previous asterid-wide and larger angiosperm dating papers (Wikström, Savolainen & Chase, 2001; Bremer, Friis & Bremer, 2004; Janssens *et al*., 2009; Magallón & Castillo, 2009; Bell, Soltis & Soltis, 2010). As we did not have an acceptable date (especially for the upper boundary) to use for the crown of Lamiaceae, we used previously published dates for the crown of the order Lamiales. The minimum age of 49 Mya was based on the oldest well-accepted fossil of Lamiaceae (Kar, 1996). The standard deviation (SD) of the exponential prior we imposed corresponded to an upper boundary of 107 Mya, and was chosen based on the upper confidence interval crown estimate for the Lamiales in Janssens *et al*. (2009). Confidence intervals (for upper boundaries) from other large dating papers involving the asterids (Bremer *et al*., 2004; Magallón & Castillo, 2009; Bell *et al*., 2010) all fall within the upper (and lower) boundary prior we imposed on the root, so we consider this boundary quite conservative. Although Lamiaceae are not well represented in the fossil record (Harley *et al*., 2004), there are enough accepted fossils in the family to use them confidently as calibration points in this study. The Nepetoideae crown was constrained (not monophyletic) with a log-normal prior having an offset of 49 Myr, a mean of 2.6 and an SD of 0.5. The 49 Myr offset is based on an Early Eocene hexacolpate fossil identified by Kar (1996). Hexacolpate pollen is extremely rare in angiosperms, but is a synapomorphy for subfamily Nepetoideae (Harley *et al*., 2004). Kar identified the fossil as *Ocimum* L., which is in Nepetoideae, but based upon

the comments of Harley *et al*. (2004) we considered it prudent to place the fossil at the crown of Nepetoideae as opposed to elsewhere (crown of Ocimeae). The assigned mean of 2.6 to the offset of 49 Myr allows for the possibility that the Coniacian hexacolpate fossil described by Boltenhagen (1976a, b) is truly Nepetoideae. This fossil was listed as 'pending' by Muller (1981) because of its temporal distance from other pollen fossils of Nepetoideae. We constrained the MRCA of *Melissa* and *Lepechinia* with a log-normal distribution having an offset of 28.4 Mya, a mean of 1.5 and an SD of 0.5. The offset was based on a fossil fruit of *Melissa* from the Early–Mid Oligocene (Reid & Chandler, 1926 Martínez-Millán, 2010). Based upon the findings of Drew & Sytsma (2011), we constrained the MRCA of *Melissa* and *Lepechinia* to be monophyletic.

Calibration points for nuclear DNA

For the nuclear DNA analysis we constrained the crown of Mentheae with a truncated normal distribution that had an age range of 37.9–53.8 Myr, a mean of 45.4 Myr and an SD of 10. This range reflects the 95% confidence interval from the crown node of Mentheae from our plastid DNA analysis. Subtribe Salviinae was constrained to be monophyletic in order to root the analysis appropriately. The truncated normal distribution we used was similar to a uniform distribution, but differed in assigning a slightly diminished probability at the edges of the age ranges. The MRCA of *Melissa* and *Lepechinia* was constrained using the same priors as in the plastid DNA analysis, but this clade was not constrained as monophyletic in the nuclear DNA analysis.

Evolution of dioecy

We explored the evolutionary transitions in breeding systems in *Lepechinia* in terms of number of shifts, directionality of change, timing and contingent evolution with other characters using the dated chronograms uncovered in BEAST analyses. *Lepechinia* and outgroups were scored based on whether species were hermaphroditic, gynodioecious or dioecious using information from the literature and personal observations. This breeding system character was maintained as unordered in all analyses, thus allowing shifts between any state in both directions. We also scored each species for floral number many (> 50 per inflorescence) vs. few (< 50 per inflorescence), floral size [small (< 9 mm) vs. large (> 9 mm)] and floral colour (white vs. non-white) in order to test for correlative evolution with breeding system. We implemented maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) optimization of character evolution. MP reconstruction utilized the 'trace character' option in MacClade (Maddison & Maddison, 2005) with the resolving option of 'all most parsimonious states at each node'. ML and BI [Markov chain Monte Carlo (MCMC) – Pagel, 1999] reconstructions were implemented in BayesTraits v.1.0 (Pagel & Meade, 2007) using MultiState and a random set of 100 Bayesian posterior probability (PP) trees. We used the branch scaling parameter (k) to adjust the weight of branch lengths in the model and allow it to take its maximum likelihood (Pagel, 1994). We used the hyperprior approach (the rjhp command) and explored combinations of hyperprior values (exponential or gamma, mean and variance) and rate parameter values to find acceptance rates when running the Markov chains of between 20 and 40% (as recommended by Pagel, Meade & Barker, 2004; Pagel & Meade, 2007, 2008). All subsequent analyses used rjhp gamma 0 10 0 10 and a rate parameter of 65. All Bayesian analyses used 25 million generations with sampling every 1000 generations and a burn-in of 20 000. ML analyses used 25 independent runs (mltries = 25). Ancestral reconstruction of character evolution under ML or BI with the 100 random PP trees was represented by pie charts indicating state probabilities at each node in the nuclear DNA chronogram.

We tested for correlated evolution between breeding system and each of the three floral characters (number, size, colour) using BayesTraits (Pagel & Meade, 2007) under ML using the same methods and trees as described above. Because of the near equivalency of floral features with either gynodioecy or dioecy, we used a two-state character for breeding system (hermaphrodism vs. gynodioecy/dioecy). We implemented the BayesDiscrete module, which investigates correlated evolution between a pair of discrete binary traits by comparing the fit (log likelihood) of two models. The first is a model in which two traits such as breeding system and floral colour evolve independently on the tree. This creates two rate coefficients per trait or four rate coefficients that must be estimated. The other model allows the traits to evolve in a correlated fashion such that the rate of change in one trait depends on the background state of the other. The dependent model has four states, one for each combination of the two binary traits or eight rate coefficients that must be estimated. To determine whether breeding system is correlated with another trait, we compared the likelihood estimate of the independent model [$L(I)$] with the likelihood estimate of the dependent model [$L(D)$]. Support for correlated evolution is obtained when $L(D)$ is significantly greater than $L(I)$ using the formula $-2[L(D) - L(I)]$ to a χ^2 distribution with four degrees of freedom (eight vs. four estimated rate coefficients in the dependent

vs. independent model, respectively). We determined whether each of the eight estimated transition parameters in the dependent model is significantly greater than zero. Individual transition parameters were restricted to zero and the likelihood score of this seven-parameter dependent model was compared with the likelihood score of the full parameter–parameter-dependent model using a χ^2 distribution with 1 d.f.

Finally, we tested a number of hypotheses of contingent evolution between breeding system and putatively correlated characters in BayesDiscrete using the phylogenetic framework for *Lepechinia*: for example, white flowers evolve more often in dioecious relative to hermaphroditic clades. We tested these specific hypotheses of contingent evolution by restricting two rates being equal (e.g. rates of evolution of white flowers from non-white flowers in a hermaphroditic clade and in a dioecious clade are equal). This seven-parameter dependent model can be compared with the full eight-parameter model as described above with a 1-d.f. χ^2 distribution test. A significant likelihood ratio would indicate, for example, that the state of the second character (hermaphroditic vs. dioecious) influences the evolution of white flowers (Pagel, 1994; Friedman & Barrett, 2008).

