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

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ABSTRACT

The subfamily Lamioideae (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 Lamioideae 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.

Keywords Synandreae, Brazoria, Macbridea, Physostegia, Synandra, Warnockia, Biogeography, Phylogeny, Nuclear markers, North America, Stachydeae
INTRODUCTION

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

All five genera of Synandreae are herbs with relatively large flowers (for Lamiaceae), which are sessile or short-pedicellate in racemoid inflorescences. Corolla color ranges from white (Macbridea alba, Synandra, and some Physostegia species) to lavender (Macbridea caroliniana, Brazoria, Warnockia, and most Physostegia species). The anther thecae either narrow apically to a sharp point (Synandra) or bear one or more teeth along the suture. Monotypic Synandra hispidula (2n=18) is a biennial of mesic woodlands in the eastern United States, mostly in the Appalachian region (Harley et al., 2004). It differs from the rest of the tribe in having long-petiolate, cordate-ovate leaves. Macbridea (2n=18) comprises two species of
rhizomatous perennial herbs of wetlands and pine savannas in the southeastern United States (Harley et al., 2004) (Fig. 1). Macbridea flowers are tightly packed into terminal and sub-terminal capitate glomerules, unlike the elongate inflorescences of the other four genera, and its three-lobed calyx is distinctive. Brazoria ($2n=28$) comprises three species of annuals of sandy soils in eastern and central Texas (Fig. 1), with an erect and deeply bifid upper corolla lip (Turner, 1996). Monotypic Warnockia scutellarioides ($2n=20$) is an annual of calcareous soils in Texas, southern Oklahoma, and northwestern Mexico (Coahuila) (Turner, 1996) (Fig. 1).

Physostegia ($2n=38$ and 76), with 12 species of perennials, is the most widespread genus of Synandreae, ranging from Northern Canada to Northern Mexico and growing in diverse habitats and a wide range of soil conditions (Cantino, 1982). Physostegia virginiana is often grown as an ornamental and has become naturalized in some areas. Physostegia is the only genus of Synandreae with an actinomorphic, five-lobed calyx.

Bentham (1848) described subtribe Melittidinae (“Melittieae”), comprising the monotypic European genus Melittis and the North American genera Brazoria, Synandra, Macbridea, and Physostegia. Bentham (1876) and Briquet (1895-1897) added the Asian genus Chelonopsis to this subtribe but transferred Brazoria to Scutellariinae and Prunellinae, respectively. Cantino (1985a) and Abu-Asab and Cantino (1987) considered Melittidinae to include Brazoria, and Turner (1996) segregated Warnockia from Brazoria. However, morphological and karyological studies (Cantino 1982; 1985a) and investigation of leaf anatomy (Abu-Asab and Cantino, 1987), palynology (Abu-Asab and Cantino, 1994), and pericarp structures (Ryding, 1994) were unable to provide synapomorphies supporting the monophyly of Melittidinae. Furthermore, molecular phylogenetic studies demonstrated the non-monophyly of Melittidinae (Scheen et al., 2008; Scheen et al., 2010; Bendiksby et al., 2011; Salmaki et al.,
Scheen et al. (2008) found that, rather than grouping with the North American endemics, *Melittis melissophyllum* grouped with *Stachys*, and *Chelonopsis* grouped with the Asian genus *Gomphostemma*. These studies also demonstrated the monophyly of a group comprising the North American endemics (*Brazoria*, *Warnockia*, *Synandra*, *Macbridea*, and *Physostegia*). Since *Melittis* is not part of this clade, it could no longer be named Melittidinae and was instead named tribe Synandreae (Scheen et al., 2008). Since the study by Scheen et al. (2008) was based on chloroplast and nuclear ribosomal DNA markers, the goal of the current study is to investigate the phylogenetic relationships among the members of Synandreae based on low-copy nuclear markers.

