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Evolutionary relationships within the lamioid tribe Synandreae (Lamiaceae) based on multiple low-copy nuclear loci

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The subfamily Lamiioideae (Lamiaceae) comprises ten tribes, of which only Stachydeae and Synandreae include New World members. Previous studies have investigated the phylogenetic relationships among the members of Synandreae based on plastid and nuclear ribosomal DNA loci. In an effort to re-examine the phylogenetic relationships within Synandreae, the current study incorporates data from four low-copy nuclear loci, *PHOT1*, *PHOT2*, *COR*, and *PPR*. Our results confirm previous studies based on chloroplast and nuclear ribosomal markers in supporting monophyly of tribe Synandreae, as well as sister relationships between *Brazoria* and *Warnockia*, and between that pair of genera and a monophyletic *Physostegia*. However, we observe incongruence in the relationships of *Macbridea* and *Synandra*. The placement of Synandreae within Lamiioideae is poorly resolved and incongruent among different analyses, and the sister group of Synandreae remains enigmatic. Comparison of the colonization and migration patterns corroborates a single colonization of the New World by Synandreae during the mid-Miocene. This is in contrast to the only other lamioid tribe that includes New World members, Stachydeae, which colonized the New World at least twice—during the mid-Miocene and Pliocene. Edaphic conditions and intolerance of soil acidity may be factors that restricted the distribution of most genera of Synandreae to southeastern and south-central North America, whereas polyploidy could have increased the colonizing capability of the more wide-ranging genus, *Physostegia*.

1 **Evolutionary relationships within the lamioid tribe Synandreae (Lamiaceae)**
2 **based on multiple low-copy nuclear loci**

3

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18 **ABSTRACT**

19 The subfamily Lamioideae (Lamiaceae) comprises ten tribes, of which only Stachydeae and
20 Synandreae include New World members. Previous studies have investigated the phylogenetic
21 relationships among the members of Synandreae based on plastid and nuclear ribosomal DNA
22 loci. In an effort to re-examine the phylogenetic relationships within Synandreae, the current
23 study incorporates data from four low-copy nuclear loci, *PHOT1*, *PHOT2*, *COR*, and *PPR*. Our
24 results confirm previous studies based on chloroplast and nuclear ribosomal markers in
25 supporting monophyly of tribe Synandreae, as well as sister relationships between *Brazoria* and
26 *Warnockia*, and between that pair of genera and a monophyletic *Physostegia*. However, we
27 observe incongruence in the relationships of *Macbridea* and *Synandra*. The placement of
28 Synandreae within Lamioideae is poorly resolved and incongruent among different analyses, and
29 the sister group of Synandreae remains enigmatic. Comparison of the colonization and migration
30 patterns corroborates a single colonization of the New World by Synandreae during the mid-
31 Miocene. This is in contrast to the only other lamioid tribe that includes New World members,
32 Stachydeae, which colonized the New World at least twice—during the mid-Miocene and
33 Pliocene. Edaphic conditions and intolerance of soil acidity may be factors that restricted the
34 distribution of most genera of Synandreae to southeastern and south-central North America,
35 whereas polyploidy could have increased the colonizing capability of the more wide-ranging
36 genus, *Physostegia*.

37

38 **Keywords** Synandreae, *Brazoria*, *Macbridea*, *Physostegia*, *Synandra*, *Warnockia*,

39 Biogeography, Phylogeny, Nuclear markers, North America, Stachydeae

40 INTRODUCTION

41 The angiosperm family Lamiaceae has a worldwide distribution, comprising ~7200 species in
42 approximately 240 genera (Bentham, 1876; Harley et al., 2004). Lamiaceae is subdivided into
43 seven subfamilies, of which Lamioideae, the second largest, exhibits an impressive ecological
44 and taxonomic diversity (Scheen et al., 2010; Bendiksby et al., 2011; Roy and Lindqvist, 2015).
45 Most members of Lamioideae have been classified into ten tribes, with the majority of the
46 species inhabiting Eurasia and Africa. However, approximately 113 species are native to the
47 New World, and they are members of just two tribes: Stachydeae and Synandreae (Scheen et al.,
48 2010; Roy et al., 2013; 2015). Considerable molecular phylogenetic work has recently been
49 performed in Stachydeae (Lindqvist and Albert 2002; Salmaki et al., 2013, Roy et al., 2013,
50 2015), and it has been suggested that the New World members of the genus *Stachys* colonized
51 the Americas twice, first during the mid-Miocene and later during the early Pliocene (Roy et al.,
52 2013; 2015). The focus of the current study is Synandreae, the other lamioid tribe represented in
53 the New World, comprising five genera: *Synandra* Nutt., *Macbridea* Elliott ex Nutt., *Brazoria*
54 Engelm & A. Gray, *Warnockia* M.W. Turner, and *Physostegia* Benth.

55 All five genera of Synandreae are herbs with relatively large flowers (for Lamiaceae),
56 which are sessile or short-pedicellate in racemoid inflorescences. Corolla color ranges from
57 white (*Macbridea alba*, *Synandra*, and some *Physostegia* species) to lavender (*Macbridea*
58 *caroliniana*, *Brazoria*, *Warnockia*, and most *Physostegia* species). The anther thecae either
59 narrow apically to a sharp point (*Synandra*) or bear one or more teeth along the suture.
60 Monotypic *Synandra hispidula* ($2n=18$) is a biennial of mesic woodlands in the eastern United
61 States, mostly in the Appalachian region (Harley et al., 2004). It differs from the rest of the tribe
62 in having long-petiolate, cordate-ovate leaves. *Macbridea* ($2n=18$) comprises two species of

63 rhizomatous perennial herbs of wetlands and pine savannas in the southeastern United States
64 (Harley et al., 2004) (Fig. 1). *Macbridea* flowers are tightly packed into terminal and sub-
65 terminal capitate glomerules, unlike the elongate inflorescences of the other four genera, and its
66 three-lobed calyx is distinctive. *Brazoria* ($2n=28$) comprises three species of annuals of sandy
67 soils in eastern and central Texas (Fig. 1), with an erect and deeply bifid upper corolla lip
68 (Turner, 1996). Monotypic *Warnockia scutellarioides* ($2n=20$) is an annual of calcareous soils in
69 Texas, southern Oklahoma, and northwestern Mexico (Coahuila) (Turner, 1996) (Fig. 1).
70 *Physostegia* ($2n=38$ and 76), with 12 species of perennials, is the most widespread genus of
71 Synandreae, ranging from Northern Canada to Northern Mexico and growing in diverse habitats
72 and a wide range of soil conditions (Cantino, 1982). *Physostegia virginiana* is often grown as an
73 ornamental and has become naturalized in some areas. *Physostegia* is the only genus of
74 Synandreae with an actinomorphic, five-lobed calyx.

75 Bentham (1848) described subtribe Melittidinae (“Melittieae”), comprising the
76 monotypic European genus *Melittis* and the North American genera *Brazoria*, *Synandra*,
77 *Macbridea*, and *Physostegia*. Bentham (1876) and Briquet (1895-1897) added the Asian genus
78 *Chelonopsis* to this subtribe but transferred *Brazoria* to Scutellariinae and Prunellinae,
79 respectively. Cantino (1985a) and Abu-Asab and Cantino (1987) considered Melittidinae to
80 include *Brazoria*, and Turner (1996) segregated *Warnockia* from *Brazoria*. However,
81 morphological and karyological studies (Cantino 1982; 1985a) and investigation of leaf anatomy
82 (Abu-Asab and Cantino, 1987), palynology (Abu-Asab and Cantino, 1994), and pericarp
83 structures (Ryding, 1994) were unable to provide synapomorphies supporting the monophyly of
84 Melittidinae. Furthermore, molecular phylogenetic studies demonstrated the non-monophyly of
85 Melittidinae (Scheen et al., 2008; Scheen et al., 2010; Bendiksby et al., 2011; Salmaki et al.,

86 2013; Roy and Lindqvist, 2015). Scheen et al. (2008) found that, rather than grouping with the
87 North American endemics, *Melittis melissophyllum* grouped with *Stachys*, and *Chelonopsis*
88 grouped with the Asian genus *Gomphostemma*. These studies also demonstrated the monophyly
89 of a group comprising the North American endemics (*Brazoria*, *Warnockia*, *Synandra*,
90 *Macbridea*, and *Physostegia*). Since *Melittis* is not part of this clade, it could no longer be named
91 Melittidinae and was instead named tribe Synandreae (Scheen et al., 2008). Since the study by
92 Scheen et al. (2008) was based on chloroplast and nuclear ribosomal DNA markers, the goal of
93 the current study is to investigate the phylogenetic relationships among the members of
94 Synandreae based on low-copy nuclear markers.

95 With the availability of improved technologies and universal primers, there has been a
96 shift from plastid and ribosomal loci towards the use of low-copy nuclear genes (Mort and
97 Crawford, 2004) in investigations of interspecific phylogenetic relationships because they often
98 have a higher rate of evolution, leading to higher resolution in species-level phylogenies.
99 Furthermore, maternally inherited plastid DNA, as a single linkage group, can only provide the
100 genealogical history of one parent and thus cannot provide any information on hybrid species
101 histories. Although nuclear ribosomal DNA (ITS, ETS and 5S-NTS) is biparentally inherited,
102 these data do not always provide reliable markers for the reconstruction of hybrid speciation and
103 resolution of phylogenetic histories due to concerted evolution and homogenization (Wendel et
104 al., 1995). Hence, the true evolutionary relationships among closely related taxa may be
105 confounded. Also, in situations where speciation has taken place rapidly, as may be the case
106 within Synandreae, genomic DNA may not have undergone enough divergence to resolve a
107 phylogeny with only one locus (Seehausen, 2004). In such cases, multiple independent nuclear
108 loci may provide the variability necessary to make a more accurate estimation of phylogenetic

109 relationships (Sang, 2002; Hughes et al., 2006). However, low-copy nuclear genes are not devoid
110 of shortcomings. Some of the issues encountered while dealing with low-copy nuclear loci
111 include presence of paralogous copies, incomplete lineage sorting, and gene tree/species tree
112 incongruence due to hybridization and introgression. Hence, these factors should always be taken
113 into account when drawing conclusions on evolutionary relationships.