RESULTS

Plastid DNA analyses

The combined plastid DNA data matrix consisted of 6396 aligned characters after excluding 300 nucleotides because of ambiguous alignment. The majority of the data set came from *ycf1* with an aligned length of 4932 bp, of which 81 bp were excluded because of ambiguity. The *ycf1-rps15* spacer region accounted for 774 aligned positions, of which 156 were excluded. Almost all of the excluded positions in the spacer were the result of a difficult-to-align poly A/T region approximately 50 nucleotides downstream from the *ycf1* gene. The *trnL-trnF* region had 990 aligned characters, 63 of which were excluded because of difficulties in alignment. Of the 6396 retained characters in the plastid DNA data set, 2840 were variable and 1481 (23.2%) were potentially parsimony informative.

Relationships among subtribes Lycopinae, Menthinae, Nepetinae, Prunellinae and Salviinae (see also Supporting Information, Figs S1, S2) are the same as discussed in Drew & Sytsma (2011, 2012). Although the purpose of this study is not explicitly phylogenetic, and relationships in *Lepechinia* will be explored in detail in the near future (B. T. Drew & K. J. Sytsma, unpubl. data), a few relationships in *Lepechinia* should be noted (Figs 2; see also Supporting Information, Figs S1, S2). *Lepechinia mexicana* is sister to the recently described *L. yecorana*. These two species form a clade with *Neoeplingia* and *Chaunostoma* that is well supported as sister to the rest of *Lepechinia*. In the core *Lepechinia*, a clade of six mostly North American species [*L. meyenii* is endemic to South America, and *Lepechinia schiedeana* (Schltdl.) Vatke has a small part of its distribution in northern Columbia and western Venezuela] is sister to a large clade that contains the vast majority of South American *Lepechinia* spp. (Fig. 2). Although the South American clade is well supported, the relationships within it are mostly weakly resolved.

Nuclear DNA analyses

The combined nrDNA data matrix was 1154 aligned characters (ITS-732; ETS-422). After excluding 39 (ITS-36; ETS-3) nucleotides because of ambiguous alignment, the combined nrDNA data matrix consisted of 1115 aligned characters. Of those, 496 were variable and 314 (28.2%) were potentially parsimony informative. The GBSSI gene region (from exon 7 to exon 11) was 1566 aligned characters in length. Alignment of *Lepechinia* (and *Melissa officinalis* L.) was straightforward for the introns of the GBSSI region, but there were several areas where it was impossible to align *Lepechinia* + *Melissa* with *Salvia* and/or *Hedeoma* Pers. In instances of ambiguous alignment, the sequences of questionable taxa were separated and excluded. In all, 366 nucleotide positions were excluded from the GBSSI partition. Of the remaining 1200 characters, 395 were variable and 155 (12.9%) were potentially parsimony informative. The PPR data partition contained 1121 characters (462 variable; 191 potentially parsimony informative – 17.0%), none of which were excluded. In all, the nuclear analysis consisted of 3436 included characters. The three species that were cloned for ITS all showed evidence of incomplete concerted evolution (Wendel, Schnabel & Seelanan, 1995) in the form of pseudogenes that were inferred by irregularities and/or gaps in the 5.8S region. These clones were excluded from subsequent analyses. All remaining ITS clones clustered in monophyletic groupings with their respective directly sequenced analogues in the ML analysis (results not shown). For our BEAST runs we selected one of the non-pseudogene clones at random to include in our analyses. The cloned taxa from the *PPR-AT3G09060* region also clustered all into individual monophyletic groupings (see also Supporting Information, Fig. S4). Two taxa, *Hedeoma piperitum* and *Zhumeria majdae*, had some clones containing deletions of one or two nucleotides. As *PPR-AT3G09060* is coding and indels should be in threes,