With the availability of improved technologies and universal primers, there has been a shift from plastid and ribosomal loci towards the use of low-copy nuclear genes (Mort and Crawford, 2004) in investigations of interspecific phylogenetic relationships because they often have a higher rate of evolution, leading to higher resolution in species-level phylogenies. Furthermore, maternally inherited plastid DNA, as a single linkage group, can only provide the genealogical history of one parent and thus cannot provide any information on hybrid species histories. Although nuclear ribosomal DNA (ITS, ETS and 5S-NTS) is biparentally inherited, these data do not always provide reliable markers for the reconstruction of hybrid speciation and resolution of phylogenetic histories due to concerted evolution and homogenization (Wendel et al., 1995). Hence, the true evolutionary relationships among closely related taxa may be confounded. Also, in situations where speciation has taken place rapidly, as may be the case within Synandreae, genomic DNA may not have undergone enough divergence to resolve a phylogeny with only one locus (Seehausen, 2004). In such cases, multiple independent nuclear loci may provide the variability necessary to make a more accurate estimation of phylogenetic
relationships (Sang, 2002; Hughes et al., 2006). However, low-copy nuclear genes are not devoid of shortcomings. Some of the issues encountered while dealing with low-copy nuclear loci include presence of paralogous copies, incomplete lineage sorting, and gene tree/species tree incongruence due to hybridization and introgression. Hence, these factors should always be taken into account when drawing conclusions on evolutionary relationships.

In this study, we have analyzed data from four low-copy nuclear loci: two PHOT gene duplicates (PHOT1 and PHOT2), COR (cold acclimation protein), and the PPR (pentatricopeptide repeat) region AT3G09060. The PHOT genes are responsible for encoding the blue and ultra-violet-A light receptor of plants involved in the process of phototropism (Christie et al., 1998), chloroplast relocation (Jarillo et al., 2001; Kagawa et al., 2001), and the regulation of stomatal openings (Kinoshita et al., 2001). Two PHOT loci are present in most angiosperms (PHOT1 and PHOT2), resulting from a duplication event predating the divergence between monocots and tricolpates (Briggs et al., 2001). The two PHOT gene duplicates have accumulated a sufficiently large number of nucleotide substitutions since their divergence to be distinct from each other, which is important for overcoming orthology/paralogy issues when being utilized in phylogenetic analyses (Fitch, 1970). The two paralogs have been shown to be so variable that their intron regions are unalignable with each other and hence can be treated as two separate markers. Due to the presence of many small, relatively conserved exon regions, separated by variable introns, it has been suggested that the amount of information that can be collected from these loci is high relative to the effort that is applied to work with them (Yuan and Olmstead, 2008). Also, through the investigation of these two paralogs, the mode of intron evolution can be observed across closely related species, such as members of Synandreae. All these factors make the PHOT gene duplicates ideal for use in our current study. The COR locus also consists of
intron regions flanked by exons that provide conserved primer binding sites (EPIC markers; Curto et al., 2012; Thomson et al., 2008). Curto and colleagues (2012) have shown from their study of *Micromeria* (Lamiaceae) that this locus can be phylogenetically informative, providing a substantial amount of variation among closely related species. Lastly, the PPR gene family encodes a group of proteins with short helical repeats that are arranged in stacks, forming extended surfaces (Geddy and Brown, 2007; Barkan and Small, 2014). Previous studies (Yuan et al., 2009, 2010; Crowl et al., 2014) and our own study on Lamioideae (Roy and Lindqvist, 2015) have shown the PPR loci to be useful markers to reconstruct phylogenetic relationships involving rapidly radiating taxa. In addition to the low copy nuclear markers, we also incorporated chloroplast DNA (cpDNA) data from previous studies (Scheen et al., 2008; Scheen et al, 2010; Bendiksby et all, 2011) for four regions (*matK, rps16, trnL* intron, and *trnL*-F spacer) to generate a more comprehensive multispecies coalescent tree.

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

**METHODS**

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

Leaf material was collected from specimens held at the following herbaria: BISH, C, GH, LL, O, TEX, UNA, UPS, and US (herbarium acronyms follow Holmgren et al., 1990). All taxon names in this study follow the “World checklist of Lamiaceae and Verbenaceae” (Govaerts et al., 2013).
DNA was extracted from silica-dried leaves or from herbarium specimen leaf fragments using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. DNA sequence data were collected for a total of 16 of the 19 species of Synandreae, including accessions from all five genera (Table 1). Furthermore, 22 additional lamioid species were selected based on previous studies (Scheen et al. 2010; Bendiksby et al. 2011). *Scutellaria hirta* was included as an outgroup since many studies have shown Scutellarioideae and Lamioideae to be closely related (Wagstaff et al., 1998, Scheen et al., 2010; Bendiksby et al., 2011; Li et al., 2012; Chen et al., 2014).