114 In this study, we have analyzed data from four low-copy nuclear loci: two *PHOT* gene
115 duplicates (*PHOT1* and *PHOT2*), *COR* (cold acclimation protein), and the *PPR*
116 (pentatricopeptide repeat) region AT3G09060. The *PHOT* genes are responsible for encoding the
117 blue and ultra-violet-A light receptor of plants involved in the process of phototropism (Christie
118 et al., 1998), chloroplast relocation (Jarillo et al., 2001; Kagawa et al., 2001), and the regulation
119 of stomatal openings (Kinoshita et al., 2001). Two *PHOT* loci are present in most angiosperms
120 (*PHOT1* and *PHOT2*), resulting from a duplication event predating the divergence between
121 monocots and tricolpates (Briggs et al., 2001). The two *PHOT* gene duplicates have accumulated
122 a sufficiently large number of nucleotide substitutions since their divergence to be distinct from
123 each other, which is important for overcoming orthology/paralogy issues when being utilized in
124 phylogenetic analyses (Fitch, 1970). The two paralogs have been shown to be so variable that
125 their intron regions are unalignable with each other and hence can be treated as two separate
126 markers. Due to the presence of many small, relatively conserved exon regions, separated by
127 variable introns, it has been suggested that the amount of information that can be collected from
128 these loci is high relative to the effort that is applied to work with them (Yuan and Olmstead,
129 2008). Also, through the investigation of these two paralogs, the mode of intron evolution can be
130 observed across closely related species, such as members of Synandreae. All these factors make
131 the *PHOT* gene duplicates ideal for use in our current study. The *COR* locus also consists of

132 intron regions flanked by exons that provide conserved primer binding sites (EPIC markers;
133 Curto et al., 2012; Thomson et al., 2008). Curto and colleagues (2012) have shown from their
134 study of *Micromeria* (Lamiaceae) that this locus can be phylogenetically informative, providing
135 a substantial amount of variation among closely related species. Lastly, the *PPR* gene family
136 encodes a group of proteins with short helical repeats that are arranged in stacks, forming
137 extended surfaces (Geddy and Brown, 2007; Barkan and Small, 2014). Previous studies (Yuan et
138 al., 2009, 2010; Crowl et al., 2014) and our own study on Lamiioideae (Roy and Lindqvist, 2015)
139 have shown the *PPR* loci to be useful markers to reconstruct phylogenetic relationships
140 involving rapidly radiating taxa. In addition to the low copy nuclear markers, we also
141 incorporated chloroplast DNA (cpDNA) data from previous studies (Scheen et al., 2008; Scheen
142 et al, 2010; Bendiksby et al., 2011) for four regions (*matK*, *rps16*, *trnL* intron, and *trnL-F*
143 spacer) to generate a more comprehensive multispecies coalescent tree.

144 The goals of this study included 1) assessing the monophyly of tribe Synandreae, 2)
145 further clarifying relationships within Synandreae, 3) investigating the historical biogeography of
146 Synandreae, including its introduction into the New World, and 4) comparing the migration and
147 diversification patterns of Synandreae with those of tribe Stachydeae, the only other lamioid tribe
148 with endemic New World species.

149

150 **METHODS**

151 **Taxon sampling, DNA extraction, amplification, and sequencing**

152 Leaf material was collected from specimens held at the following herbaria: BISH, C, GH, LL, O,
153 TEX, UNA, UPS, and US (herbarium acronyms follow Holmgren et al., 1990). All taxon names
154 in this study follow the “World checklist of Lamiaceae and Verbenaceae” (Govaerts et al., 2013).

155 DNA was extracted from silica-dried leaves or from herbarium specimen leaf fragments using
156 the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's
157 instructions. DNA sequence data were collected for a total of 16 of the 19 species of Synandreae,
158 including accessions from all five genera (Table 1). Furthermore, 22 additional lamioid species
159 were selected based on previous studies (Scheen et al. 2010; Bendiksby et al. 2011). *Scutellaria*
160 *hirta* was included as an outgroup since many studies have shown Scutellarioideae and
161 Lamioideae to be closely related (Wagstaff et al., 1998, Scheen et al., 2010; Bendiksby et al.,
162 2011; Li et al., 2012; Chen et al., 2014).

163 For amplification of the two *PHOT* loci, we used primers previously published by Yuan
164 and Olmstead (2008). For the *PHOT1* locus, we utilized the primers 10F ('5'-
165 ATTGGAGTSCAAYTAGATGGAAG-'3') and 12R ('5'-TCCACAAGTCCTCTGGTTTCT-
166 '3'). For the *PHOT2* locus, due to difficulty in amplification of the entire locus, we amplified
167 two separate fragments and treated them initially as two separate loci, labeling them *PHOT2A*
168 and *PHOT2B*. For the amplification of *PHOT2A*, we utilized the primers 10F ('5'-
169 GATGGAAGTGATMATKTGGAAC-'3') and 12R ('5'-AGCCCACAGGTCYTCTGGTCTC-
170 '3'), whereas *PHOT2B* was amplified with primers 12F ('5'-
171 GAGACCAGARGACCTGTGGGCT-'3') and 14R ('5'- GATTRTCCATTGCTTTCATGGC-
172 '3'). The *COR* locus was amplified using the following primers previously published by Curto et
173 al. (2012): forward primer ('5'-CTCGAATGTGTTCCCTGCAG-'3') and reverse primer ('5'-
174 CACATCCCTCTTAGTCCCATAC-'3'). Amplification and sequencing of *PPR* is described in
175 Roy and Lindqvist (2015). All loci were amplified using a GeneAmp PCR System 9700
176 (Applied Biosystems, Foster City, CA, USA) using a touchdown method with the following
177 thermocycling profile: hold for 10 min at 95°C; 10 cycles of 1 min at 95°C, 1 min at 60°C and

178 decreasing the temperature by 1°C every cycle, 1 min at 72°C; followed by 35 cycles of 1 min at
179 94°C, 1 min at 50°C, 1 min at 72°C; and a final extension of 1 min at 72°C. In certain cases when
180 this touchdown method failed to amplify our locus of interest, a modified touchdown method
181 was used, where the annealing temperature started at 55°C and decreased by 1°C every cycle.
182 PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany)
183 following the manufacturer's instructions.

184 All PCR products generated were further cloned using the Qiagen PCR cloning kit
185 (Qiagen, Hilden, Germany) following the manufacturer's instructions, with the exception that we
186 used 25 µL competent cells to transform each ligation reaction. Transformed clones were
187 incubated overnight at 37°C. Up to 12 positive clones were picked per individual, with the
188 average number of clones varying between 2 and 4 per locus. PCR reactions were prepared in 25
189 µL volumes with the AmpliTaq DNA Polymerase buffer II kit (Applied Biosystems, Foster City,
190 CA, USA) using 2.5 µL buffer, 2.5 µL MgCl₂, 1.0 µL dNTP, 0.6 µL each of M13F and M13R
191 primers and 0.2 µL of AmpliTaq polymerase. All PCR products were examined by gel
192 electrophoresis on 1% agarose gels, and positive PCR amplified products were sequenced in one
193 direction using SP6 or T7 primers at the University of Washington High Throughput Genomics
194 Center, Seattle, USA.

195

196 **Phylogenetic tree reconstruction**

197 All sequences generated were edited and assembled in the program Sequencher v.4.7 (Gene
198 Codes, Ann Arbor, Michigan, USA) and aligned with ClustalX v.2 (Larkin et al., 2007) or
199 MAFFT (EMBL-EBI); the alignments were manually adjusted in BioEdit (Hall, 1999). Gaps
200 were treated as missing, and indels were not coded. We evaluated evidence of recombination

201 using the Phi test (Bruen et al., 2006) in Splitstree v.4.13.1 (Huson, 1998). Initial Bayesian and
202 maximum likelihood (ML) analyses were performed on the two *PHOT2* regions, *PHOT2A* and
203 *PHOT2B* (see above), separately, but since their topologies were compatible, the datasets
204 generated from these two regions were concatenated in the program WINCLADA (Nixon, 1999)
205 before running further phylogenetic analyses. Phylogenetic relationships were examined for the
206 three loci, *PHOT1*, *PHOT2* and *COR*, separately using Bayesian inference conducted in either
207 MrBayes v.3.1.2 or 3.2.2 (Huelsenbeck and Ronquist, 2001) using Cipress XSEDE (Miller et al.,
208 2010). We used substitution models that best fit the data as determined by the Bayesian
209 Information Criterion (BIC) using the program jModeltest v.1.1 (Posada, 2008). The HKY+G
210 model was used for the *PHOT1* loci. The TPM1uf+G and TrN+G models were initially selected
211 for *PHOT2*, and *COR*, respectively, but since these two models are not implemented in MrBayes
212 3.2.2, we utilized the next model (HKY+G) proposed with the highest score. We also conducted
213 ML analyses using the RAxML Blackbox webserver (Stamatakis et al., 2008), or through the
214 RAxML HPC Blackbox in the Cipress portal (Miller et al., 2010). We rooted the *COR* and
215 *PHOT1* trees with *Scutellaria hirta* (not shown in figures), however, due to lack of sequence data
216 for *S. hirta* for the *PHOT2* loci, we used *Gomphostemma javanicum* to root the *PHOT2* tree.
217 Apart from analyzing our individual datasets, we also concatenated the three low-copy nuclear
218 loci and conducted Bayesian and ML analyses on this dataset. For this purpose, we selected the
219 GTR+G model and used *S. hirta* as the outgroup. Phylogenetic analyses of *PPR* alone are
220 described in Roy and Lindqvist (2015).