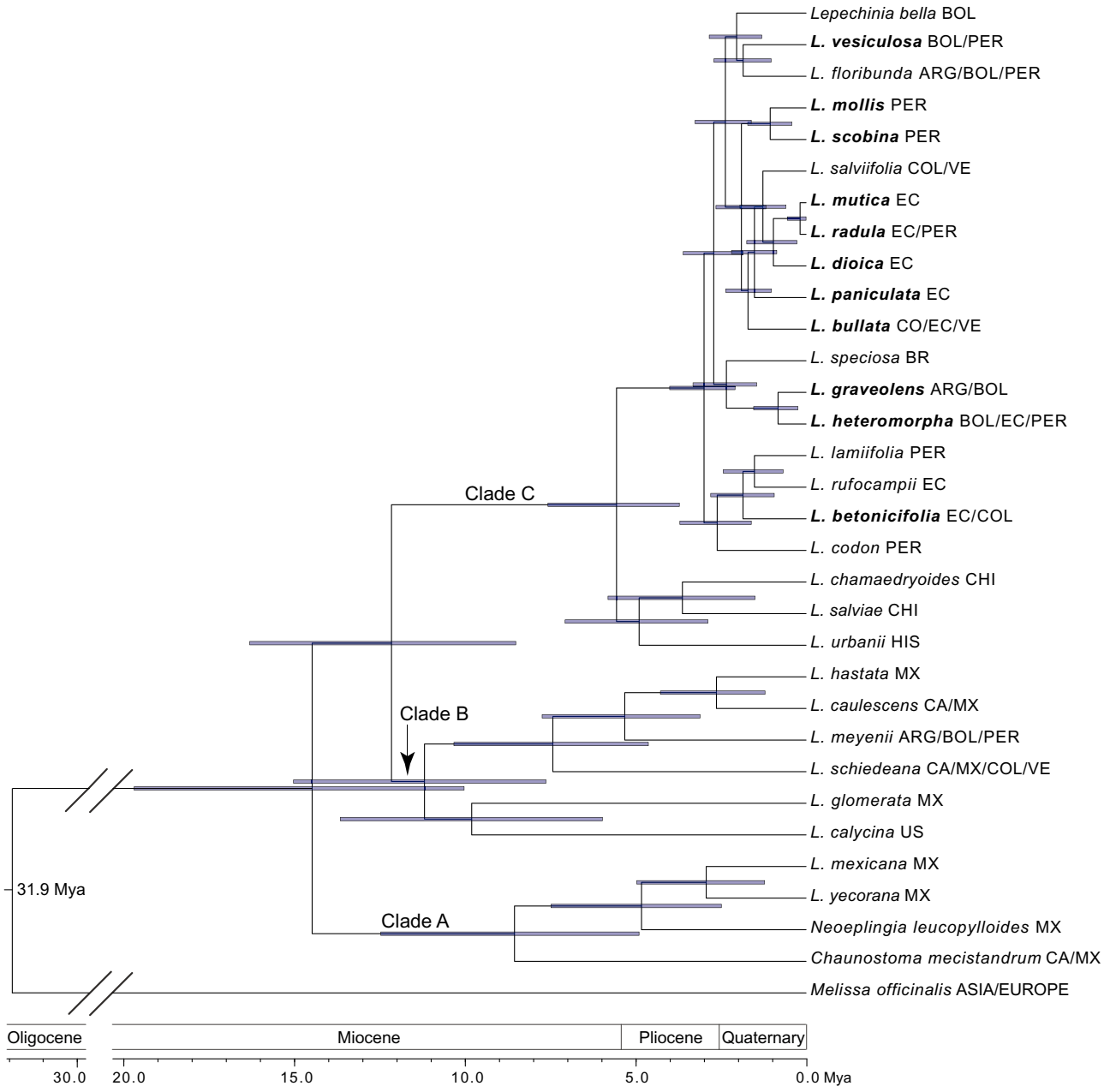

Figure 2. Chronogram showing results from plastid DNA (*ycf1*, *ycf1-rps15* spacer, *trnL-F*) as inferred using BEAST. Bars indicate 95% confidence intervals. Taxa in bold are species of section *Parviflorae* as defined by Hart (1983). Country abbreviations: ARG, Argentina; BOL, Bolivia; BR, Brazil; CA, Central America; CHI, Chile; COL, Colombia; EC, Ecuador; HIS, Hispaniola; MX, Mexico; PER, Peru; US, USA; VE, Venezuela.

these clones are presumably non-functional paralogues. Again, for the *PPR-AT3G09060* region we chose one cloned sample from each taxon at random to include in our analyses.

In the nuclear data set (Fig. 3), two well-supported main clades are evident: (1) a clade of Californian and Meso-American species and (2) a clade of Meso-American and South American species. In clade 2, a clade of mostly Meso-American distribution is sister to a clade of wholly South American distribution (with the exception of *Lepechinia urbanii* Epling from Hispaniola). *Lepechinia speciosa* and *L. salviae* (from eastern Brazil and Chile, respectively) formed a weakly (PP = 0.87) supported grade at the base of a

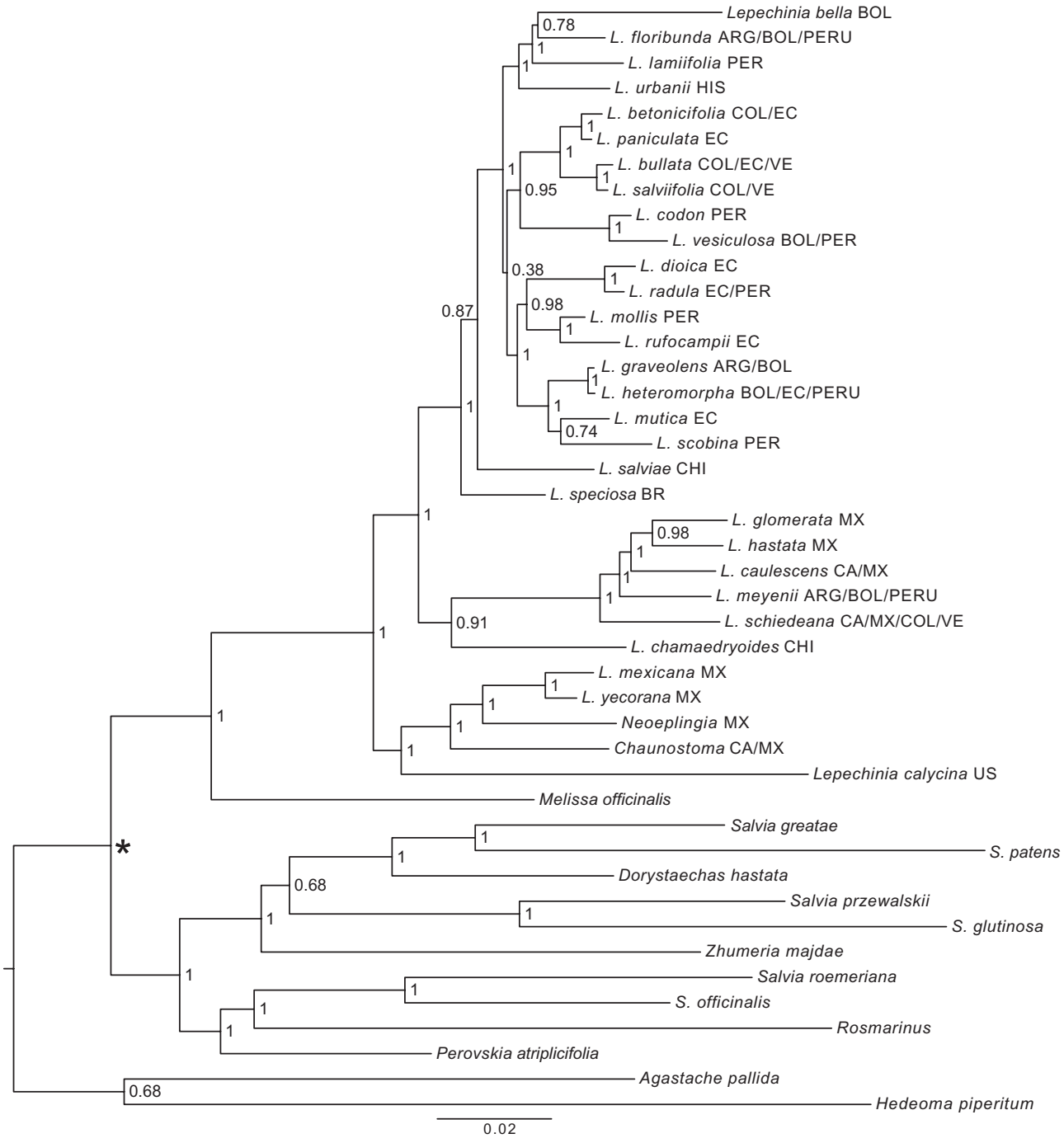

Figure 3. Phylogram of subtribe Salviinae based on results from nuclear DNA (ETS, ITS, GBSSI and *PPR-AT3G09060*) with *Hedeoma* and *Agastache* as the outgroup. Posterior probability values are given near nodes. Asterisk indicates the subtribe Salviinae crown (which was constrained as monophyletic). Country abbreviations: ARG, Argentina; BOL, Bolivia; BR, Brazil; CA, Central America; CHI, Chile; COL, Colombia; EC, Ecuador; HIS, Hispaniola; MX, Mexico; PER, Peru; US, USA; VE, Venezuela.

strongly supported clade of South American species. *Lepechinia urbanii* is a gynodioecious species that bears resemblance to *L. salviifolia* (Kunth) Epling and *L. codon* Epling. The placement of this species with herbaceous hermaphroditic members of *Lepechinia* (Figs 3, 4) is curious, and is at odds with unpublished results based on nuclear gene sequences (Drew, 2011).