For amplification of the two PHOT loci, we used primers previously published by Yuan and Olmstead (2008). For the PHOT1 locus, we utilized the primers 10F (‘5’-ATTGGAGTSCAAYTAGATGGAAG-‘3’) and 12R (‘5’-TCCACAAGTCCTCTGGTTTCT-‘3’). For the PHOT2 locus, due to difficulty in amplification of the entire locus, we amplified two separate fragments and treated them initially as two separate loci, labeling them PHOT2A and PHOT2B. For the amplification of PHOT2A, we utilized the primers 10F (‘5’-GATGGAAGTGATMATKTGGAAC-‘3’) and 12R (‘5’-AGCCCACAGGTCYTCTGGTCTC-‘3’), whereas PHOT2B was amplified with primers 12F (‘5’-GAGACCAGRGACCTGTGGGCT-‘3’) and 14R (‘5’-GATTTRTCCATTGCTTTCATGGC-‘3’). The COR locus was amplified using the following primers previously published by Curto et al. (2012): forward primer (‘5’-CTCGAATGTGTTCCTGCAG-‘3’) and reverse primer (‘5’-CACATCCCTCTTAGTCCCATAC-‘3’). Amplification and sequencing of *PPR* is described in Roy and Lindqvist (2015). All loci were amplified using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using a touchdown method with the following thermocycling profile: hold for 10 min at 95°C; 10 cycles of 1 min at 95°C, 1 min at 60°C and...
decreasing the temperature by 1°C every cycle, 1 min at 72°C; followed by 35 cycles of 1 min at
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
this touchdown method failed to amplify our locus of interest, a modified touchdown method
was used, where the annealing temperature started at 55°C and decreased by 1°C every cycle.
PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany)
following the manufacturer’s instructions.

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

**Phylogenetic tree reconstruction**

All sequences generated were edited and assembled in the program Sequencher v.4.7 (Gene
Codes, Ann Arbor, Michigan, USA) and aligned with ClustalX v.2 (Larkin et al., 2007) or
MAFFT (EMBL-EBI); the alignments were manually adjusted in BioEdit (Hall, 1999). Gaps
were treated as missing, and indels were not coded. We evaluated evidence of recombination
using the Phi test (Bruen et al., 2006) in Splitstree v.4.13.1 (Huson, 1998). Initial Bayesian and maximum likelihood (ML) analyses were performed on the two PHOT2 regions, PHOT2A and PHOT2B (see above), separately, but since their topologies were compatible, the datasets generated from these two regions were concatenated in the program WINCLADA (Nixon, 1999) before running further phylogenetic analyses. Phylogenetic relationships were examined for the three loci, PHOT1, PHOT2 and COR, separately using Bayesian inference conducted in either MrBayes v.3.1.2 or 3.2.2 (Huelsenbeck and Ronquist, 2001) using Cipress XSEDE (Miller et al., 2010). We used substitution models that best fit the data as determined by the Bayesian Information Criterion (BIC) using the program jModeltest v.1.1 (Posada, 2008). The HKY+G model was used for the PHOT1 loci. The TPM1uf+G and TrN+G models were initially selected for PHOT2, and COR, respectively, but since these two models are not implemented in MrBayes 3.2.2, we utilized the next model (HKY+G) proposed with the highest score. We also conducted ML analyses using the RAxML Blackbox webserver (Stamatakis et al., 2008), or through the RAxML HPC Blackbox in the Cipress portal (Miller et al., 2010). We rooted the COR and PHOT1 trees with Scutellaria hirta (not shown in figures), however, due to lack of sequence data for S. hirta for the PHOT2 loci, we used Gomphostemma javanicum to root the PHOT2 tree. Apart from analyzing our individual datasets, we also concatenated the three low-copy nuclear loci and conducted Bayesian and ML analyses on this dataset. For this purpose, we selected the GTR+G model and used S. hirta as the outgroup. Phylogenetic analyses of PPR alone are described in Roy and Lindqvist (2015).