221

222 **Coalescence analysis, network analysis, and ancestral area reconstruction**

223 We implemented a multispecies coalescence model within the BEAST v.1.8.0 software package
224 (Drummond and Rambaut, 2007) to further explore phylogenetic signals from our nuclear loci.
225 *BEAST applies Bayesian MCMC analysis of the sequence data, jointly exploring gene trees
226 and species trees to estimate the species tree posterior distribution under the assumption of the
227 coalescence model. For this purpose, we incorporated sequences from the low-copy nuclear loci
228 *PPR* (Roy and Lindqvist, 2015), as well as from a concatenated dataset comprising sequences
229 from the cpDNA regions *matK*, *rps16*, *trnL* intron, and *trnL-F* spacer obtained from previously
230 published studies (Scheen et al., 2008; Scheen et al., 2010; Bendiksby et al, 2011), for all the
231 members of Synandreae. We pruned these datasets, keeping only members of Synandreae and
232 the taxa common to all of the loci. The nuclear loci were treated as unlinked. A relaxed
233 molecular clock model for all the loci and HKY+G models of nucleotide substitution were
234 applied for the nuclear loci, and the GTR+G model for the cpDNA regions. The tree prior was
235 set to exponential, and other priors were kept to default values. Analyses were done for 10
236 million generations sampling every 10,000 generations. A relative proportion of the posterior
237 samples from each Markov chain were discarded as burn-in, and trees were summarized in
238 TreeAnnotator v.1.8.0 (Drummond and Rambaut, 2007). The resulting trees were then visualized
239 in FigTree v. 1.4.0 (Rambaut, 2008). We also implemented a phylogenetic network method to
240 analyze signals of reticulate evolution and character conflicts in our datasets. The network was
241 created with Neighbor-Net (Huson and Bryant, 2006) in SplitsTree v.4.13.1 (Huson, 1998) using
242 uncorrected p-distances. For this purpose, we utilized *PHOT1* and *PPR*, the two nuclear loci that
243 have the highest representation in the various lamioid tribes, generating a concatenated dataset in
244 WINCLADA (Nixon, 1999). For distance calculations, we chose the most parameterized model
245 available in SplitsTree v.4.13.1 with an HKY85 model, transitions: transversions weighted 2:1,

246 gamma model of rate heterogeneity, and base frequencies estimated empirically. For our
247 ancestral area reconstruction, we used the program S-DIVA (Statistical Dispersal-Vicariance
248 Analysis; Yu et al., 2010) as implemented in the program RASP v.2.1. The program implements
249 a Bayesian approach to dispersal-vicariance analysis (DIVA; Ronquist, 1997), following the
250 method suggested by Nylander et al. (2008), which estimates optimized areas over a set of trees
251 and accounts for uncertainty in the phylogenetic estimate. The distribution range for the various
252 species was selected based on present day distributions of the species of *Synandreae* according to
253 information contained in the World Checklist of Lamiaceae and Verbenaceae (Goevarts et al.,
254 2013). Following the geographical zones defined by Brummit (2001), six geographical areas
255 were defined, and each included species of *Synandreae* assigned to one or more of these areas:
256 A: southeastern US except Texas; B: east-central US; C: Texas; D: Mexico; E: southern Canada,
257 North Dakota and Northwest Territories; F: western Canada and north-central US and G: Old
258 World. The S-DIVA analysis was performed using the tree file generated after the burn-in period
259 from the MrBayes run consisting of 1000 random trees utilizing the concatenated dataset
260 mentioned above comprising of *PHOT1*, *PHOT2* and *COR*. Since this dataset had multiple
261 cloned sequences for each species forming their own clades, we further trimmed this dataset by
262 including only one representative sequence for each species to form our final dataset. This file
263 was converted into a condensed tree file in RASP. We ran S-DIVA with the default settings,
264 except maximum number of areas was set to 2. We did not select the “allow reconstruction”
265 button, and this allowed the program to calculate the proportions of inferred alternative most-
266 parsimonious ancestral ranges at each node in a tree accounting for topological as well as
267 dispersal-vicariance uncertainties. We mapped the ancestral areas onto the 50% majority rule
268 consensus tree derived from our Bayesian analysis of the above dataset.

269 **RESULTS**

270 Our complete datasets, including gaps, generated from our current study consisted of 564
271 characters for *PHOT1*, 1816 characters in the concatenated *PHOT2* dataset, and 352 characters
272 for *COR*, totaling ~2.7 Kb characters (the raw alignment files in FASTA format for the three loci
273 are provided in Supplemental Information). Our results indicated correlation within the overall
274 topologies of the 50% majority rule Bayesian consensus trees and maximum likelihood (ML)
275 trees for all these datasets (Figs. 1a, 1b, and 1c, respectively). DNA sequence data were collected
276 for a total of 71 samples for the *PHOT1* locus, representing 34 species of Lamioideae, including
277 17 species of Synandreae. For the *PHOT2* locus, we generated a total of 51 sequences,
278 comprising 25 lamiod species including 14 species representing all of the genera of Synandreae.
279 For the *COR* locus, 64 sequences were included, representing 26 lamiod species and 15 species
280 of Synandreae. The sampling for our three new datasets differs due to limitations in the
281 availability of material and success with DNA extraction and amplification. However, based on
282 the topological congruence in the overall placement of the various species, we expect that the
283 few missing species will group with other members of their respective genera included in the
284 analyses.

285 Among these three new datasets, the *PHOT1* phylogeny (Fig. 1a) is based on the most
286 comprehensive sampling of taxa across most of the lamiod tribes. This dataset includes
287 representative taxa from the tribes Pogostemoneae, Gomphostemmatae, Marrubieae,
288 Leucadeae, Phlomideae, Stachydeae and Synandreae, and the unplaced genera *Galeopsis* and
289 *Betonica*. Using *Scutellaria hirta* as the outgroup (not shown), the *PHOT1* phylogeny infers
290 *Achyrospermum radicans* (Pogostemoneae) as sister to all other included Lamioideae (posterior
291 probability value PP=1.00; bootstrap value BS=100). *Gomphostemma javanicum*

292 (Gomphostemmatae) is sister to the remaining lamioid tribes, but the latter clade is poorly
293 supported. Although the inter-relationships of the remaining tribes, along with *Galeopsis* and
294 *Betonica*, are unresolved or poorly supported, the tribes themselves, including Synandreae, are
295 strongly supported as monophyletic. In the *PHOT2* combined phylogeny (Fig. 1b),
296 *Gomphostemma* was treated as the outgroup in the absence of *Scutellaria* or any representatives
297 of Pogostemoneae. In this tree, *Acrotome* (Leucadeae) and *Ballota* (Marrubieae) form a clade,
298 sister to the rest of the Lamioideae. Within the latter clade, members of Stachydeae are
299 monophyletic (PP=1.00; BS=100) and group with a poorly supported clade comprising *Betonica*,
300 Phlomideae, and a strongly supported Synandreae. In the *COR* tree (Fig. 1c), which used
301 *Scutellaria hirta* as the outgroup (not shown), Gomphostemmatae emerges as sister to a
302 trichotomy comprising the clades Phlomideae-Stachydeae (PP=0.98; BS<80), Marrubieae-
303 Leucadeae (PP=0.99; BS<80) and Synandreae (PP=1.00; BS=95). Our concatenated dataset of
304 the three loci *PHOT1*, *PHOT2*, and *COR* (Fig. 2) corroborates the *PHOT1* results in placing
305 *Achyrospermum radicans* in the basal split of the lamioid tree (PP=1.00; BS=100). Members of
306 Marrubieae form a strongly supported clade (PP=1.00; BS=99) sister to a monophyletic
307 Leucadeae (PP=1.00; BS=99). The tribe Stachydeae is inferred to be paraphyletic, although this
308 paraphyly is poorly supported in both the ML and Bayesian analyses.

309 The position of Synandreae within Lamioideae remains poorly resolved. It is inferred to
310 be sister to Stachydeae based on *PHOT1* (Fig. 1a) sister to *Phlomis fruticosa* based on *PHOT2*
311 (Fig. 1b), in a trichotomy with the Phlomideae-Stachydeae clade and the Marrubieae-Leucadeae
312 clade based on *COR* (Fig. 1c), and as sister to a clade composed of some taxa from Stachydeae
313 and Phlomideae in the concatenated tree (Fig. 2). All individual gene trees (Figs. 1a-c), as well as
314 the phylogeny resulting from the concatenated dataset (Fig. 2), strongly support the monophyly of

315 Synandreae (PP=1.00 and BS=100 in *PHOT1*, *PHOT2*, and the concatenated dataset; PP=1.00
316 and BS=95 in the *COR* tree).