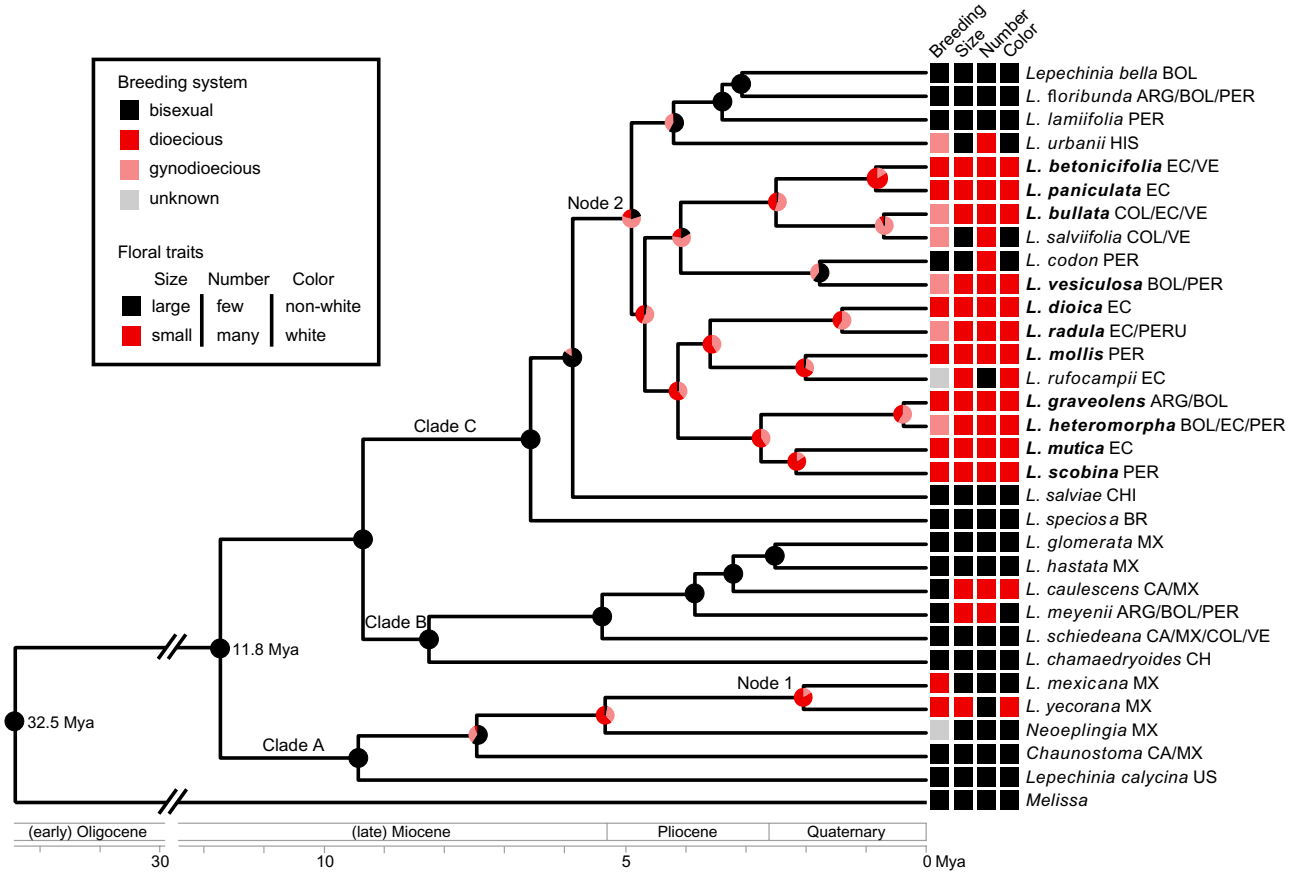

Figure 4. Chronogram based on nuclear DNA (ETS, ITS, GBSSI, and *PPR-AT3G09060*) showing *Lepechinia* and related genera, showing mapped ancestral state reconstructions (using ML) for the character 'breeding system'. Blocks to the right of taxa indicate breeding system and possible contingent floral traits. Labeled clades (A, B, C) and nodes (1, 2) are discussed in the text. Taxa in bold are species of section *Parviflorae* as defined by Hart (1983). Country abbreviations: ARG, Argentina; BOL, Bolivia; BR, Brazil; CA, Central America; CHI, Chile; COL, Colombia; EC, Ecuador; HIS, Hispaniola; MX, Mexico; PER, Peru; US, USA; VE, Venezuela.

Combined analyses

Preliminary analyses of the nuclear vs. plastid DNA phylogenies showed obvious and widespread incongruence. Because of the topological inconsistencies between the two data sets no attempt was made to combine them. In general, the trees from the nuclear phylogenetic analysis seemed more plausible based on a priori expectations with respect to morphological similarity. In the plastid phylogenetic analysis, taxa tended to cluster in accordance with geography. To some extent geographical clustering should be expected, but some species probably only formed clades in the plastid analysis as a result of recent hybridization (e.g. *Lepechinia chamaedryoides* and *L. salviae*). A more detailed look at the discrepancies between the two genomes is forthcoming (B. T. Drew & K. J. Sytsma, unpubl. data; Drew, 2011).

Molecular dating

Results from chronograms showing diversification of *Melissa* and *Lepechinia* based on the plastid DNA and nuclear DNA BEAST analyses are shown in Figures 2 and 4. The MRCA of *Melissa* and *Lepechinia* (a calibration point), is early-Oligocene with a date of 32.6mya (95% CIs 29.5–36.7). Both the plastid DNA and nuclear DNA analyses (Figs 2, 4) show that *Lepechinia* has diversified within the past 15 Myr. The crown of *Lepechinia* is estimated to have originated near the beginning of the mid-late Miocene (Table 3). Diversification in *Lepechinia* began to accelerate *c.* 7–8 Mya and the three major clades of *Lepechinia* (Figs 2, 4) were present at around this time. Most lineages in *Lepechinia* date to within the past 10 Myr, with a notable acceleration since the Pliocene. The crown of *Lepechinia* is somewhat older in the plastid DNA chronogram, but the date is similar to the date

Table 3. Ages of selected nodes from Figures 2, 4

	Plastid			Nuclear		
Ages of selected nodes:	95% HPD lower	Mean	95% HPD upper	95% HPD lower	Mean	95% HPD upper
Melissa and *Lepechinia* MRCA	29.6	31.9	35	29.6	32.5	36.1
Lepechinia Crown	10.0	14.5	19.7	7.8	11.8	16.3
Meso-American Clade (clade B)	7.6	11.2	15.0	5.3	8.3	11.8
Core South American Clade Crown (clade C)	3.7	5.6	7.6	4.1	6.6	9.4
MRCA of Clade B and C	8.5	12.1	16.3	6.1	9.4	13.2
Andean *Lepechinia* Crown	2.1	3	4.0	3.1	4.9	6.9

HPD, high posterior density; MRCA, most recent common ancestor.

obtained from the nuclear data. In both instances, the majority of South American taxa have diversified within the past 5 Myr, especially in the Pleistocene.