Coalescence analysis, network analysis, and ancestral area reconstruction
We implemented a multispecies coalescence model within the BEAST v.1.8.0 software package (Drummond and Rambaut, 2007) to further explore phylogenetic signals from our nuclear loci. *BEAST applies Bayesian MCMC analysis of the sequence data, jointly exploring gene trees and species trees to estimate the species tree posterior distribution under the assumption of the coalescence model. For this purpose, we incorporated sequences from the low-copy nuclear loci PPR (Roy and Lindqvist, 2015), as well as from a concatenated dataset comprising sequences from the cpDNA regions matK, rps16, trnL intron, and trnL-F spacer obtained from previously published studies (Scheen et al., 2008; Scheen et al., 2010; Bendiksy et al, 2011), for all the members of Synandreae. We pruned these datasets, keeping only members of Synandreae and the taxa common to all of the loci. The nuclear loci were treated as unlinked. A relaxed molecular clock model for all the loci and HKY+G models of nucleotide substitution were applied for the nuclear loci, and the GTR+G model for the cpDNA regions. The tree prior was set to exponential, and other priors were kept to default values. Analyses were done for 10 million generations sampling every 10,000 generations. A relative proportion of the posterior samples from each Markov chain were discarded as burn-in, and trees were summarized in TreeAnnotator v.1.8.0 (Drummond and Rambaut, 2007). The resulting trees were then visualized in FigTree v. 1.4.0 (Rambaut, 2008). We also implemented a phylogenetic network method to analyze signals of reticulate evolution and character conflicts in our datasets. The network was created with Neighbor-Net (Huson and Bryant, 2006) in SplitsTree v.4.13.1 (Huson, 1998) using uncorrected p-distances. For this purpose, we utilized PHOT1 and PPR, the two nuclear loci that have the highest representation in the various lamioid tribes, generating a concatenated dataset in WINCLADA (Nixon, 1999). For distance calculations, we chose the most parameterized model available in SplitsTree v.4.13.1 with an HKY85 model, transitions: transversions weighted 2:1,
gamma model of rate heterogeneity, and base frequencies estimated empirically. For our ancestral area reconstruction, we used the program S-DIVA (Statistical Dispersal-Vicariance Analysis; Yu et al., 2010) as implemented in the program RASP v.2.1. The program implements a Bayesian approach to dispersal-vicariance analysis (DIVA; Ronquist, 1997), following the method suggested by Nylander et al. (2008), which estimates optimized areas over a set of trees and accounts for uncertainty in the phylogenetic estimate. The distribution range for the various species was selected based on present day distributions of the species of Synandreae according to information contained in the World Checklist of Lamiaceae and Verbenaceae (Goevarts et al., 2013). Following the geographical zones defined by Brummit (2001), six geographical areas were defined, and each included species of Synandreae assigned to one or more of these areas: A: southeastern US except Texas; B: east-central US; C: Texas; D: Mexico; E: southern Canada, North Dakota and Northwest Territories; F: western Canada and north-central US and G: Old World. The S-DIVA analysis was performed using the tree file generated after the burn-in period from the MrBayes run consisting of 1000 random trees utilizing the concatenated dataset mentioned above comprising of \textit{PHOT1}, \textit{PHOT2} and \textit{COR}. Since this dataset had multiple cloned sequences for each species forming their own clades, we further trimmed this dataset by including only one representative sequence for each species to form our final dataset. This file was converted into a condensed tree file in RASP. We ran S-DIVA with the default settings, except maximum number of areas was set to 2. We did not select the “allow reconstruction” button, and this allowed the program to calculate the proportions of inferred alternative most-parsimonious ancestral ranges at each node in a tree accounting for topological as well as dispersal-vicariance uncertainties. We mapped the ancestral areas onto the 50% majority rule consensus tree derived from our Bayesian analysis of the above dataset.
RESULTS