317 The five genera of Synandreae (*Synandra*, *Macbridea*, *Brazoria*, *Warnockia*, and
318 *Physostegia*) are each resolved as monophyletic in all trees, except in the *COR* analysis where
319 *Synandra* and *Macbridea* are unresolved with respect to each other. In phylogenies based on the
320 *PHOT1*, *PHOT2*, and concatenated datasets *Brazoria* and *Warnockia* are strongly supported as
321 sister groups (concatenated: PP=0.99; BS=90), and this clade is in turn sister to *Physostegia*
322 (concatenated: PP=1.00; BS=100). In the *COR* tree, *Brazoria*, *Warnockia*, and *Physostegia* form
323 a trichotomy, and the clade comprising these three genera receives only moderate support. All
324 the individual gene trees, as well as the concatenated dataset, strongly support the monophyly of
325 *Physostegia* (concatenated: PP=1.00; BS=100). Relationships among *Physostegia* species are
326 poorly resolved in the individual gene trees. In the phylogeny from the concatenated dataset,
327 relationships within *Physostegia* are better resolved but still poorly supported, with one
328 exception: all species of *Physostegia* are resolved into two main clades (clades A and B in Fig.
329 2), which are well supported in the Bayesian analyses, although not in the ML analyses. Clade A
330 (PP=0.93, BS=75) comprises *P. longisepala* (clone1), *P. ledinghamii*, *P. correlli*, *P. virginiana*,
331 *P. pulchella*, and *P. angustifolia*, whereas clade B (PP=0.94, BS=71) comprises *P. godfreyi*, *P.*
332 *digitalis*, *P. parviflora*, *P. leptophylla*, *P. longisepala* (clone 2), and *P. purpurea*.

333 The multispecies coalescence-based tree from the *BEAST analysis of all markers (Fig.
334 3), corroborates results from previous findings (Scheen et al., 2010; Bendiksby et al., 2011; Roy
335 and Lindqvist, 2015), as well as those from our individual gene trees (Figs. 1a-c) and
336 concatenated dataset (Fig. 2), supporting *Synandra* as sister to *Macbridea*, which together are

337 sister to the remaining Synandreae (PP= 1.00). *Warnockia* and *Brazoria* form a clade (PP=0.93),
338 which is sister to a robustly supported *Physostegia* (PP=1.00).

339 The neighbor-net network analysis of the two loci *PHOT1* and *PPR* (Fig. 4) corroborates
340 the clustering of species into their respective tribes and an isolated phylogenetic position of
341 Synandreae separate from the remaining Lamioideae. Within Synandreae, *Synandra* and
342 *Macbridea* are close relatives and separate from its other members of which *Brazoria*, and
343 *Warnockia* are most closely related. No infrageneric phylogenetic structure is resolved among
344 the members of *Physostegia* included here.

345 The ancestral area reconstruction (not shown) infers southeastern US and Texas to be the
346 ancestral areas for the entire Synandreae clade, as well as for various subgroups of the
347 *Physostegia* clade.

348

349 **DISCUSSION**

350 Phylogenetic relationships among Synandreae and their position within Lamioideae were until
351 recently only investigated with cpDNA and nrDNA markers (Scheen et al., 2008; 2010;
352 Bendiksby et al., 2011). Our current study reconstructs evolutionary relationships in this group
353 based on multiple low-copy nuclear DNA markers. Although our results corroborate many of the
354 findings from previous research (Scheen et al., 2008; Scheen et al., 2010; Bendiksby et al.,
355 2011), we observe some instances of incongruence. Since low-copy loci are biparentally
356 inherited, there is a possibility that either the paternal or maternal gene copy in hybrid progeny
357 was randomly selected, resulting in conflicting patterns in the placement of some of the taxa in
358 the individual gene trees. Our phylogenetic network from the two loci *PHOT1* and *PPR* also
359 shows signatures of reticulation events throughout the phylogeny, including at the base where the

360 different tribes split (Fig. 4). As has been noted in previous studies, the signatures of ancestral
361 gene flow that may have taken place in deep time could have eroded after a long history of
362 divergence, and a substantially larger amount of data are required to precisely pinpoint those
363 loci, which could have introgressed from one species to another (Leache et al., 2014).

364

365 **Monophyly of tribe Synandreae: Chromosomal evolution and intergeneric relationships**

366 All gene trees (Fig. 1a-c), as well as the tree from the concatenated dataset (Fig. 2), unanimously
367 corroborate the monophyly of the New World tribe Synandreae, although its relationship with
368 other lamioid members, and its sister group, remain enigmatic. This clade of North American
369 (NA) endemics is distinguished from most other lamioid genera by the absence of thick-walled
370 cells in the exocarp (Ryding, 1994). The five member genera—*Synandra*, *Macbridea*, *Brazoria*,
371 *Warnockia*, and *Physostegia*—are also characterized by the presence of villous stamens (Harley
372 et al., 2004) and by the anther thecae either narrowing apically to a sharp point (*Synandra*) or
373 bearing one or more teeth along the suture (the other four genera), though it is not clear whether
374 these two character states are homologous.

375 Our findings unanimously corroborate the monophyly of *Brazoria* and *Warnockia*, which
376 together are sister to *Physostegia*, a relationship also found by Scheen et al. (2008). *Brazoria* and
377 *Warnockia* were recently recognized as separate genera by Turner (1996), having long been
378 treated as congeneric. *Brazoria*, *Warnockia*, and *Physostegia* share distinctive saclike idioblasts
379 in the leaf mesophyll, a feature not found in *Synandra* and unknown elsewhere in the family
380 (Abu-Asab and Cantino, 1987; Lersten and Curtis, 1998), thus an unambiguous synapomorphy.

381 The strongly supported sister-relationship between *Synandra* and *Macbridea*, which form
382 a clade that is sister to the rest of Synandreae, was also encountered in a nuclear phylogeny based

383 on the *PPR* locus alone (Roy and Lindqvist, 2015), but not in studies based on cpDNA and
384 nrDNA regions (Scheen et al., 2008, 2010; Bendiksby et al., 2011). In these latter studies,
385 *Synandra* emerged as sister to the rest of Synandreae. There is non-molecular support for both
386 phylogenetic hypotheses. Previous chromosomal studies (Cantino, 1985a) demonstrated that
387 *Macbridea* and *Synandra* have the same chromosome number ($2n=18$). They also share a derived
388 androecial character—the outer thecae of the anterior stamens are fused (for pictures of this
389 feature in *Synandra*, see Cantino [1985b]). Chromosome numbers based on $x=9$ are uncommon
390 in subfamily Lamioideae and may be a synapomorphy for a clade comprising *Synandra* and
391 *Macbridea* (Cantino, 1985a). However, in leaf shape, texture, and indumentum, *Macbridea* is
392 much more similar to *Brazoria*, *Warnockia*, and *Physostegia* than to *Synandra* (Cantino, 1982).
393 The leaves in the former four genera are usually lanceolate to elliptical or oblanceolate (rarely
394 ovate and never cordate), narrowing to a cuneate to rounded base, have a firm, semi-succulent
395 texture, are glabrous or at most sparsely puberulent, and at least the upper leaves are sessile. In
396 contrast, the leaves in *Synandra* are broadly ovate-cordate, membranaceous, villous, and
397 petiolate below the inflorescence. Furthermore, Cantino (1990) suggested that absence of
398 anomocytic stomata is a synapomorphy of a clade comprising *Macbridea*, *Brazoria* (including
399 *Warnockia*), and *Physostegia*. It is thus evident that *Macbridea* shares conflicting sets of
400 apparent synapomorphies with *Synandra*, on the one hand, and the *Brazoria-Warnockia-*
401 *Physostegia* clade, on the other. A possible explanation for both this character distribution and
402 the inconsistency between cpDNA and low-copy nuclear loci in the placement of *Macbridea* is a
403 scenario involving ancient hybridization between the ancestors of these genera.

404 *Synandra*, *Macbridea*, *Warnockia*, *Brazoria*, and *Physostegia* are characterized by base
405 chromosome numbers $x=9$ ($2n=18$), $x=9$ ($2n=18$), $x=10$ ($2n=20$), $x=14$ ($2n=28$), and $x=19$

406 ($2n=38, 76$), respectively (Cantino, 1985a). Although it has been suggested (Gill, 1981) that the
407 ancestral chromosome number in Lamiaceae is $x=7$, a base number of $x=9$ in the ancestor of
408 Synandreae could have evolved through aneuploid increase. Similarly, chromosome evolution
409 within Synandreae may have occurred through a series of aneuploidy events (Scheen et al., 2008)
410 from $x=9$ to $x=10$, $x=14$ and $x=19$ in the ancestors of *Warnockia*, *Brazoria*, and *Physostegia*,
411 respectively. Increasing chromosome numbers in these genera in comparison to *Synandra* and
412 *Macbridea* has been shown to be positively correlated with a decrease in the chromosome sizes
413 (Cantino, 1985a). Alternatively, the origin of the base chromosome number in *Physostegia* has
414 been posited to be a result of fusion of unreduced gametes ($x=9$ and $x=10$) or of polyploidization
415 and merger of normal gametes (Scheen et al., 2008). Hence, the chromosome number of $2n=38$
416 in some *Physostegia* species may indicate tetraploidy, while species like *P. ledinghamii* and *P.*
417 *leptophylla* may be octoploids ($2n=76$; Cantino, 1985a). If this hypothesis is correct, *Warnockia*
418 is a good candidate to be one of the progenitors of *Physostegia*, based on its chromosome
419 number ($2n=20$) and overall morphological similarity. The other progenitor, with $2n=18$, is most
420 likely extinct. One can hypothesize that this missing parent of *Physostegia* was the source of its
421 actinomorphic, 5-lobed calyx, a feature not found in any other extant genus of Synandreae.
422 *Macbridea* and *Synandra* would seem to be candidates for the missing parent based solely on
423 their chromosome number. However, there is no morphological evidence for a link between
424 *Synandra* and *Physostegia*. *Macbridea* and *Physostegia* do share a few character states that are
425 not found in *Warnockia*: a rhizomatous perennial habit, mid-stem leaves lacking capitate-
426 glandular hairs, and filaments roughly equal in length (Turner, 1996), suggesting that *Macbridea*
427 might be the other progenitor of *Physostegia*. However, all three of these character states are so

428 widespread in Lamioideae that they could easily be plesiomorphic in Synandreae and thus do not
429 provide convincing evidence for a special relationship between *Macbridea* and *Physostegia*.