Evolution of dioecy

Using unordered character state transitions, the shift from hermaphroditism to gynodieocy and/or dioecy occurred multiple times in *Lepechinia*, with the possibility of up to two reversions back to hermaphroditism (Fig. 4). The newly discovered occurrence of dioecy in a subclade from northern Mexico represents one distinct transition (node 1 in Fig. 4). Assessment of the breeding system of the Mexican *Neoeplingia* is needed to know whether it also belongs to this dioecious subclade (because the breeding system of *Neoeplingia* is unknown, the probability of gynodioecy and dioecy as ancestral states is inflated in clade A, Fig. 4). All but four species in a South American subclade exhibit either gynodioecy or dioecy (node 2 in Fig. 4). BayesMultistate indicates that this node has a 59 and 21% probability of being gynodioecious and dioecious, respectively. Allowing the additional assumption (see Discussion) that gynodiocey is a precursor to dioecy (as suggested by Hart, 1985a), up to five separate shifts to dioecy can be invoked just in this South American subclade. However, the complex pattern of all three breeding system states in this subclade might suggest that the transition away from hermaphroditism allows for frequent shifts between gynodioecy and dioecy, with two reversals back to hermaphroditism (in *L. codon* and in the lineage leading *to L. bella, L. floribunda* (Benth.) Epling, and *L. lamiifolia* (Benth.) Epling).

To evaluate correlative evolution of dioecy and other floral features typically shown to be associated with it, BayesDiscrete implemented a binary coding of breeding systems (hermaphroditism vs. gynodioecy/dioecy; hereafter referred to as dioecy). Results under MCMC and ML were generally similar and only the ML results are displayed here. All three floral features (floral number, size and colour) significantly evolve in a correlative fashion, with dioecy with floral colour the strongest (Fig. 4, see column 2 in Table 4). Rates of transitions for the eight estimated parameters for each of the three comparisons are shown in Figure 5. Although each of the three characters evolves in a correlative manner with dioecy, there appears not to be enough power (i.e. number of shifts) in most tests of contingent evolution to show significance (see columns 3–6 in Table 4). For example, the contingent test that small flowers evolve more often in dioecious clades, as hypothesized, is not significant ($P = 0.199$; Table 4), despite the fact that the estimated transition rate towards small flowers from dioecious clades ($q_{34} = 488$, see Fig. 5A) is 24 times greater than from hermaphroditic clades ($q_{12} = 19$, see Fig. 5A). Additional insights into the ordering of steps to arrive at the highly correlated suite of characters with dioecy (many, small white flowers) are seen in Figure 5. From the ancestral large hermaphroditic flowers, the only pathway to small dioecious flowers involves acquisition first of dioecy, then of small flowers (see dashed arrows, Fig. 5A). The transition from few hermaphroditic flowers to many dioecious flowers first requires the evolution of many flowers, then dioecy (see dashed arrows, Fig. 5B). Lastly, from non-white hermaphroditic flowers, the only possible route to white dioecious flowers is first through evolution of dioecy and then white flowers (see dashed arrows, Fig. 5C).

DISCUSSION

Gene tree concordance

While supporting most of the same major geographical groups, the plastid and nuclear phylogenetic analyses differed markedly with respect to some relationships in *Lepechinia*. The most striking differences between the two topologies were observed in the

Table 4. Likelihood ratio (LR) values for tests of correlated evolution between breeding system and three floral characters using BayesTraits

Trait	Likelihood ratio of dependent vs. independent model	Trait (state 1) evolves more often in dioecious clades	Dioecy evolves more often in clades with trait (state 1)	Dioecy lost more often in clades with opposite trait (state 0)	Opposite trait (state 0) evolves more often in bisexual clades
Floral size (0 = large, 1 = small)	12.42*	1.65	0.67	2.26	0.12
Floral number (0 = few, 1 = many)	11.86*	3.16	0.99	0.87	4.55*
Floral colour (0 = non-white, 1 = white)	14.22†	0.85	0.11	1.23	0.02

*$P < 0.05$; †$P < 0.01$.

placement of *L. chamaedryoides* (Chile) and *L. calycina* (USA). In the plastid tree, *L. chamaedryoides* is sister to another Chilean species, *L. salviae*, and is included in a larger clade that contains all *Lepechinia* endemic to South America (except *L. meyenii*). In the nuclear phylogenetic analysis *L. chamaedryoides* is part of a clade consisting of mostly North American taxa. *L. chamaedryoides* and *L. salviae* are known to hybridize [*L. subhastata* (Benth.) Epling; Epling 1948] and the placement of *L. chamaedryoides* in the plastid tree is likely a result of plastid capture. Additionally, in terms of leaf size, shape and floral architecture, *L. chamaedryoides* is more similar to some *Lepechinia* spp. in Mexico (e.g. *L. schiedeana* and *L. mexicana*) than species in South America [with the notable exceptions of *L. tomentosa* (Benth.) Epling and *L. marica* Epling & Mathias, both from central Peru]. The discrepancy between the placement of *L. calycina* in the nuclear and plastid trees is more problematic. In the plastid tree, *L. calycina* is sister to *L. glomerata* Epling (southern Mexico) and is part of a clade of Mexican/Central–South American taxa. This clade is sister to a clade containing the bulk of South American *Lepechinia* spp. In the nuclear analyses, *L. calycina* is sister to a (different) clade of Mexican/Central America species, and this clade in turn is sister to a clade consisting of the aforementioned Mexican/Central–South American taxa and the rest of the South American species. It seems the best way to account for this discrepancy is to invoke a hybridization and concomitant plastid capture event between *L. calycina* and *L. glomerata* in the ancient past. The two species are not found to be sister species in any other gene tree in this study except for the plastid.

In this study, data from the GBSSI and PPR gene regions independently support (albeit weakly; see also Supporting Information, Figs S4, S5) a relationship showing *L. calycina* and a group of Mexican/Central American (*L. mexicana*, *L. yecorana*, *Neoeplingia* and *Chaunostoma*) taxa as sister to the remainder of *Lepechinia*, whereas the nrDNA data (see also Supporting Information, Fig. S3) is ambiguous in this regard. The three nuclear regions (ITS-ETS, PPR and GBSSI) had somewhat different topologies (see also Supporting Information, Figs S3–S5), but the major clades of interest in this paper (*Lepechinia*, *Lepechinia* + *Melissa*, South American dioecious/gynodioecious *Lepechinia*) were recovered in all three estimations. In general, differences between the topologies in the three nuclear DNA regions were only weakly (BS < 60%), if at all, supported. As much of the plastid DNA/nuclear gene incongruence stems from putative hybridization/plastid capture events (B. T. Drew & K. J. Sytsma, unpubl. data; Drew, 2011) and the three unlinked nuclear markers used here