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

Among these three new datasets, the PHOT1 phylogeny (Fig. 1a) is based on the most comprehensive sampling of taxa across most of the lamioid tribes. This dataset includes representative taxa from the tribes Pogostemoneae, Gomphostemmateae, Marrubieae, Leucadeae, Phomideae, Stachydeae and Synandreae, and the unplaced genera Galeopsis and Betonica. Using Scutellaria hirta as the outgroup (not shown), the PHOT1 phylogeny infers Achyrospermum radicans (Pogostemoneae) as sister to all other included Lamioideae (posterior probability value PP=1.00; bootstrap value BS=100). Gomphostemma javanicum
(Gomphostemmateae) is sister to the remaining lamioid tribes, but the latter clade is poorly supported. Although the inter-relationships of the remaining tribes, along with Galeopsis and Betonica, are unresolved or poorly supported, the tribes themselves, including Synandreae, are strongly supported as monophyletic. In the PHOT2 combined phylogeny (Fig. 1b), Gomphostemma was treated as the outgroup in the absence of Scutellaria or any representatives of Pogostemoneae. In this tree, Acrotome (Leucadeae) and Ballota (Marrubieae) form a clade, sister to the rest of the Lamioideae. Within the latter clade, members of Stachydeae are monophyletic (PP=1.00; BS=100) and group with a poorly supported clade comprising Betonica, Phlomideae, and a strongly supported Synandrea. In the COR tree (Fig. 1c), which used Scutellaria hirta as the outgroup (not shown), Gomphostemmateae emerges as sister to a trichotomy comprising the clades Phlomideae-Stachydeae (PP=0.98; BS<80), Marrubieae-Leucadeae (PP=0.99; BS<80) and Synandreae (PP=1.00; BS=95). Our concatenated dataset of the three loci PHOT1, PHOT2, and COR (Fig. 2) corroborates the PHOT1 results in placing Achyrospermum radicans in the basal split of the lamioid tree (PP=1.00; BS=100). Members of Marrubieae form a strongly supported clade (PP=1.00; BS=99) sister to a monophyletic Leucadeae (PP=1.00; BS=99). The tribe Stachydeae is inferred to be paraphyletic, although this paraphyly is poorly supported in both the ML and Bayesian analyses.

The position of Synandreae within Lamioideae remains poorly resolved. It is inferred to be sister to Stachydeae based on PHOT1 (Fig. 1a) sister to Phlomis fruticosa based on PHOT2 (Fig. 1b), in a trichotomy with the Phlomideae-Stachydeae clade and the Marrubieae-Leucadeae clade based on COR (Fig. 1c), and as sister to a clade composed of some taxa from Stachydeae and Phlomideae in the concatenated tree (Fig. 2). All individual gene trees (Figs. 1a-c), as well as the phylogeny resulting from the concatenated dataset (Fig. 2), strongly support the monophyly of
Synandreae (PP=1.00 and BS=100 in PHOT1, PHOT2, and the concatenated dataset; PP=1.00 and BS=95 in the COR tree).

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

The multispecies coalescence-based tree from the *BEAST analysis of all markers (Fig. 3), corroborates results from previous findings (Scheen et al., 2010; Bendiksby et al., 2011; Roy and Lindqvist, 2015), as well as those from our individual gene trees (Figs. 1a-c) and concatenated dataset (Fig. 2), supporting Synandra as sister to Macbridea, which together are
sister to the remaining Synandreae (PP= 1.00). *Warnockia* and *Brazoria* form a clade (PP=0.93), which is sister to a robustly supported *Physostegia* (PP=1.00).

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

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

**DISCUSSION**

Phylogenetic relationships among Synandreae and their position within Lamioideae were until recently only investigated with cpDNA and nrDNA markers (Scheen et al., 2008; 2010; Bendiksby et al., 2011). Our current study reconstructs evolutionary relationships in this group based on multiple low-copy nuclear DNA markers. Although our results corroborate many of the findings from previous research (Scheen et al., 2008; Scheen et al., 2010; Bendiksby et al., 2011), we observe some instances of incongruence. Since low-copy loci are biparentally inherited, there is a possibility that either the paternal or maternal gene copy in hybrid progeny was randomly selected, resulting in conflicting patterns in the placement of some of the taxa in the individual gene trees. Our phylogenetic network from the two loci *PHOT1* and *PPR* also shows signatures of reticulation events throughout the phylogeny, including at the base where the
different tribes split (Fig. 4). As has been noted in previous studies, the signatures of ancestral
gene flow that may have taken place in deep time could have eroded after a long history of
divergence, and a substantially larger amount of data are required to precisely pinpoint those
loci, which could have introgressed from one species to another (Leache et al., 2014).