430 Our phylogeny based on the concatenated dataset assembles all *Physostegia* species into
431 two clades (labeled A and B in Fig. 2). Although we are aware of no morphological
432 synapomorphies for either of these clades, previous morphological studies (Cantino, 1982) have
433 suggested interspecific relationships that receive support in some of our analyses. For example, a
434 close relationship between *P. pulchella* and *P. angustifolia* is indicated (within clade A in Fig. 2
435 and strongly supported in Fig. 3), corroborating Cantino's (1982) morphology-based studies.
436 One of the two octoploid species, *P. leptophylla*, which was speculated to be a polyploid
437 derivative of a hybrid between *P. purpurea* and *P. virginiana* in previous studies (Cantino,
438 1982; Scheen et al., 2008), groups with both of these species in one of our analyses (Fig. 1a) and
439 with *P. purpurea* in others (Figs. 1b, 1c, 2). However, our results provide only modest support
440 for this hypothesis because *P. leptophylla* also groups with *P. longisepala* in three analyses (Fig.
441 1a, 1b, 2) and with *P. digitalis* and *P. parviflora* in the multi-locus coalescence-based analysis
442 (Fig. 3). Cantino (1981) and Scheen et al. (2008) also hypothesized a hybrid origin for the other
443 octoploid species, *P. ledinghamii*, involving *P. virginiana* and *P. parviflora* as parents. Although
444 *P. ledinghamii* and *P. virginiana* group within the same clade (A) in the concatenated phylogeny
445 (Fig. 2), our study does not support a close relationship among these three species. On the other
446 hand, a close relationship is suggested between *P. ledinghamii* and *P. longisepala* (Fig. 1b and
447 2), a relationship also shown in Scheen et al.'s (2008) study, where these two species grouped
448 closely in the 5S-NTS tree. This relationship, however, is not supported by cpDNA, morphology,
449 or geographic distribution. It is also worth noting that a second *P. longisepala* clone groups with
450 *P. leptophylla* (clade B in Fig. 2) It is possible that the different phylogenetic positions of these

451 two *P. longisepala* clones reflect paternal ancestries of the involved species, but further studies
452 with greater number of clones and individuals are needed to support such a hypothesis.

453

454 **Biogeography of Synandreae: Migration and diversification**

455 *Synandra* and *Macbridea*, which together form a sister clade to the other three genera of
456 Synandreae, are largely confined to southeastern USA, but the range of *Synandra* also extends
457 north into northern West Virginia and central Ohio and Indiana (Cantino, 1985b). *Brazoria* and
458 *Warnockia* are found in south-central US; *Brazoria* is endemic to the eastern half of Texas and
459 *Warnockia* occurs mostly in central Texas, with a few outliers in eastern Texas, southern
460 Oklahoma and Coahuila in northern Mexico (Turner, 1996). The most widespread genus,
461 *Physostegia* with 12 species (Cantino, 1982), is extensively distributed across North America,
462 stretching from northern Canada to northern Mexico. However, seven out of the 12 species occur
463 in a region comprising southeastern Texas and southwestern Louisiana, suggesting that this area
464 is the center of diversity for this genus (Cantino, 1982). Our ancestral area reconstruction (Figure
465 not shown) supports a scenario in which southeastern US, including Texas, is the area where the
466 most recent common ancestor (MRCA) of Synandreae most likely evolved. From this original
467 center of diversity, migration and diversification took place northward and westward.

468 Roy and Lindqvist (2015) investigated the biogeography of the tribes of Lamioideae, and
469 their fossil-based molecular dating suggests that the MRCA of Synandreae diversified in the
470 New World (NW) from Old World (OW) relatives sometime during the Mid-Miocene epoch.
471 Since Synandreae appear to be phylogenetically isolated from other lamioid groups, and no well-
472 supported extant sister group of Synandreae has been determined (Scheen et al., 2008, 2010;
473 Bendiksby et al., 2011; Roy and Lindqvist, 2015), it is likely that several lineages,

474 phylogenetically intercalated between Synandreae and the other extant Lamioideae, have
475 undergone extinction. The Miocene epoch was characterized by extreme climatic optima, with
476 major long-term cooling strongly affecting the distribution and establishment of modern
477 terrestrial biomes (Kurschner et al., 2008). Atmospheric carbon dioxide variations during the
478 Miocene led to changes in the structure and productivity of terrestrial biomes by affecting their
479 photosynthesis (Flower and Kennett, 1994). East Antarctic ice sheet growth and polar cooling
480 also had large effects on global carbon cycling and on the terrestrial biosphere, including
481 aridification of mid-latitude continental regions (Kurschner et al., 2008). Such cool-dry cycles of
482 the Miocene epoch could have caused the extinction of some of the closest OW relatives of
483 Synandreae. Numerous biogeographic studies have emphasized the origins and diversification
484 patterns of widely disjunct plant groups in the Northern Hemisphere (NH) (Tiffney and
485 Manchester, 2001; Wen, 1999, 2001; Donoghue et al., 2001; Donoghue and Smith, 2004), and
486 three different biogeographic patterns have been hypothesized for their current distributions. The
487 first pattern suggests that there was an extinction of a once-continuous Arcto-Tertiary, Tethyan
488 or boreal flora, giving rise to the current disjunct distributions of some genera (Mao et al., 2012;
489 Sun et al., 2001). The second pattern posits that a majority of genera showing disjunct
490 distributions had their origin on the Qinghai Tibetan Plateau (QTP) and adjacent regions in Asia,
491 later migrating to and colonizing other NH regions (including the Arctic), where they gave rise to
492 derivative species (Xu et al., 2010; Zhang and Fritsch, 2010; Zhang et al., 2009). The third
493 pattern assumes the origin of the groups in other regions of the world with subsequent
494 diversifications on the QTP after the arrival of their ancestors there (Liu et al., 2002; Tu et al.,
495 2010). The absence of a clear extant sister group of Synandreae, presumably due to extinction, is
496 most consistent with the first pattern.

497

498 **Comparison with Stachydeae: Exploring causes for the restricted distribution of most**
499 **genera of Synandreae**

500 Stachydeae and Synandreae, the only two lamioid tribes that include NW members,
501 independently colonized the NW via separate migratory events. Members of Stachydeae
502 (belonging to the genus *Stachys*) colonized the NW twice, once during the mid-Miocene and the
503 other during the Pliocene, whereas Synandreae colonized the NW only once during the mid-
504 Miocene (Roy et al., 2013). While the nearest OW ancestors of NW Stachydeae can be
505 confidently inferred, with African and East Asian *Stachys* species grouping closely with the
506 temperate NA and Hawaiian taxa (Lindqvist and Albert, 2002; Roy et al., 2013; 2015), the
507 closest extant OW relatives of Synandreae have not been determined. These two tribes contrast
508 sharply in their pattern of diversification within the NW. NW Stachydeae rapidly migrated and
509 radiated in different parts of temperate NA, Mesoamerica, and South America, and they also
510 successfully colonized and diversified in the Hawaiian archipelago, giving rise to one of the
511 largest clades endemic to the islands (Lindqvist and Albert, 2002; Roy et al., 2013; 2015).
512 Members of Synandreae, on the other hand, split into 19 species in five genera but did not spread
513 outside of North America, with most species restricted to the southeastern and south-central US.
514 The range of one species of *Brazoria* extends into northern Mexico, and one species of
515 *Physostegia* has reached northern Canada.

516 A number of factors, both biological and ecological, could have led to the disparities in
517 the colonization and diversification patterns of the members of these two groups of NW
518 endemics. Polyploidy seems to be one of the leading factors contributing to the widespread
519 distribution of NW Stachydeae ($2n=32-84$), as well the genus *Physostegia* ($2n=38, 76$) within

520 Synandreae. Numerous studies have been performed on polyploid genome evolution, and these
521 have shown that phenomena such as substantial intra-genomic rearrangement and altered gene
522 regulatory relationships can lead to a certain degree of evolutionary flexibility, allowing for
523 improved success in colonization and establishment in novel habitats (Levin, 2002; Soltis and
524 Soltis, 2000; Wendel, 2000; Wendel and Doyle, 1998; Tate et al., 2005). The high-polyploid
525 members of NW Stachydeae and the Hawaiian mints seem to have rapidly radiated and
526 established themselves in novel habitats, carving out new niches, likely as a result of
527 hybridization and polyploidization (Roy et al. 2013; 2015). This includes *Stachys* species derived
528 from both the Miocene and Pliocene colonizations of the NW. We observe similar trends within
529 the genus *Physostegia*, the only polyploid genus of Synandreae, which has been more successful
530 in colonizing a broad geographic range within temperate NA than its diploid relatives, which
531 have remained largely limited to warm-temperate southeastern and south-central NA.