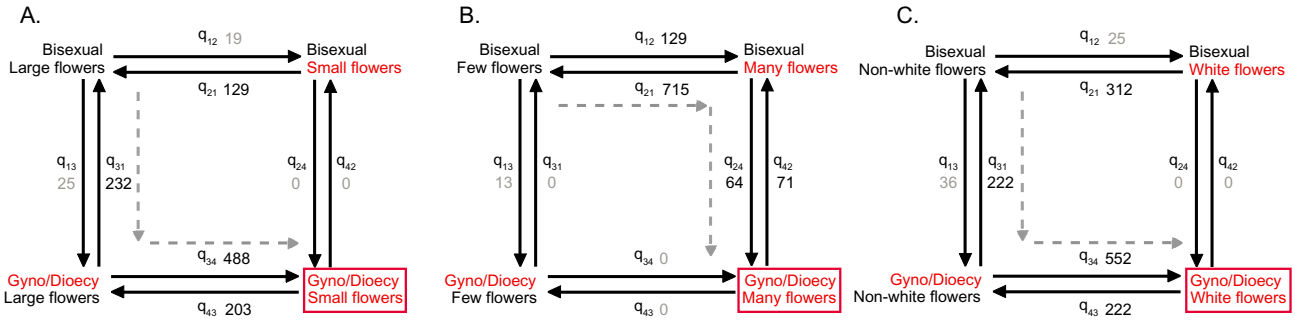

Figure 5. Rate parameters for the eight possible transitions between breeding system and three floral characters showing correlated evolution in *Lepechinia*: (A) floral size; (B) number; and (C) colour. Rate parameters were generated under a model of dependent evolution and those not significantly greater than zero are grey. Character states generally considered correlated with dioecy are shown in red. Hypothesized evolutionary routes toward dioecy and correlated floral traits are shown as dashed arrows.

were more or less consistent, we consider the nuclear phylogenetic analysis to be a closer approximation to the true phylogeny and will focus on those results for the discussion unless otherwise stated.

Relationships among South American *Lepechinia*

Two well-supported main clades were recovered in *Lepechinia* (Figs 3, 4). One clade consists of species ranging from northern Central America to California (Fig. 4, clade A), and the other clade contains mostly South American taxa. In the latter, two main clades are evident. One contains species that are mostly Meso-American in distribution and the other contains taxa found solely in South America (with the exception of *Lepechinia urbanii* from the major island in the Caribbean, Hispaniola, which contains the countries of the Dominican Republic and Haiti). The Meso-American clade (Fig. 4, clade B) has a Chilean species, *L. chamaedryoides*, as sister to the rest of the species. This relationship is only moderately (PP = 0.91) supported, however. The three species found in this clade that occur in South America, *L. chamaedryoides*, *L. meyenii* and *L. schiedeana*, are quite different morphologically from the other *Lepechinia* spp. native to South America (with the exceptions of *L. tomentosa* and *L. marica*). *Lepechinia meyenii* and *L. schiedeana* (*L. schiedeana* is mostly Mexican in distribution and only reaches northern Colombia and extreme western Venezuela) are prostrate subshrubs that grow at high elevations and *L. chamaedryoides* is an oddball in the genus, in that it has small leaves and large flowers and grows near sea level. *Lepechinia chamaedryoides* is distinct enough that it was placed in its own section, *Campanulatae*, by Epling (1948). The three South American species of *Lepechinia* in this mostly Mexican clade seem to represent an independent lineage of *Lepechinia* in South America that was derived, at least in part, from Mexican ancestry. A future study incorporating additional South American and Mexican accessions will explore this connection in greater detail.

In the core South American *Lepechinia* clade (Fig. 4, clade C), *L. speciosa* from Brazil and *L. salviae* from Chile form a grade at the base of a clade containing the remaining Andean taxa (Figs 3, 4). The Chilean sister relationship to the Andean taxa of this group is intriguing, and shows a pattern similar to that seen in *Chuquiraga* Juss. (Ezcurra, 2002), *Puya* Molina (Jabaily & Sytsma, 2010), *Calceolaria* L. (Cosacov *et al.*, 2009) and subfamily Bromelioideae (Givnish *et al.*, 2011). In the plastid DNA phylogenetic analysis, *L. salviae* is sister (with *L. urbanii* from Hispaniola and the Chilean *L. chamaedryoides*) to the remainder of the South American *Lepechinia* spp. As previously stated, however, the placement of *L. chamaedryoides* in the plastid DNA tree is probably the result of hybridization with *L. salviae* (Epling, 1948). Among the Andean *Lepechinia* spp. there are several well-supported clades, but the relationships between those clades are not well resolved.

The placement of *L. urbanii* (from Hispaniola) as sister to a clade of Peruvian/Bolivian taxa is curious. *Lepechinia urbanii* is gynodioecious and shrubby and has relatively small flowers in dense panicles, whereas the other three taxa in the clade are hermaphroditic and herbaceous and have large flowers that are not aggregated. Upon further investigation, this relationship was heavily influenced by the nrDNA data, and could be a result of incomplete lineage sorting in the nrDNA region of *L. urbanii*. An analysis using low copy nuclear markers (Drew, 2011) shows *L. urbanii* to be in a clade with other Northern South American gynodioecious (and dioecious) taxa as

expected. We plan to further investigate the nrDNA sequences of *L. urbanii* via cloning.

Divergence time analysis

In both plastid DNA and nuclear DNA analyses, the crown of the core Andean *Lepechinia* clade dates to within the past 5 Myr. The plastid DNA chronogram indicates a more recent Andean radiation than the nuclear DNA chronogram, but this result may well be influenced by hybridization and or plastid capture – events as described above that appear to occur in *Lepechinia*. Both analyses clearly show that most cladogenesis in Andean *Lepechinia* has occurred since the Pliocene, with much occurring in the Pleistocene. The recent diversifications in South American *Lepechinia* appear to be associated with Pliocene/Pleistocene orogenic (Van der Hammen, Werner & Van Dommelen, 1973; Hooghiemstra, Wijninga & Cleef, 2006; Antonelli *et al*., 2009) and glaciation/cooling events of the Pleistocene (Van Der Hammen, 1974; Simpson, 1975; Hooghiemstra, 1989), as has been argued for major diversifications seen in other Andean clades such as *Lupinus* L. (Drummond, 2008), *Puya* (Jabaily, 2009; Jabaily & Sytsma, 2012) and core Tillansioideae and epiphytic Bromelioideae (Givnish *et al*., 2011).

Most *Lepechinia* spp. today grow in open habitats. Tropical species are usually encountered in naturally occurring light gaps or near road-cuts or in other areas of anthropogenic disturbance and are almost always found in clusters that receive full sunlight. Subtropical and Mediterranean species are typically found in open and at least seasonally dry habitats. In California, an area well studied for *Lepechinia*, populations are known to expand following fire (Boyd & Mistretta, 2006). The specific ecological envelope is not well known for species in other areas, but it is possible that the historical geographical distribution of *Lepechinia* may be related to fire ecology, although the present distribution of *Lepechinia* does not coincide with fire-dependent ecosystems. Most South American *Lepechinia* spp. (especially section *Parviflorae*) are only abundant in areas that have experienced human disturbance. In the distant past, fire may have mimicked present human disturbance conditions. In the more recent past (post-Miocene but pre-human), the distribution of *Lepechinia* has probably been dependent on openings created by natural disturbances and/or treeline fluctuations. In this regard, it is noteworthy that the crown of *Lepechinia* (*c*. 12 Mya) dates to around the beginning of the mid Miocene (Figs 2, 4), a period when the earth began to get substantially cooler and drier (Woodruff, Savin & Douglas, 1981; Zachos *et al*., 2001). It appears that that this cooling/drying trend aided in diversification and spread of *Lepechinia*, in some areas (e.g. California) by causing an increase in fire frequency and in other areas by fostering more open environments. The Miocene cooling trend, in concert with the rise of the northern Andes in the mid Miocene (Hoorn, 1994; Hoorn *et al*., 1995, 2010), may have aided the diversification of *Lepechinia* in South America by leading to more open habitats, conditions that most extant members of *Lepechinia* need to thrive.