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

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

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

The strongly supported sister-relationship between *Synandra* and *Macbridea*, which form
a clade that is sister to the rest of Synandreae, was also encountered in a nuclear phylogeny based
on the PPR locus alone (Roy and Lindqvist, 2015), but not in studies based on cpDNA and

nrDNA regions (Scheen et al., 2008, 2010; Bendiksby et al., 2011). In these latter studies,

*Synandra* emerged as sister to the rest of Synandreae. There is non-molecular support for both

phylogenetic hypotheses. Previous chromosomal studies (Cantino, 1985a) demonstrated that

*Macbridea* and *Synandra* have the same chromosome number ($2n=18$). They also share a derived

androecial character—the outer thecae of the anterior stamens are fused (for pictures of this

feature in *Synandra*, see Cantino [1985b]). Chromosome numbers based on $x=9$ are uncommon

in subfamily Lamioideae and may be a synapomorphy for a clade comprising *Synandra* and

*Macbridea* (Cantino, 1985a). However, in leaf shape, texture, and indumentum, *Macbridea* is

much more similar to *Brazoria*, *Warnockia*, and *Physostegia* than to *Synandra* (Cantino, 1982).

The leaves in the former four genera are usually lanceolate to elliptical or oblanceolate (rarely

ovate and never cordate), narrowing to a cuneate to rounded base, have a firm, semi-succulent

texture, are glabrous or at most sparsely puberulent, and at least the upper leaves are sessile. In

contrast, the leaves in *Synandra* are broadly ovate-cordate, membranaceous, villous, and

petiolate below the inflorescence. Furthermore, Cantino (1990) suggested that absence of

anomocytic stomata is a synapomorphy of a clade comprising *Macbridea*, *Brazoria* (including

*Warnockia*), and *Physostegia*. It is thus evident that *Macbridea* shares conflicting sets of

apparent synapomorphies with *Synandra*, on the one hand, and the *Brazoria-Warnockia-

*Physostegia* clade, on the other. A possible explanation for both this character distribution and

the inconsistency between cpDNA and low-copy nuclear loci in the placement of *Macbridea* is a

scenario involving ancient hybridization between the ancestors of these genera.

*Synandra*, *Macbridea*, *Warnockia*, *Brazoria*, and *Physostegia* are characterized by base

chromosome numbers $x=9$ ($2n=18$), $x=9$ ($2n=18$), $x=10$ ($2n=20$), $x=14$ ($2n=28$), and $x=19$
(2n=38, 76), respectively (Cantino, 1985a). Although it has been suggested (Gill, 1981) that the ancestral chromosome number in Lamiaceae is x=7, a base number of x=9 in the ancestor of Synandreae could have evolved through aneuploid increase. Similarly, chromosome evolution within Synandreae may have occurred through a series of aneuploidy events (Scheen et al., 2008) from x=9 to x=10, x=14 and x=19 in the ancestors of Warnockia, Brazoria, and Physostegia, respectively. Increasing chromosome numbers in these genera in comparison to Synandra and Macbridea has been shown to be positively correlated with a decrease in the chromosome sizes (Cantino, 1985a). Alternatively, the origin of the base chromosome number in Physostegia has been posited to be a result of fusion of unreduced gametes (x=9 and x=10) or of polyploidization and merger of normal gametes (Scheen et al., 2008). Hence, the chromosome number of 2n=38 in some Physostegia species may indicate tetraploidy, while species like P. ledinghamii and P. leptophylla may be octoploids (2n=76; Cantino, 1985a). If this hypothesis is correct, Warnockia is a good candidate to be one of the progenitors of Physostegia, based on its chromosome number (2n=20) and overall morphological similarity. The other progenitor, with 2n=18, is most likely extinct. One can hypothesize that this missing parent of Physostegia was the source of its actinomorphic, 5-lobed calyx, a feature not found in any other extant genus of Synandreae. Macbridea and Synandra would seem to be candidates for the missing parent based solely on their chromosome number. However, there is no morphological evidence for a link between Synandra and Physostegia. Macbridea and Physostegia do share a few character states that are not found in Warnockia: a rhizomatous perennial habit, mid-stem leaves lacking capitate-glandular hairs, and filaments roughly equal in length (Turner, 1996), suggesting that Macbridea might be the other progenitor of Physostegia. However, all three of these character states are so
widespread in Lamioideae that they could easily be plesiomorphic in Synandreae and thus do not provide convincing evidence for a special relationship between *Macbridea* and *Physostegia*.