532 Abiotic factors could also have played an important role in the current restricted
533 distribution of Synandreae. Glacial climates were extremely variable, and it has been postulated
534 that terrestrial organisms respond individually to climate changes (Huntley & Webb, 1989). A
535 consensus opinion gleaned from palaeoecological studies is that individual species respond to
536 changing environments through their geographical distributions (Webb, 1992). Glacial
537 conditions have helped shape the modern distributions of most plant and animal species (Willis
538 and Whittaker, 2000). Local flora and fauna during glaciations could have survived only within
539 certain protected localities, referred to as “refugia” (Provan and Bennett, 2008). These refugia
540 provided stable microclimates for species to persist. Southeastern US has been highlighted as a
541 refugium for numerous other species (reviewed by Soltis et al., 2006). The geographic
542 distribution of plant species in southeastern USA has been mainly shaped in an east to west

543 pattern by three geographical factors—the Apalachicola River discontinuity, the Tombigbee
544 River discontinuity, and the Appalachian Mountains discontinuity—leading to endemism and
545 climatically determined glacial refugia (Soltis et al., 2006), especially during the Pliocene and
546 Pleistocene. Swenson and Howard (2005) cited instances of contact zones in Alabama, where
547 closely related species or populations emerging from glacial refugia in Florida and eastern
548 Texas/western Louisiana intermingled. However, due to differential tolerance of climatic and
549 edaphic conditions, species emerging from these refugia became fragmented in their
550 distributions, the less tolerant species thriving only within isolated pockets of favorable abiotic
551 conditions. The spread of *Physostegia*, the most widespread genus of Synandreae, may be due in
552 part to its ability to grow in a broad range of edaphic conditions. Cantino (1982) stated that this
553 genus is tolerant of a wide range of soil acidity conditions. As a result of millions of years of
554 weathering and acidification, southeastern NA is largely characterized by acidic, infertile soils
555 leading to relatively small areas of rich, circum-neutral soils (Manthey et al., 2011). Hence, other
556 genera of Synandreae, which are not as tolerant of acidic soil conditions, may have remained
557 restricted to such pockets of fertile soil, resulting in their current, more limited, ranges. However,
558 further studies are required to document and substantiate this hypothesis, and to investigate other
559 possible causes, such as anthropogenic alterations of habitat conditions, loss of pollinators, and
560 competition with invasive species, that may also have influenced the current restricted
561 distributions of most species of Synandreae.

562

563 **Supplemental Information**

564 The raw alignment files in FASTA format for the three loci, *PHOT1*, *PHOT2*, and *COR*.

565

566 **Additional Information and Declarations**

567 **Competing Interests**

568 Charlotte Lindqvist is an Academic Editor for PeerJ. The authors declare that there are no
569 competing interests.

570

571 **Author Contributions**

572 Tilottama Roy conceived and designed the experiments, performed the experiments, analyzed
573 the data, wrote the paper, reviewed drafts of the paper.

574 Nathan S. Catlin and Drake M. G. Garner performed the experiments, contributed
575 reagents/materials.

576 Philip D. Cantino wrote the paper and reviewed drafts of the paper.

577 Anne-Cathrine Scheen conceived and designed the experiments, contributed reagents/materials,
578 reviewed drafts of the paper.

579 Charlotte Lindqvist conceived and designed the experiments, analyzed the data, wrote the paper,
580 reviewed drafts of the paper.

581

582 **DNA Deposition**

583 The DNA sequences have been deposited in GenBank with the following accessions numbers:

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Table 1 (on next page)

List of taxa and voucher information.

Abbreviations of herbaria follow the Index Herbariorum.

1 **Table 1.** List of taxa and voucher information. Herbaria abbreviations follow the Index Herbariorum.

Taxon names	Voucher information	Geographic distribution
<i>Achyrospermum radicans</i> Gürke in H.G. A. Engler	E. Farkas & T. Pocs 86604 (UPS)	Tanzania
<i>Acrotome inflata</i> Benth.	G.L. Maggs & L. Guarino 1072 (UPS)	Namibia
<i>Acrotome pallescens</i> Benth.	I. Ortendahl 105 (UPS)	Namibia
<i>Ballota nigra</i> L. subsp. <i>ruderalis</i> (Sw.) Briq.	M. Bendiksby & A.-C. Scheen (O)	Greece
<i>Ballota pseudodictamnus</i> (L.) Benth.	M. Bendiksby & A.-C. Scheen 0420 (O)	Greece
<i>Betonica macrantha</i> K. Koch	D. McNeal et al. 161 (C)	Georgia
<i>Brazoria arenaria</i> Lundell	M.W. Turner 25 (TEX)	USA
<i>Brazoria enquistii</i> M.W. Turner	M.W. Turner 61 (TEX)	Texas, USA
<i>Brazoria truncata</i> (Benth.) Engelm. & A.Gray var. <i>truncata</i>	D.S. Corell 1605 (GH)	Texas, USA
<i>Craniotome furcata</i> (Link) Kuntze	O. Polunin et al. 5638 (UPS)	Nepal
<i>Eremostachys labiosa</i> Bunge.	V. Goloskokov s.n., 15 May 1963 (C)	CCCP, Kazakhstan
<i>Eriophyton wallichii</i> Benth.	Stainton et al. 7748 (UPS)	Nepal
<i>Eurysolen gracilis</i> Prain.	R. Geesink, P. Hiepko & C. Phengklai 8240 (C)	Thailand
<i>Galeopsis pyrenaica</i> Bartl.	P. Montserrat & al. 141487 (C)	Spain
<i>Gomphostemma javanicum</i> (Blume) Benth.	R.G. Olmstead 93-38	S. China to SE Asia
<i>Leonotis nepetifolia</i> (L.) R. Br. var. <i>nepetifolia</i>	R. Abdallah et al. 493 (UPS)	Tanzania

<i>Leucas inflata</i> Benth.	V. Goloskokov s.n., 15 May 1963 (C)	Ethiopia
<i>Macbridea caroliniana</i> (Walter) S.F.Blake	R.K. Godfrey & R.M. Tryon 741 (GH)	USA
<i>Marrubium peregrinum</i> L.	A. Strid 33875 (C)	Greece
<i>Phlomis fruticosa</i> L.	C. Mathiesen & J.M. Taylor 81 (National Collection of <i>Phlomis</i> , UK)	Sardegna (Italy) to Transcaucasus
<i>Phlomis tuberosa</i> L.	C. Mathiesen & J.M. Taylor 88 (National Collection of <i>Phlomis</i> , UK)	EC Europe to China and Mongolia
<i>Phyllostegia kaalaensis</i> St. John	S. Perlman 6117 (BISH)	Hawaii/O'ahu
<i>Physostegia angustifolia</i> Fernald	C.L. Lundell & A.A. Lundell 16031 (US)	Texas, USA
<i>Physostegia correllii</i> (Lundell) Shinnery	D.S. Corell & I.M. Johnston 19427 (LL)	Texas, USA
<i>Physostegia digitalis</i> Small	P.D. Cantino 1076 (GH)	Texas, USA
<i>Physostegia godfreyi</i> P.D.Cantino	R.K. Godfrey 77073 (GH)	Florida, USA
<i>Physostegia ledinghamii</i> (Boivin) P.D.Cantino	V.L. Harms 34491 (GH)	Saskatchewan, Canada
<i>Physostegia longisepala</i> P.D.Cantino	L.E. Brown 13523 (TEX)	Texas, USA
<i>Physostegia leptophylla</i> Small	P.D. Cantino 1026 (GH)	Florida, USA
<i>Physostegia parviflora</i> Nutt. ex A.Gray	M. Mooar 13667 (GH)	Montana, USA
<i>Physostegia pulchella</i> Lundell	Wm.F. Mahler 8530 (GH)	Texas, USA
<i>Physostegia virginiana</i> (Walter) S.F.Blake	P.D. Cantino 1007 (GH)	Florida, USA
<i>Scutellaria hirta</i> Sm.	M. Bendiksby & A.-C. Scheen 0411 (O)	Greece

<i>Stachys chamissonis</i> Benth.	C. Lindqvist 10-02 (UB)	W. Canada to W. USA
<i>Stachys sylvatica</i> L.	C. Lindqvist & V.A. Albert 358 (UNA)	Macaronesia, Europe to W Himalaya (cultivar)
<i>Stenogyne kamehamehae</i> Wawra.	S. Permlan 6933 (BISH)	Hawaii
<i>Synandra hispidula</i> (Michx.) Baill.	V.E. McNeilus 97-143 (GH)	Tennessee, USA
<i>Warnockia scutellarioides</i> (Engelm. & A.Gray) M.W.Turner	M.W. Turner 67 (TEX)	Texas, USA