Evolution of dioecy in *Lepechinia*

Hart (1985b) used a cladistics analysis of morphological characters in an effort to trace the evolution of dioecy in *Lepechinia*. His results indicated that dioecy evolved only once when breeding system was used as a character in the morphological matrix. However, when the breeding system character was excluded from the analysis, dioecy arose independently at least three times (and up to five). Hart (1985b) placed all small white-flowered dioecious/gynodioecious *Lepechinia* spp. (he did not know that dioecy occurred in Mexico) into what he considered a monophyletic section *Parviflorae*. The results shown here (Figs 2–4), however, clearly show that Hart's section *Parviflorae* is not monophyletic and that the evolution of breeding system in *Lepechinia* is far more complex than Hart envisioned. Using BayesTraits and the conservative approach of keeping all state transitions unordered for the breeding system character, dioecy arose once in Mexico and at least twice in the Andes (Fig. 4). Node 2 represents the ancestor of all gynodioecious and dioecious (and some bisexual) species in the Andes (Fig. 4). BayesTraits provides a strong gynodioecious signal for the state at node 2, with subsequent shifts to dioecy and even back to gynodioecy and hermaphroditism. Dioecious taxa are sister to each other or to gynodioecious taxa; likewise, gynodioecious and hermaphroditic taxa are sister to each other. These results indicate that the transitions in breeding systems in *Lepechinia* do not necessarily involve gynodioecy as an intermediary stage to dioecy as proposed by Hart (1985a). However, these results should be viewed as preliminary on two counts. First, the aforementioned issues with hybridization and our incomplete taxon sampling provide here only a first estimation of breeding system evolution in South American *Lepechinia*. Second, the South American taxa are sometimes difficult to categorize as either gynodioecious or dioecious and may well show some plasticity (e.g. fruits on apparently male individuals of a dioecious species; B. T. Drew, pers. observ.). Supporting this observation is that both gynodioecious and dioecious species share suites of correlated floral features (see Fig. 5). Additionally (as previously mentioned), the placement of the gynodioecious species *L. urbanii* requires further investigation.

The BayesDiscrete analyses of contingent evolution in the suite of characters associated with breeding systems provide some notable insights. The character suite comprising many, small and white flowers is strongly correlated with gynodioecy and dioecy (and thus combined here as 'gyno/dioecy') in a phylogenetic context (Table 4, Figs 4, 5). It appears that the only transition route to a gyno/dioecious species possessing small and white flowers is first gaining gyno/dioecy from a hermaphrodite with large and non-white flowers (Fig. 5A, C). Subsequent to gaining gyno/dioecy, the transitions to small and white flowers are possible. Conversely, the only transition route to a gyno/dioecious species possessing many flowers is gaining many flowers from a hermaphrodite with few flowers, and then evolving gyno/dioecy (Fig. 5B). We lack the statistical power in this small sample (Table 4) to support clear contingent evolutionary hypotheses (e.g. dioecy evolves at a higher rate in many flower clades than few flower clades). However, the differences in rates of transitions (Fig. 5) are striking and suggest that most of the contingent hypotheses based on generalized correlative trait evolution in dioecious species (Givnish, 1980; Renner & Ricklefs, 1995; Sakai *et al*., 1995; Weiblen *et al*., 2000; Vamosi *et al*., 2003; Vamosi & Vamosi, 2004; Vary *et al*., 2011) have a biological basis in *Lepechinia*.

FUTURE DIRECTIONS

Although this study represents the most comprehensive sampling in *Lepechinia* to date, questions still remain. It is obvious that additional low copy nuclear (LCN) markers will be needed to tease apart relationships fully in *Lepechinia*, and we plan to incorporate more LCN and additional taxon sampling in *Lepechinia* in the near future. This upcoming study will examine biogeographical relationships in the genus, focusing on where *Lepechinia* originated and evaluating how many independent radiations to South America have occurred. Also, with additional sampling of key South American and Mexican taxa, we will explore the evolution of dioecy in *Lepechinia* in greater detail. Finally, there appears to be widespread hybridization and/or plastid capture among some South American *Lepechinia* spp. More intraspecies sampling and an expanded set of LCN will permit further exploration of the roles that hybridization and/or plastid capture have had in the diversification of South American *Lepechinia*.

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APPENDIX 1

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 previously submitted loci (where applicable): *ycf1* & *ycf1-rpl15* spacer region, *trnL-F*, ITS, ETS, respectively. Abbreviations: RBG-Edinburgh, Royal Botanic Garden-Edinburgh; RSABG, Rancho Santa Ana Botanical Garden; UCBG, UC-Berkeley Botanical Garden.