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

**Biogeography of Synandreae: Migration and diversification**

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

Roy and Lindqvist (2015) investigated the biogeography of the tribes of Lamioideae, and their fossil-based molecular dating suggests that the MRCA of Synandreae diversified in the New World (NW) from Old World (OW) relatives sometime during the Mid-Miocene epoch. Since Synandreae appear to be phylogenetically isolated from other lamioid groups, and no well-supported extant sister group of Synandreae has been determined (Scheen et al., 2008, 2010; Bendiksby et al., 2011; Roy and Lindqvist, 2015), it is likely that several lineages,
phylogenetically intercalated between Synandreae and the other extant Lamioideae, have undergone extinction. The Miocene epoch was characterized by extreme climatic optima, with major long-term cooling strongly affecting the distribution and establishment of modern terrestrial biomes (Kurschner et al., 2008). Atmospheric carbon dioxide variations during the Miocene led to changes in the structure and productivity of terrestrial biomes by affecting their photosynthesis (Flower and Kennett, 1994). East Antarctic ice sheet growth and polar cooling also had large effects on global carbon cycling and on the terrestrial biosphere, including aridification of mid-latitude continental regions (Kurschner et al., 2008). Such cool-dry cycles of the Miocene epoch could have caused the extinction of some of the closest OW relatives of Synandreae. Numerous biogeographic studies have emphasized the origins and diversification patterns of widely disjunct plant groups in the Northern Hemisphere (NH) (Tiffney and Manchester, 2001; Wen, 1999, 2001; Donoghue et al., 2001; Donoghue and Smith, 2004), and three different biogeographic patterns have been hypothesized for their current distributions. The first pattern suggests that there was an extinction of a once-continuous Arcto-Tertiary, Tethyan or boreal flora, giving rise to the current disjunct distributions of some genera (Mao et al., 2012; Sun et al., 2001). The second pattern posits that a majority of genera showing disjunct distributions had their origin on the Qinghai Tibetan Plateau (QTP) and adjacent regions in Asia, later migrating to and colonizing other NH regions (including the Arctic), where they gave rise to derivative species (Xu et al., 2010; Zhang and Fritsch, 2010; Zhang et al., 2009). The third pattern assumes the origin of the groups in other regions of the world with subsequent diversifications on the QTP after the arrival of their ancestors there (Liu et al., 2002; Tu et al., 2010). The absence of a clear extant sister group of Synandreae, presumably due to extinction, is most consistent with the first pattern.
Comparison with Stachydeae: Exploring causes for the restricted distribution of most genera of Synandreae

Stachydeae and Synandreae, the only two lamioid tribes that include NW members, independently colonized the NW via separate migratory events. Members of Stachydeae (belonging to the genus *Stachys*) colonized the NW twice, once during the mid-Miocene and the other during the Pliocene, whereas Synandreae colonized the NW only once during the mid-Miocene (Roy et al., 2013). While the nearest OW ancestors of NW Stachydeae can be confidently inferred, with African and East Asian *Stachys* species grouping closely with the temperate NA and Hawaiian taxa (Lindqvist and Albert, 2002; Roy et al., 2013; 2015), the closest extant OW relatives of Synandreae have not been determined. These two tribes contrast sharply in their pattern of diversification within the NW. NW Stachydeae rapidly migrated and radiated in different parts of temperate NA, Mesoamerica, and South America, and they also successfully colonized and diversified in the Hawaiian archipelago, giving rise to one of the largest clades endemic to the islands (Lindqvist and Albert, 2002; Roy et al., 2013; 2015).

Members of Synandreae, on the other hand, split into 19 species in five genera but did not spread outside of North America, with most species restricted to the southeastern and south-central US. The range of one species of *Brazoria* extends into northern Mexico, and one species of *Physostegia* has reached northern Canada.