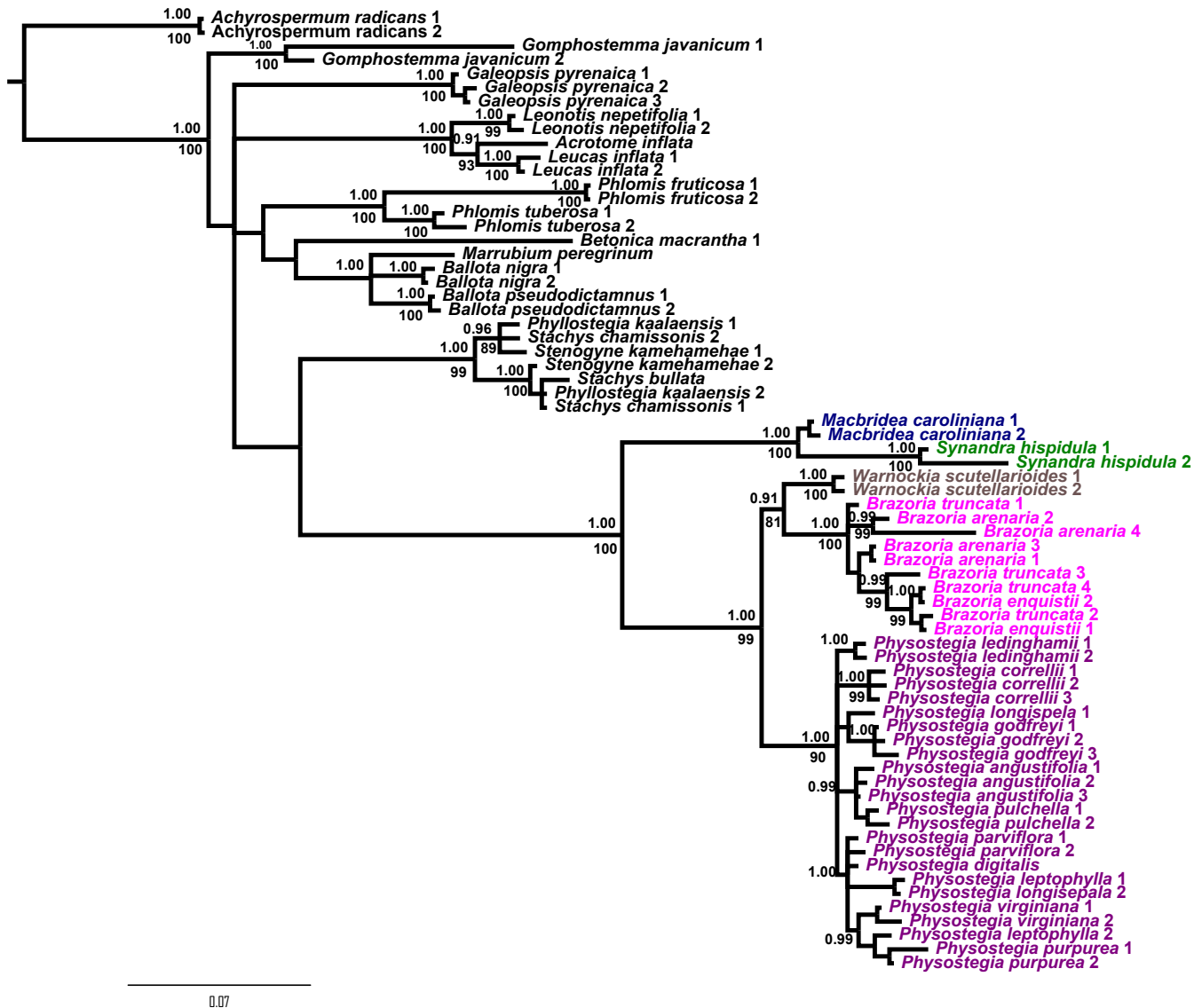
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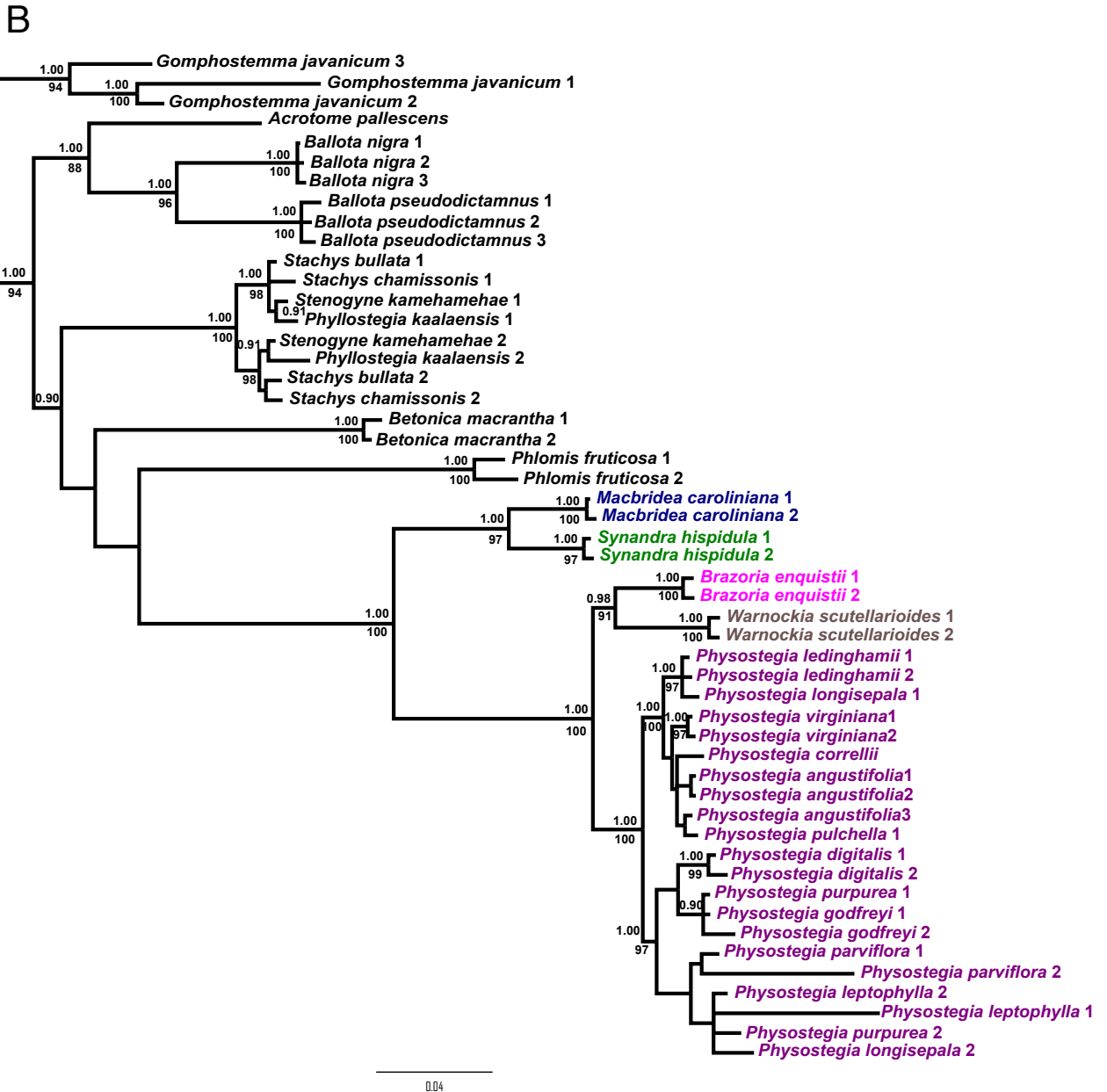
Figure 1(on next page)

Phylogenetic gene trees.

Bayesian 50% majority rule consensus trees obtained from analyses of (A) *PHOT1*, (B) *PHOT2*, and (C) *COR*, respectively. Bayesian posterior probability values ≥ 0.9 and maximum likelihood bootstrap support values ≥ 80 are shown above and below the nodes, respectively. Numbers following taxon names refer to different clones from PCR products.

A





C

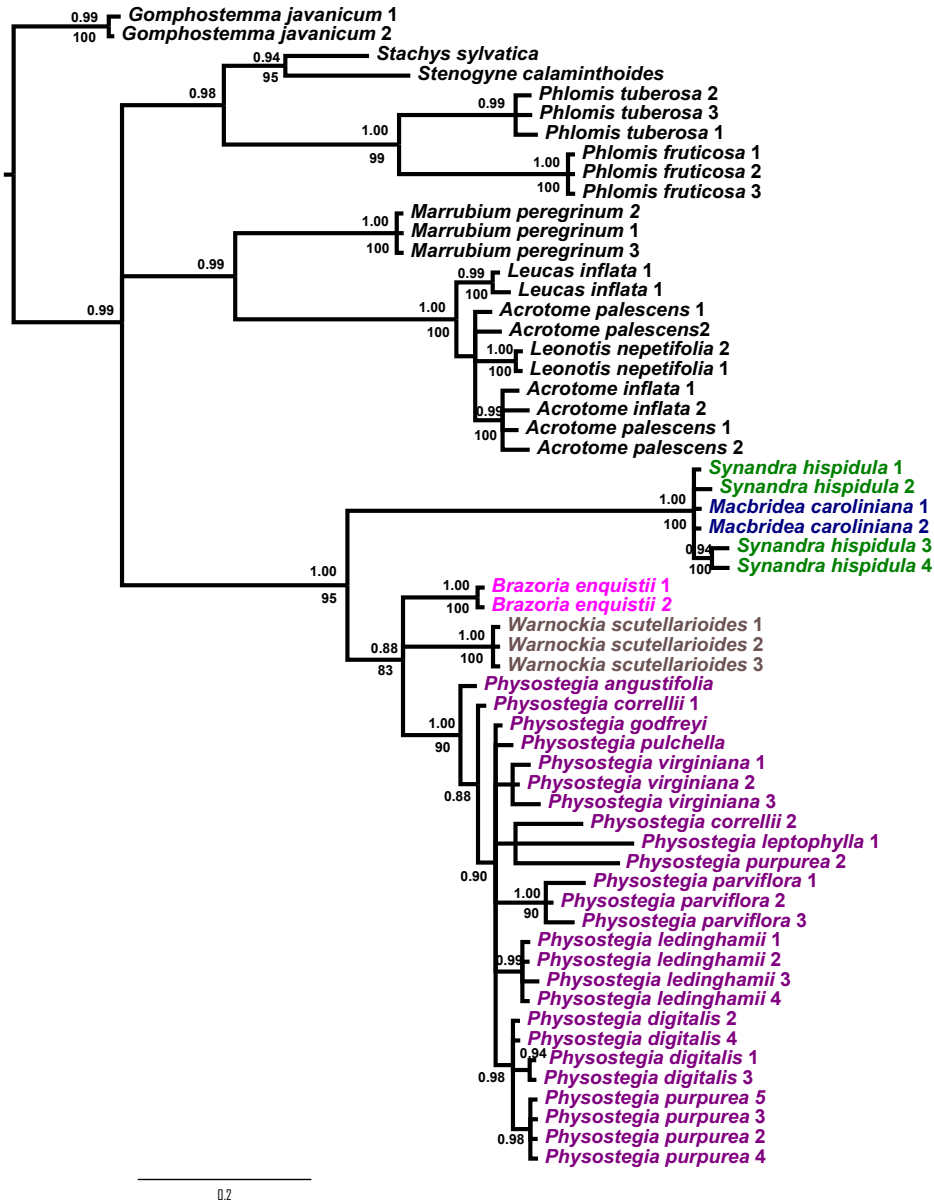


Figure 2 (on next page)

Phylogenetic tree of concatenated nuclear loci.