Agastache pallida (Lindl.) Cory, Mexico, *B. Drew 118* (WIS); JF289001, JF301357;
Bystropogon origanifolius L'Hér., cultivated-USA, *B. Drew s.n.* (WIS);
Caryopteris incana (Thunb. ex Houtt.) Miq., cultivated-UCBG 1989.0459, *Erskine et al. SICH395* (UC); JF289003, JF301359;
Cedronella canariensis (L.) Webb & Berthel., Canary Islands, cultivated-UCBG 2004.0788, *Royl 6859* (UC); JF289004, JF301360;
Chaunostoma mecistandrum Donn. Sm., El Salvador, *J.A. Monterrosa & R. A. Carballo 213* (MO); JF289005, JF301361, JF301342, JF301311;
Clinopodium vulgare L., Portugal, *Riina 1579* (WIS);
Collinsonia canadensis L., USA, cultivated-UCBG 1984.0696, *Raiche s.n.* (UC); JF289010, JF301364;
Cunilla microcephala Benth., Uruguay, *K. Sytsma 7247* (WIS); JF289013, DQ667491;
Dorystaechas hastata Boiss. & Heldr. ex Benth., cultivated RBG-Edinburgh 1972–0177D, *J. Walker s.n.* (WIS); JF289014, AY570454, DQ667252, JF301312;
Elsholtzia ciliata (Thunb.) Hyl., USA, *B. Drew 210* (WIS); JF289017, JF301367;
Glechoma hederacea L., USA, *B. Drew 69* (WIS); JF289018, JF301368;
Glechon marifolia Benth., Uruguay, *K. Sytsma 7214* (WIS); JF289019, DQ667489;
Hedeoma piperitum Benth., Mexico, *B. Drew 92* (WIS); JF289020, JF301369, JF301343, JF301313;
Horminum pyrenaicum L., cultivated-RBG-Edinburgh 1997-2109a, *J. Walker s.n.* (WIS); JF289022, AY570456, JF301314;
Hyptis laniflora Benth., Mexico, *B. Drew 41* (WIS); JF289024, JF301370;
Isodon dawoensis (Hand.-Mazz.) H.Hara, cultivated-UCBG 90.066, *Erskine et al. 392* (UC); JF289025, JF301372;
Lallemantia canescens Fisch. & C.A.Mey., cultivated-DBG 940037 (KHD); JF289026, JF301373;
Lamium maculatum L., cultivated-UW-Madison Botanical Garden, *B. Drew 75* (WIS); JF289027, JF301374;
Lavandula angustifolia Mill., cultivated-UW-Madison Greenhouse, *J. Walker 2565* (WIS); JF289028, AY570457;
Lepechinia bella Epling, Bolivia, *R. Jabaily s.n.* (WIS);
Lepechinia betonicifolia (Lam.) Epling, Ecuador, *B. Drew 224* (WIS);
Lepechinia bullata (Kunth) Epling, Ecuador, *B. Drew 223* (WIS);
Lepechinia calycina (Benth.) Epling ex Munz, USA, *B. Drew 20* (WIS);
Lepechinia caulescens (Ortega) Epling, Mexico, *B. Drew 149* (WIS);
Lepechinia chamaedryoides (Balb.) Epling, Chile, cultivated-RSABG, *J. Walker 2537* (WIS); JF289031, AY570459, DQ667231, JF301317;
Lepechinia codon Epling, Peru, *B. Drew 177* (WIS);
Lepechinia dioica J. A. Hart, Ecuador, B. Drew 232 (WIS);
Lepechinia floribunda (Benth.) Epling, Peru, *B. Drew 172* (WIS);
Lepechinia glomerata Epling, Mexico, *B. Drew 155* (WIS); JF289032, JF301377, JF301346, JF301318;
Lepechinia graveolens (Regel) Epling, Bolivia, (M);
Lepechinia hastata (A. Gray) Epling, Mexico, *B. Drew 44* (WIS); JF289033, JF301378, JF301347, JF301319;
Lepechinia heteromorpha (Briq.) Epling, *Peru 192* (WIS);
Lepechinia lamiifolia (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 meyenii (Walp.) Epling, Peru, *B. Drew 173* (WIS);
Lepechinia mollis Epling, Peru, *B. Drew 182* (WIS);
Lepechinia mutica (Benth.) Epling, Ecuador, *B. Drew 229* (WIS);
Lepechinia paniculata (Kunth) Epling, Ecuador, *B. Drew 241* (WIS);
Lepechinia radula (Benth.) Epling, Ecuador, *B. Drew 237* (WIS);
Lepechinia rufocampii Epling & Mathias, Ecuador, *B. Drew 245* (WIS);
Lepechinia salviae (Lindl.) Epling, Chile, *R. Jabaily s.n.* (WIS);
Lepechinia salviifolia (Kunth) Epling, Colombia, *R. Jabaily s.n.* (WIS); JF289038, JF301383, JF301352, JF301324;
Lepechinia schiedeana (Schltdl.) Vatke, Mexico, *B. Drew 157* (WIS);
Lepechinia scobina Epling, Peru, *B. Drew 184* (WIS);
Lepechinia speciosa (A. St.-Hil. ex Benth.) Epling, Brazil, *Cordeno 3060* (WIS);

Lepechinia urbanii Epling, Dominican Republic, *B. Drew 135* (WIS);
Lepechinia vesiculosa (Benth.) Epling, Peru, *B. Drew 175* (WIS);
Lepechinia yecorana Henrickson, Fishbein, & T. Van Devender, Mexico, *Henrickson 24 691* (WIS);
Lycopus uniflorus Michx., USA, *J. Walker 2586* (WIS); JF289040, DQ667488;
Melissa officinalis L., cultivated-UW-Madison, *B. Drew 70* (WIS); JF289042, JF301386, JF301353, JF301325;
Mentha arvensis L., USA, *B. Drew 82* (WIS); JF289043, JF301387;
Monarda citriodora Cerv. ex Lag., Mexico, *B. Drew 114* (WIS); JF289045, JF301388;
Monardella villosa Benth., USA, *B. Drew 66* (WIS); JF289046, JF301389;
Neoeplingia leucophylloides Ramamoorthy, Hiriart & Medrano, Mexico, *B. Drew 129* (WIS); JF289047, JF301390, JF301354, JF301327;
Nepeta cataria L., USA, *B. Drew 72* (WIS); JF289048, JF301391;
Ocimum basilicum L., cultivated-UW-Madison Greenhouse, *J. Walker 2557* (WIS); JF289049, AY570462;
Origanum vulgare L., USA, *B. Drew 77* (WIS); JF289050, JF301392;
Perovskia atriplicifolia Benth., cultivated-UW-Madison Botanical Garden, *J. Walker 2524* (WIS); JF289051, AY570464, DQ667223, JF301328;
Plectranthus cremnus B.J. Conn, cultivated-UCBG *3.0347 s.n.* (UC); JF289052, JF301393;
Prunella vulgaris L., USA, *J. Walker 3225* (WIS); JF289055, DQ667508;
Rhabdocaulon strictus (Benth.) Epling, Uruguay, *Sytsma 7218* (WIS); JF289056, JF301396;
Rhododon ciliatus (Benth.) Epling, USA, *Singhurst s.n* (TEX); JF289057, JF301397;
Rosmarinus officinalis L., cultivated-UW-Madison Greenhouse, *J. Walker 2558* (WIS); JF289058, AY570465, DQ667241, JF301329;
Salvia glutinosa L., cultivated-U.S.A, *J. Walker 2568* (WIS); JF289061, AY570480;
Salvia greatae Brandegee, USA, *J. Walker 2511* (WIS); JF289062, AY570481, DQ667215, JF301331;
Salvia officinalis L., cultivated-UCBG 7.0083, *M. Palma s.n.* (UC); JF289065, JF301398, JF301355, JF301332;
Salvia patens Cav., cultivated-RBG-Edinburgh 1973–9197, *J. Walker s.n.* (WIS); JF289066, DQ667442, DQ667253, JF301333;
Salvia przewalskii Maxim., cultivated-RBG-Edinburgh 1993-2067A, *J. Walker s.n.* (WIS); JF289068, DQ667443, DQ667254, JF301339;
Salvia roemeriana Scheele, USA, *J. Walker 2515* (WIS); JF289069, AY570491, DQ667211, JF301340;
Thymbra capitata Cav., cultivated-UCBG 96.0817 (UC); JF289071, JF301401;
Zhumeria majdae Rech. f. & Wendelbo, *Terme 14573* (E); JF289072, DQ667524, DQ667335, JF301341;
Ziziphora clinopodioides Lam., cultivated-DBG 980177 (KHD); JF289073, JF301402.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogram showing results from plastid DNA (*ycf1*, *ycf1-rps15* spacer, *trnL-F*) ML analysis.
Figure S2. Chronogram of tribe Mentheae based on plastid DNA (*ycf1*, *ycf1-rps15* spacer, *trnL-F*) data.
Figure S3. Maximum likelihood phylogram as inferred from the nuclear ribosomal gene regions ITS and ETS.
Figure S4. Maximum likelihood phylogram of the *PPR-AT3G09060* nuclear gene region (including all cloned accessions).
Figure S5. Maximum likelihood phylogram as inferred from between exons 7 and 11 of the GBSSI gene region.