A number of factors, both biological and ecological, could have led to the disparities in the colonization and diversification patterns of the members of these two groups of NW endemics. Polyploidy seems to be one of the leading factors contributing to the widespread distribution of NW Stachydeae ($2n = 32-84$), as well the genus *Physostegia* ($2n=38$, 76) within
Synandreae. Numerous studies have been performed on polyploid genome evolution, and these have shown that phenomena such as substantial intra-genomic rearrangement and altered gene regulatory relationships can lead to a certain degree of evolutionary flexibility, allowing for improved success in colonization and establishment in novel habitats (Levin, 2002; Soltis and Soltis, 2000; Wendel, 2000; Wendel and Doyle, 1998; Tate et al., 2005). The high-polyploid members of NW Stachydeae and the Hawaiian mints seem to have rapidly radiated and established themselves in novel habitats, carving out new niches, likely as a result of hybridization and polyploidization (Roy et al. 2013; 2015). This includes *Stachys* species derived from both the Miocene and Pliocene colonizations of the NW. We observe similar trends within the genus *Physostegia*, the only polyploid genus of Synandreae, which has been more successful in colonizing a broad geographic range within temperate NA than its diploid relatives, which have remained largely limited to warm-temperate southeastern and south-central NA.

Abiotic factors could also have played an important role in the current restricted distribution of Synandreae. Glacial climates were extremely variable, and it has been postulated that terrestrial organisms respond individually to climate changes (Huntley & Webb, 1989). A consensus opinion gleaned from palaeoecological studies is that individual species respond to changing environments through their geographical distributions (Webb, 1992). Glacial conditions have helped shape the modern distributions of most plant and animal species (Willis and Whittaker, 2000). Local flora and fauna during glaciations could have survived only within certain protected localities, referred to as “refugia” (Provan and Bennett, 2008). These refugia provided stable microclimates for species to persist. Southeastern US has been highlighted as a refugium for numerous other species (reviewed by Soltis et al., 2006). The geographic distribution of plant species in southeastern USA has been mainly shaped in an east to west
pattern by three geographical factors—the Apalachicola River discontinuity, the Tombigbee River discontinuity, and the Appalachian Mountains discontinuity—leading to endemism and climatically determined glacial refugia (Soltis et al., 2006), especially during the Pliocene and Pleistocene. Swenson and Howard (2005) cited instances of contact zones in Alabama, where closely related species or populations emerging from glacial refugia in Florida and eastern Texas/western Louisiana intermingled. However, due to differential tolerance of climatic and edaphic conditions, species emerging from these refugia became fragmented in their distributions, the less tolerant species thriving only within isolated pockets of favorable abiotic conditions. The spread of Physostegia, the most widespread genus of Synandreae, may be due in part to its ability to grow in a broad range of edaphic conditions. Cantino (1982) stated that this genus is tolerant of a wide range of soil acidity conditions. As a result of millions of years of weathering and acidification, southeastern NA is largely characterized by acidic, infertile soils leading to relatively small areas of rich, circum-neutral soils (Manthey et al., 2011). Hence, other genera of Synandreae, which are not as tolerant of acidic soil conditions, may have remained restricted to such pockets of fertile soil, resulting in their current, more limited, ranges. However, further studies are required to document and substantiate this hypothesis, and to investigate other possible causes, such as anthropogenic alterations of habitat conditions, loss of pollinators, and competition with invasive species, that may also have influenced the current restricted distributions of most species of Synandreae.
Supplemental Information

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

Additional Information and Declarations

Competing Interests

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

Author Contributions

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

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

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

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

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

DNA Deposition

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

KT716867-KT717052
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Table 1 (on next page)

List of taxa and voucher information.

Abbreviations of herbaria follow the Index Herbariorum.
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<th>Taxon names</th>
<th>Voucher information</th>
<th>Geographic distribution</th>
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<td>Achyrospermum radicans Gürke in H.G. A. Engler</td>
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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 $\geq 0.9$ and maximum likelihood bootstrap support values $\geq 80$ are shown above and below the nodes, respectively. Numbers following taxon names refer to different clones from PCR products.
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.
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 $\geq 0.8$ are labeled.
Figure 4 (on next page)

Phylogenetic network.

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