Bayesian 50% majority rule consensus tree obtained from analyses of the concatenated dataset. Nodes supported by Bayesian posterior probability values (PP) ≥ 0.9 and maximum likelihood bootstrap support (BS) ≥ 80 are labeled with pink dots. The green stars represent two nodes (clades A and B) discussed in the text, which have a PP ≥ 0.9 but a BS < 80 . Numbers following taxon names refer to different clones from PCR products.

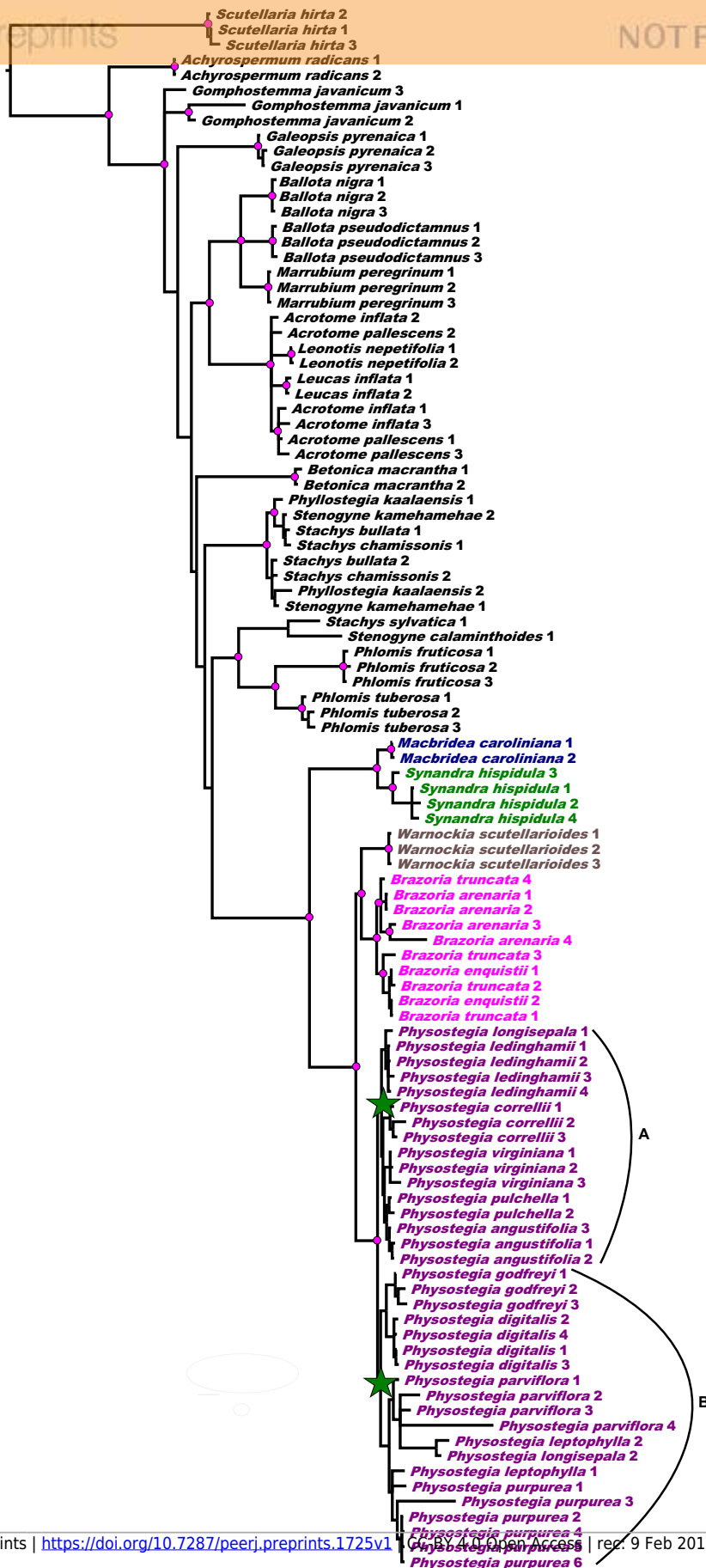


Figure 3(on next page)

Multi-locus coalescent tree.

The coalescence-based tree is inferred from a *BEAST analysis of the nuclear loci, as well as the concatenated chloroplast DNA data set. Only nodes with Bayesian posterior probability values ≥ 0.8 are labeled.

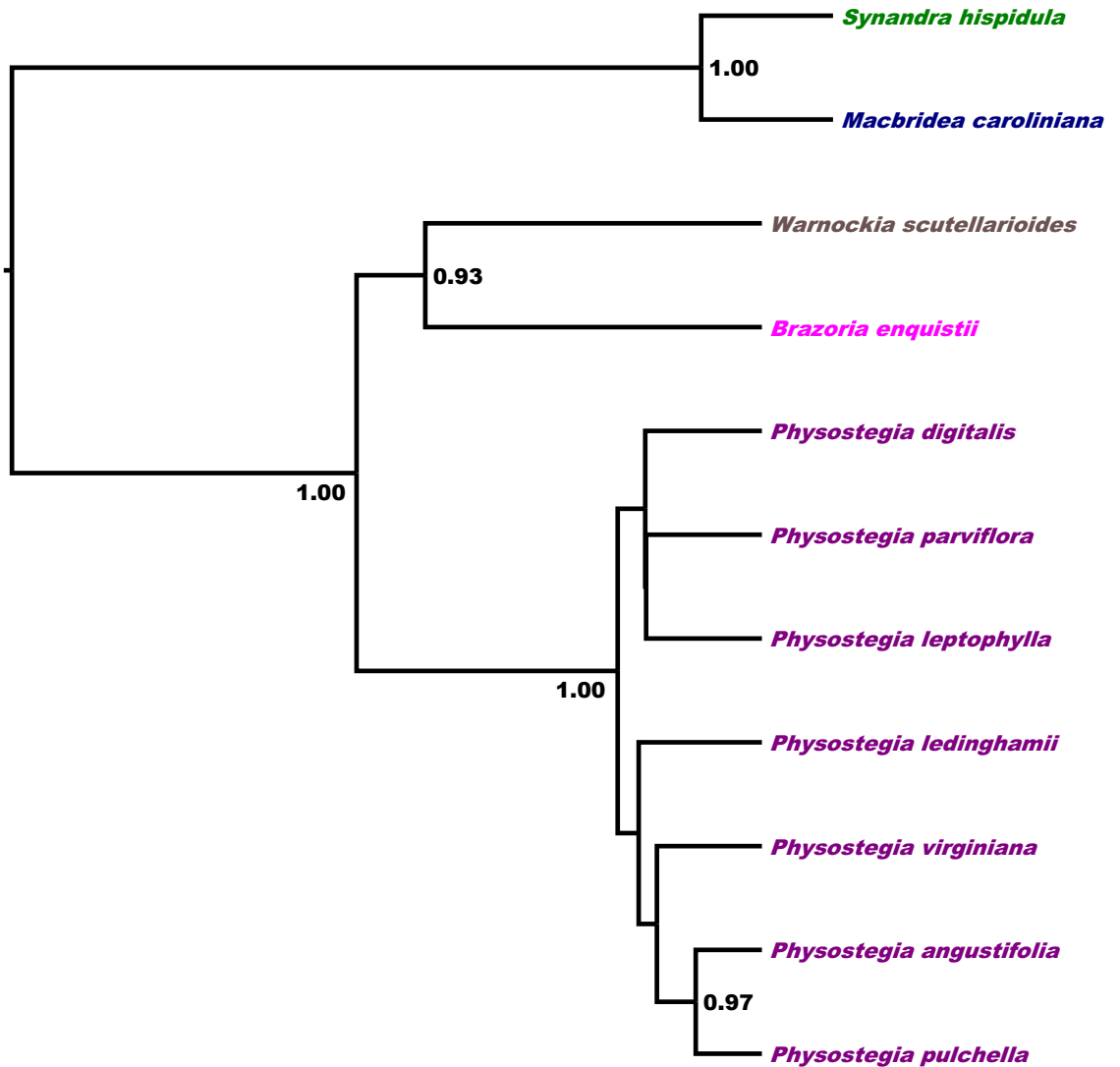


Figure 4(on next page)

Phylogenetic network.

NeighborNet analysis of the concatenated data set for *PHOT1* and *PPR* loci.

